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Research Article

In Vitro Effect of Probiotic Mix and Fibrolytic Enzyme Mixture on Digestibility of Paddy Straw

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Abstract | The study was undertaken to evaluate the effects of probiotic mix and fibrolytic enzyme mixture on in-vitro fermentation and digestibility using whole rumen flora from sheep. The feed additives viz. probiotic mix and fibrolytic enzyme mixture were incorporated @ 3g, 6g, 9g, 12g, 15g/kg dry matter of complete feedfor adult sheep. The rations were subjected to in- vitro analysis to evaluateDM, OM and NDF digestibilityfor determination ofoptimum level of incorporation of feed additives in the complete ration.Significant improvement in nutrient degradability of DM, OM and NDF was observed due to probiotics mix and fibrolytic enzyme mixture supplementation with maximum values at 3g and 9g kg-1DM, respectively. Another in vitro study was carried to evaluate the effect of 3g and 9g kg-1 DM of probiotics mixand fibrolytic enzyme mixture alone and in combination on rumen fermentationparametersin complete feed having paddy straw and concentrate mixture in 50:50 ratio. The probiotics mixand fibrolytic enzyme mixture alone and in combination significantly improved nutrient degradability (DM, OM and NDF), total rumen nitrogen and TVFA in comparison to control. Additions of probiotic mix in complete feed decreased rumen pH alone and in combination with enzyme mix with a significant decrease in rumen ammonia nitrogen.

Keywords | Complete feed, Fibrolytic enzyme mix, *In vitro* degradability, Paddy straw, Probiotics mix.

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INTRODUCTION

Feed additives have immense importance in livestock ration because of improvement of nutrient utilization, modify rumen fermentation and optimize performance in animal production systems. Since the use of chemicals hormones and antibiotics aslivestock feed additive are banned in many countries, "natural" additives such as probiotics, feed enzymes, herbs etc are gaining tremendous importance. These natural feed additives apart from increasing productivity, also reduce the risk oftransfer of potential human pathogens, decrease the antibiotic load and the risk of antibiotic resistance development,and limit excretion of pollutants. Manipulation of rumen microbial ecosystem in favorable direction is still a pertinent goal for animal nutritionist.Natural feed additives show a potential for manipulation of rumen fermentation.The most of importantfeed additives in this direction are "probiotics and enzymes" which have no residual effects. The "probiotics" or direct fed microbial seems to be more natural, restoring the normal and ideal flora in the digestive tract to its full capacity and results in increased animal production without any risk of human health hazards. Among probiotics, *Saccharomyces cerevisiae* (brewers and baker's yeast) and *Lactobacillus acidophilus* (lactic acid producing bacteria) have got maximum attention among nutritionists throughout world. Yeast in ruminants stabilizes the pH of rumen and therefore, favour the growth of cellulolytic

bacteria sensitive to low pH. Oxygen scavenger property of yeast in rumen helps to protect obligate anaerobes from the air ingested in rumen along with feed intake. Increase in production of total VFA, ratio of acetate to propionate and *in vitro* dry matter digestibility was reported in sheep (Ganai et al., 2015). *Lactobacillus bacillus* as a probiotic hassevaral potential benefits like growth promotion of farm animals (Tripathi and Karim, 2009), protection against pathogens (Casas and Dobrogosz, 2000), alleviation of lactose intolerance (Mustapha and Savaiano, 1996), relief of constipation, anticholesterolemic effect, reduction of gut pH by stimulating the lactic acid producing microflora, competition with pathogens for a viable nutrients (Edens, 2003) and immunomodulation (Aottouri et al., 2002).

Usage of exogenous fibrolytic enzymes has been reported to have positive effect on digestion. Many recent studies have reported increased digestion of dry matter and fibre measured *in situ* and *in vitro* on usage of fibrolytic enzymes (Khattab et al., 2013; Giraldo et al., 2014). The extent to which level the probiotic mix and enzyme mix can be incorporated in livestock ration to increase nutrient intake and utilization as well as animal performance has not been well established. Therefore, the present investigation was undertaken to study the effect of probiotics mix and fibrolytic enzyme mix at different levels on *in vitro* nutrient degradability of paddy straw and fermentation pattern in paddy straw-based complete feed using rumen liquor of sheep.

