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Effect of exogenous lignolytic enzyme-treated ragi straw on DM intake, digestibility, rumen fermentation and rumen enzymes in sheep

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ABSTRACT

The present study was conducted to evaluate the effect of exogenous lignolytic enzymes harvested from immobilized *Coriolus versicolor* and *Ganoderma lucidium* on body weight, *in vivo* digestibility, rumen fermentation and rumen enzymes in sheep. Four groups of sheep (6-each) were fed 350 g concentrate mixture to meet the energy and protein requirement as per ICAR standards. The control sheep received *ad lib.* ragi straw treated with production media devoid of enzyme (G1). Test group 1 (G2) received *ad lib.* ragi straw treated with *C. versicolor* enzyme media (Enz.1) in a 1:2.5 (w/v) ratio, group 2 (G3) received *ad lib.* straw treated with *G. lucidium* enzyme media (Enz.2) in a 1:2.5 (w/v) ratio. Group 3 (G4) received *ad lib.* amount of straw treated with a combination of Enz.1 and Enz.2 in an equal volume in a 1:2.5 (w/v) ratio. After 40 d feeding an ADG (gd^{-1}) of 112.5 and 107.5 was recorded in G2 and G3 as compared to 97.5 of control. A 5% increase in dry matter digestibility (DMD) of 77 .36±4.28 and 77.04±5.69% was obtained in G2 and G3. Treatment of straw with a combination of enzymes (G4) failed to show increase in either ADG or DMD. Rumen fermentation pattern did show any significant difference. The fiber degrading enzymes elicited enhancement of activity in sheep fed the supplemental enzymes. Applying lignin degrading enzymes as feed supplements for enhancing digestibility of crop residues holds promise in the immediate future.

Key words: Digestibility, Enzyme treatment, Lignolytic, Rumen enzymes, White rot fungi

The complete utilization of energy in crop residues by ruminants is limited on account of their high lignin content which impairs digestibility. Rumen microbes lack the enzyme machinery capable of decomposing lignocellulosic material. Studies (Hristov *et al.* 2000, Nsereko *et al.* 2002) conducted in cattle showed that enzyme supplementation can influence ruminal variables, fibrolytic activity of ruminal fluid, and microbial populations, but the effects seem to be dependent on the type of supplemented enzyme. The white rot fungi (WRF) are the most efficient to degrade lignin on account of their ligninolytic enzymes and predigestion of ligno-cellulosic materials with these enzymes enhances their digestibility for ruminants (Sridhar *et al.* 2014, Arora *et al.* 2002). Thus in the present study, we evaluated the effect of feeding exogenous lignolytic enzymes obtained from WRF on *in vivo* digestibility of ragi straw in sheep.

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MATERIALS AND METHODS

White rot fungi: The strains of white rot fungi (WRF), viz. *Coriolus versicolor* (*Polystictus versicolor*-NCIM1076) and *Ganoderma lucidum* (Leysser) Karsten -NCIM1091 were obtained from the National Collection of Industrially Important Organisms (NCIM), Pune. The stock culture was maintained on potato dextrose agar (PDA) media at 4ºC and sub cultured every five days. Young cultures were grown on potato dextrose broth for 8 days at 39 ºC. The fungal biomass from these was used as the inoculum for immobilization.

Production of lignolytic enzymes: Both the WRF were used for production of the lignolytic enzymes in submerged batch agitation culture in cotton-plugged 500 ml Erlenmeyer flasks containing 250 ml of production medium. The biomass of each of the fungi was individually homogenized and the culture used as the initial inoculum for immobilization. The production medium consisted of (g/ 100 ml of distilled water): 1.0 g glucose, 0.5 g yeast extract, 0.5 g urea (90 ml), and 10 ml of salt solution, pH 5.6. *G. lucidum and C. versicolor* were immobilized on polyurethane foam (PUF) cubes of 1.5 cm × 1.5 cm ×1.5 cm as per Krishna Prasad *et al.* (2005) with minor modifications. The enzyme rich media was harvested every

seventh day individually for both the fungi, accumulated and was used for treating the weighed quantity of ragi straw of 2.3 cm length at a concentration of 1:2.5 by spraying (selected from a range of doses tested for *in vitro* dry matter digestibility). Each day the treatment was carried out so that the required quantity was available for feeding sheep the next morning .

Experimental animals and feeding protocol: Twenty four Bannur male sheep aged around 2½ year and weighing around 23–25 kg were used in this study. The animals were healthy as verified by the clinical history and physical. They were fed with 350 g concentrate mixture to meet the energy and protein requirement as per ICAR standards. The control sheep received *ad lib.* ragi straw treated with production media devoid of enzyme (G1). Test group 1 (G2) received *ad lib.* ragi straw treated with *C. versicolor* enzyme media (Enz.1) in a 1:2.5 (w/v) ratio, group 2 (G3) received *ad lib* straw treated with *G. lucidium* enzyme media (Enz.2) in a 1:2.5 (w/v) ratio. Group 3 (G4) received *ad lib.* amount of straw treated with a combination of Enz.1 and Enz.2 in an equal volume in a 1:2.5 (w/v) ratio. The feeding trial was conducted for 40 d. Body weight changes were recorded weekly and daily feed intake was monitored. The rumen liquor was collected at 0 and 4h from all groups during the fourth week of feeding by the help of a oral tube and vacuum pump, pH noted, filtered and then centrifuged (10,000 × g for 15 min at 40°C) and stored at –80°C for estimation of enzymes. The DMI was recorded during the digestibility trial and nutrient digestibility was estimated.