MATERIAL AND METHOD

The study was carried at Division of Animal Nutrition and Mountain Research Centre for Sheep and Goat (MRCSG), Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar. The samples of paddy straw and complete feed were oven-dried, ground and passed through 1mm sieve in a Willy mill and stored in plastic bottles for further use. The proximate constituents of paddy straw, probiotic mix, fibrolytic enzyme mix and complete feed was done as per AOAC (2000) and fibre fractions as per Van Soest et al.(1991). The modified *in vitro* procedure of Tilley and Terry (1963) was used to assess *in vitro* dry matterdegradability (IVDMD), *in vitro* organic matter degradability (IVOMD) and *in vitro* nutrient detergent fibredegradability (IVNDFD) at 48 and 72 hr post-incubation and total gas production at 24 hr post-incubation.

Five levels of fibrolytic enzyme mixture and probiotic mix were selected separately, which were added to fermentation vessels (Jakhmola et al., 2010) containing 500 mg finely ground paddy straw @ 3g (T1), 6g (T2), 9g (T3), 12g (T4), 15g (T5) per kg DM of paddy straw. Three adult male Corriedale sheep were given 20 days adaptation period feeding complete feed containing paddy straw at 50% level to meet nutrient requirements as per ICAR (2013). Rumen fluid was collected from sheep before morning feeding (Solvia and Hess, 2007) by perforated tubing device under negative pressure and late squeezed through four layers of muslin cloth to get inoculums (SRL). The inoculum was transferred to pre-warmed flask (39°C) and flushed with CO_2. SRL (10 ml) was dispensed into pre-warmed (39°C) fermentation vessels with feed additives. One fermentation vessel with only paddy straw was kept as control (T_0). The fibrolytic enzyme mixture (Allenzimix HP) used in the study contains different enzymes like cellulase with 12,00,000U concentration, amylase with 52,50,000U, xylanase with 3,50,000U, Protease with 15,00,000U, Pectinase with 2,50,000U, B-Gluconase with 4,80,000U, Lipase with 120,000U, Phytase with 5,00,000U and Mannase with 100,000U concentration. The probiotic mix contains *Saccharomyces cerevisiae* 2×10^{10}cfu/g and *Lactobacillus acidophilus* 6×10^{9}cfu/g in equal ratio.

40 ml of McDougall buffer is flushed with oxygen free CO_2in each fermentation vessels. The experiment was conducted in completely randomizedblock design and each treatment run in triplicate with negative controls (SRL + buffer alone). The controlswere used to correct for fermentation residues resulting directly from SRL. Each vessel cork fitted withcontrol value was kept in incubator at 39°C for 48 hr. At the end of 48 hr incubation two drops ofsaturated HgCl2 was added in each vessel to stop microbial activity. Contents of each vessel were transferredto 1 L spout-less beakers. The vessels were thoroughly washed with neutral detergent solution and finalvolume made to 150 ml. The contents were refluxed for 1 hr at 100°C, filtered and washed through preweighed Gooch crucible (Grade 1, 50 ml capacity). This undigested residue (NDF) was oven-dried at 100°Cfor 24 hr, cooled in desiccator and weighed. Loss in DM and NDF was digested dry matter and digestedNDF. The crucibles containing residue were ignited in muffle furnace at 500°C and the ash left in crucibleafter ignition was subtracted from residual dry matter to get the organic matter content. For *in vitro* studies at72 hr, incubation of samples was done in same way as for 48 hr incubation. The reaction was stopped byadding 2 ml of 6 N HCl and 0.1 g pepsin powder (1:3000) to each vessel at the end of 48 hr incubation. Thenthe vessels were incubated for another 24 hr and procedure repeated as for 48 hr incubation, except theaddition of HgCl2 at 48 hr.The optimum levels of feed additives, chosen from experiment I, were mixed with finely ground (1 mm) complete feed containing paddy straw and concentrate mixture in 50:50 ratio. The concentrate mixturecontained maize 6, wheatbran 7.6, deoiled rice bran 9, mustardoil cake 10, soyabean 15, molasses 0.8, mineral mixture 0.8 and salt 0.4parts.

The experimental procedures for degradability of nutrients in complete feed were similar to experiment I. The pH in rumen liquor was determined immediately after the termination of incubation at 48hr using portable digital pH meter. Total volatile fatty acids was determined as per method of Barnett and Reid (1957) using Markham still distillation apparatus, total rumen nitrogen as per AOAC (2000) andammonia nitrogen by spectrophotometer method (Chaney and Marbach, 1962).

STATISTICAL ANALYSIS

The data were analyzedstatistically as per Snedecor and Cochran (1994) to draw the inference.

RESULTS AND DISCUSSION

Chemical composition of feed ingredients, supplements and experimental complete feeds

The chemical composition of paddy straw reported is presented in Table 1. Results for chemical composition were in close agreement with observations of Khattab et al. (2013) and Sheikh et al. (2014). Thechemical composition of probiotic mix was in accordance to the existing reports of Rekha et al. (2005) and Ganai et al. (2015). There has been slight improvement in nutrient content (CP, EE, NFE and minerals) of additive supplemented complete feed as also previously reported by Ganai et al. (2015) and Khattab et al. (2013).

Table 1: Chemical composition of paddy straw, complete feed and feed additives.

Chemical components (% DM)	Paddy straw	Complete feed	Probiotics mix	Fibrolytic enzyme mix
DM	84.73	89.53	99.50	98.00
CP	4.85	15.51	40.10	16.20
EE	1.37	3.15	2.5	1.50
CF	35.08	21.64	3.10	-
NFE	48.40	49.06	-	-
TA	10.31	8.64	6.3	-
AIA	4.18	3.31	-	-
NDF	79.90	68.03	-	-
ADF	61.11	42.15	-	-
HC	18.79	25.88	-	-
Cellulose	44.37	34.37	-	-
ADL	10.63	5.49	-	-
Ca	0.38	1.93	1.90	-
P	0.20	0.59	0.80	-

In vitro nutrient degradability and fermentation parameters of paddy straw

The results of *In vitro* nutrient degradability andfermentation parameters of paddy straw are presented in Table 2. There was significant (P<0.01) improvement in degradability of dry matter, OM and NDF as an effect of supplementation of enzyme mix and probiotic mix at various levels and on the basis of overall results, 9 g/kg level for enzyme mix and 3 g/kg DM level for probiotic mix were selected as optimal level. These results are comparable with the earlier report of Ganai et al. (2015) reported improved *in vitro* digestibility of DM, OM, NDF and total gas production in kids supplemented with *Saccharomyces cerevisiae*to bajra straw at dose rate of 10^8 cfu/g. Similar results were found by El-Waziry et al. (2007) recorded improvement in degradability pattern of nutrients either *in vitro* or *in sacco* due to supplementation of yeast culture in groundnut haulms and berseem hay, respectively. Similarly Chandrasekharaiah*et al.* (2016) reported that supplementation of recombinant *Butrivibriofibrisolvens* and yeast mixed culture resulted significantly improved OM and NDF degradibilty of wheat straw when compared to control. Regarding the effect of fibrolytic enzymes, Lamid et al. (2013) stated that addition of lignocellulolytic enzymes @ 5%into rice straw increase digestibility of nutrients and improve *in vitro* rumen fermentation with significantly higher production of volatile fatty acid and ammonia. Similarly Vande and Useni (2012), found increased the *in vitro* DM and NDF disappearances after 36 hours in sheep fed milled substrate consisting of a 50: 50 mixture of lucerne hay (LH) and wheat straw (WS). Mao et al. (2013) conducted *in vitro* gas test to investigate the effects of cellulase (CEL) and xylanase (XYL) on *in vitro* rumen fermentation and microbial population with rice straw as substrate,concluded that the application of CEL and XYL could improve rumen fermentation, increase rice straw digestion and affect the rumen microbial population. Thammiaha et al. (2016) who studied *in vitro* efficacy of lignin peroxidases also reported that it enhanced the digestibility of crop residues when feed to ruminants. Other workers (Chopra et al., 2007;Diaz et al., 2013; Khattab et al., 2013) have also reported improvement in degradability pattern of nutrients in different roughages either *in vitro* or *in sacco* with supplementation of enzymes possibly due to increased microbial count and activity.

In vitro nutrient degradability of experimental complete feeds

Digestibility of nutrients in *in-vitro* system was more pronounced when these optimal levels of feed additives (9g/kg DM of substrate for enzyme mix and 3g/kg DM levels for probiotic mix) alone and in combinations were incorporated in complete feed containingpaddy straw and concentrate mixture at 50:50 ratio was used as substrate (Table 3). Significant increase in degradability of DM, OM and

Table 2: In vitro DM, OM and NDF digestibility of paddy straw at 48 and 72h of incubation (%DM basis)

Feed additive	Hour of Incubation	T_0	T_1	T_2	T_3	T_4	T_5
	IVDMD						
Enzyme mix	48**	36.15±0.02^{a}	37.68±0.11^{b}	38.35±0.02^{c}	40.05±0.02^{e}	40.00±0.02^{e}	39.45±0.08^{d}
	72**	39.14±0.03^{a}	39.85±0.39^{ab}	41.02±0.30^{b}	42.55±0.42^{c}	41.00±0.72^{b}	41.07±0.38^{b}
Probiotic mix	48**	36.15±0.02^{a}	40.00±0.17^{d}	38.57±0.12^{c}	38.40±0.11^{c}	38.45±0.02^{c}	37.90±0.05^{b}
	72**	38.82±0.30^{a}	43.60±0.32^{c}	42.23±0.21^{bc}	41.40±0.90^{b}	42.12±0.30^{bc}	42.90±0.51^{bc}
	IVOMD						
Enzyme mix	48**	39.72±0.90^{a}	42.34±1.04^{ab}	42.03±0.30^{ab}	45.06±0.53^{b}	40.62±1.66^{a}	41.01±1.00^{a}
	72**	40.39±0.73^{a}	44.03±0.67^{c}	43.03±0.48^{c}	46.15±0.07^{d}	42.28±1.38^{ab}	42.04±0.39^{ab}
Probiotic mix	48*	39.72±0.90^{a}	43.31±0.03^{b}	42.21±1.26^{ab}	41.49±0.80^{ab}	41.19±0.07^{ab}	41.41±0.93^{ab}
	72**	40.95±0.82^{a}	45.16±0.33^{b}	41.87±0.57^{a}	42.23±0.78^{a}	42.53±0.38^{a}	42.47±0.30^{a}
	IVNDFD						
Enzyme mix	48**	26.12±0.04^{a}	28.45±0.02^{b}	29.52±0.03^{c}	32.10±0.05^{e}	30.87±0.18^{d}	30.91±0.47^{d}
	72**	29.46±0.27^{a}	32.47±0.04^{bc}	32.19±0.92^{b}	34.22±0.05^{c}	33.20±0.15^{bc}	32.91±0.90^{bc}
Probiotic mix	48**	26.12±0.04^{a}	31.20±0.72^{d}	30.21±0.14^{cd}	29.30±0.11^{bc}	28.68±0.16^{b}	29.37±0.63^{bc}
	72**	28.12±0.56^{a}	33.18±0.13^{d}	31.38±0.63^{bc}	30.96±0.21^{bc}	32.02±0.27^{cd}	30.51±0.47^{b}

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

Table 3: In vitro DM, OM and NDF digestibility of complete feed at 48 h of incubation (%DM basis) along with pH, TVFA, NH_3-N and Total-N.

Parametrs	C_0 Control	C_1 Probiotic mix	C_2 Enzyme mix	C_3 Enzyme+ probiotic mix
		In vitro digestibility (%)		
IVDMD**	40.27±0.02^{a}	42.47±0.03^{c}	41.80±0.11^{b}	44.17±0.03^{d}
IVOMD*	44.48±0.90^{a}	47.08±1.04^{b}	46.79±0.30^{b}	49.80±0.53^{c}
IVNDFD**	30.34±0.44^{a}	33.75±0.30^{c}	32.66±0.03^{b}	36.31±0.05^{d}
		Fermentation attributes		
pH**	6.23±0.04^{a}	6.77±0.02^{b}	6.21±0.03^{a}	6.89±0.06^{b}
TVFA (mEq/l)**	72.74±0.52^{a}	79.81±0.74^{b}	79.65±0.91^{b}	83.95±1.40^{c}
NH_3-N (mg/dl)	18.36±0.66	17.61±0.52	17.42±0.63	16.73±0.29
Total-N(mg/dl)**	107.42±0.57^{a}	115.68±0.89^{b}	115.87±0.33^{b}	117.74±1.50^{b}

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

NDF and rumen fermentation parameters were reported in supplemented feeds (C_1 and C_2) than the control (C_0) and the effect was more pronounced in combination group (C_3). These results are in line with the earlier report of Ganai et al. (2015) where in higher digestibility of DM, OM, NDF and total gas production values at supplementation of yeast to bajra straw based complete feed using goat rumen liquor in *in vitro* study. Other workers (Malik and Singh, 2009) have also reported improvement in degradability pattern of nutrients either *in vitro* or *in sacco* due to supplementation of yeast culture. Regarding the supplementation of fibrolytic enzymes, Thakur et al. (2008) reported an increase in DM and NDF degradability in total mixed rations having different roughage and concentrate ratios (75:25, 60:40 and 50:50). Ganai (2011) also reported improved digestibility of DM, OM, NDF and total gas production showing maximum values at supplementation of exogenous fibrolytic enzyme at dose rate of 2 g/kg DM. An increase in IVDMD, IVDOMD and TGP were reported as an associative effect of enzyme and yeast when supplemented together in cereal straws by Tang et al. (2008). The results of experiment undertaken in phase-I, taking into account the digestibility at 48 and 72 h incubation revealed that *in vitro* DM, OM and NDF utilization from complete feed could be improved by supplementation of feed additives at optimal level alone and

in combination.

There has been significant effect of supplementation of feed additives on the fermentative metabolites (Table 3). The supplementation of probiotic mix alone or in combination with enzyme mix, to paddy straw based complete feed had significant effect on pH of *in vitro* medium. The results reported *in vitro* studies suggested that addition of probiotic mix alone as well as in combination with fibrolytic enzymes modify rumen pH. However, fibrolytic enzymes had no effect on the pH of *in vitro* medium. These results are in accordance with reports of Kamara et al. (2002) and Garg et al. (2009) who suggested that supplementation of yeast causes an elevation in pH possibly due to utilization of lactic acid from ruminal contents, there by stabilizing pH (Dawson and Tricarica, 2002).

In contrast, some researchers have observed either no effect or even reduction in rumen pH upon yeast supplementation (Brossard et al., 2006; Ganai et al., 2015). Ganai (2011) had also reported no effect of enzyme supplementation on *in vitro* rumen pH. The variation may be attributed to different experimental conditions related to animal (species, physiological stage and level of intake), diet (composition and mode of distribution), experimental design (group or latin square) and probiotic mix (strain, dose, combination and viability).

The TVFA concentration was significantly (P<0.01) higher in feed additive supplemented complete feed as compared to un- supplemented feed, which indicated the stimulatory effects of probiotic mix and fibrolytic enzymes on fibre-degrading micro-organisms, facilitating availability of more energy. An increase in TVFA concentration upon supplementation of yeast has been reported (Garg et al., 2009; Ganai et al., 2015). Chademana and Offer (1990) reported that *S. cerevisiae*had no effect on total or individual VFAs, but others found stimulation in the proportion of propionate at the expense of acetate (Newbold et al, 1990) or even an increase in the proportion of acetate (Mustsvangwa et al., 1992). The highly variable nature of the responses in VFA proportions to probiotic mix supplementation lead to the suggestion that the effect of yeast on microbial numbers in the rumen rather than a direct effect, which might explain the gains observed in productivity (Wallace and Newbold, 1993). Regarding fibrolytic enzymes Lamid et al. (2013) reported that addition of lignocellulolytic enzymes @ 5%into rice straw improves *in vitro* rumen fermentation with significantly higher production of volatile fatty acid and ammonia. Mao et al. (2013) also reported that addition of cellulase and xylanase increased *in vitro* gas production and total volatile fatty acids with reduced ammonia nitrogen. The higher (P<0.01) level of total N upon probiotic mix and enzyme supplementation alone and in combination may be attributed to possibly higher proteolytic activity. Yoon and Stern (1996) reported similar proteolytic activity. The present results are in consistent with earlier reports of Garg et al. (2009). The concentration of rumen ammonia nitrogen was numerically lower in feed additive supplemented groups as compared to un-supplemented group but the difference was statistically non significant. The lower concentration of NH_3-N in feed additive supplemented animals may be associated with increased uptake and assimilation of NH_3-N by rumen microbes due to stimulation of bacterial growth. There have been variable earlier reports of different researchers (El-Ghani et al., 2004; Garg et al., 2009) reports reduced NH_3-N concentration (Oeztuerk, 2009) reports no effect on NH_3-N concentration and Sales (2011) and Ozsoy et al. (2013) reports increased NH_3-N concentration due to yeast supplementation. Torres et al. (2013), Mao et al. (2013) and Tang et al. (2013) reported that enzymatic treatment tends to linearly increase N-NH_3 concentration.

The study revealed a significant improvement in digestibility of nutrients and rumenfermentation in *in-vitro* rumen fluid of sheep as an effect of supplementation of probiotics mixand fibrolytic enzyme mixture supplementation with maximum values at 3g and 9g kg-1 DM respectively. However, *in vivo* trial is required before advocating these findings to sheep raisers.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors and has no financial repercussions.

AUTHORS CONTRIBUTION

All the authors contributed significantly to the paper. Author along with Ahmad and Afzal carried the trial and compiled the results. AM Ganai and HA Ahmed designed the protocol and helped in statistical analysis.

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