Analytical methods and enzyme assays: Proximate principles (AOAC 1995) and detergent fiber was estimated (Van Soest *et al.* 1991) in the straw and concentrate. Ammonia-N (Conway 1962) TVFA concentration (Barnett and Reid 1957) were estimated in rumen liquor. Ciliate protozoa were determined microscopically with the aid of a Fuchs- Rosenthal haemocytometer in MFS (methylgreen/ formalin/ salt) fixed preparations of strained rumen fluid (Onodera *et al.* 1977). Activities of carboxymethylcellulase (CM Case), micro crystalline cellulose (MCC) and xylanase were estimated as per Agarwal *et al.* (2000) along with amylase (Safarik 1991), ß-glucosidase (Shewale and Sadana 1978) and acetyl esterase (Martínez *et al.* 2007). Total proteolytic activity was measured by the procedure of Blackburn (1968) and total protein as per Lowry *et al.* (1951). All assays were performed in 3 replicates.

Statistical analysis: The data on various parameters were tabulated, mean values were calculated and deviations from the means were calculated as standard deviations of the means according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

The use of plant cell wall degrading enzymes as direct fed supplements in ruminant diets has stimulated considerable research effort in recent years (Beauchemin *et al.* 2003, Colombatto *et al.* 2003). However, results were inconsistent with many factors contributing to this variability. The potential of preparations containing exogenous endoglucanase and xylanase enzymes in increasing the performance of lambs (Rojo *et al.* 2005), ewes (Titi and Lubbadeh 2004, Giraldo *et al.* 2008), and goats (Titi and Lubbadeh 2004) were investigated. As far as our knowledge goes this is the first study with regard to feeding exogenous lignolytic enzymes harvested from WRF in sheep.

The composition of the straws showed that the crude protein content of ragi straw was enhanced by treatment with the lignolytic enzyme rich media (Table 1). The sheep of all groups readily consumed the enzyme treated straw. Concentrate feeding met the deficiency which would have arisen out of feeding sole straw based diets. After 40– days of feeding G2 and G3 showed the maximum ADG (Table 2). The DMI was highest in G3 and DMD increased in G2 and G3, compared to control (P>0.05). Treatment of straw with a combination of enzymes failed to reflect any significant change in digestibility.

The pH of the rumen liquor of sheep fed treated straw was higher (P>0.05) at 0 h and 4 h in comparison to control (Table 3). No significant change was recorded in the TVFA of the rumen fluid collected at 4h post feed offer. NH3-N and protozoa count increased in G2 and G4, showing a mild effect of the lignolytic enzyme treatment on rumen fermentation (Table 3).

The enzymes estimated in the present study are the major enzymes responsible for the degradation of lignocellulosic feed. Activities of all the enzymes (Table 4) were observed to be higher at 0 h than at 4 h post feeding. In the strategy for fiber degradation this group of enzymes is focused mainly on oligosaccharides. The composition of diet is one

Table 1. Proximate composition (%) of the straw and concentrate used for feeding the control and enzyme treated group of sheep (40 days trial)

Groups	Protein	NDF	ADF	ADL	Ash	DM	OM
G1	3.26±0.95^{d}	79.49±0.11^{b}	50.89±1.02^{a}	5.78±0.08^{a}	7.38±0.11^{ab}	91.8±0.21^{ab}	84±0.46^{b}
G2	4.09±0.32^{ab}	79.35±0.18^{b}	48.33±0.34^{ab}	5.51±0.33^{a}	7.21±0.05^{b}	93.3±0.2^{a}	86±0.02^{a}
G3	4.32±0.03^{a}	78.28±0.1^{a}	46.91±0.01^{b}	4.89±0.01^{b}	7.56±0.01^{a}	93.4±0.19^{a}	86±0.29^{a}
G4	3.69±0.29^{c}	78.5±0.51^{a}	47.94±0.64^{b}	5.49±0.40^{ab}	7.87±0.08^{a}	91.7±0.4^{ab}	83.8±0.08^{b}
Concentrate	26.41±4.30	66.19±3.66	11.56±0.05	1.15±0.08	6.76±0.12	94.36±0.22	87.8±0.09

G1, Control sheep; G2, Enz.1 in a 1:2.5 ratio; G3, Enz.2 in a 1:2.5 ratio; G4, combination of Enz.1 and Enz.2 in equal volume in a 1:2.5 ratio; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; DM, dry matter; OM, organic matter. Values are means±SE; a, b, means in the same row with different superscripts are significantly different ($p < 0.05$).

Table 2. Changes in body weight, dry matter intake, dry matter digestibility in control and treated group of sheep (40 days trial)

Parameters	G1	G2	G3	G4
Initial weight	24.5±2.95	23.95±4.64	24.73±1.47	23.93±2.19
Final weight	28.44±3.15	28.46±4.48	28.25±1.36	27.8±1.99
Total gain	3.94^{ab}	4.51^{a}	3.52^{ab}	3.87^{ab}
ADG (g/d)	97.5^{c}	112.5^{a}	107.5^{b}	97.6^{c}
DMI (g/d)	639.22	661.97	691.61	561.52
	±37.97^{b}	±25.82^{ab}	±30.05^{a}	±23.27^{c}
DM (%)	46.1±8.1^{b}	47.7±6.0^{b}	44.9±9.0^{bc}	53.0±10.9^{a}
DMD (%)	71.71	77 .36	77.04	69.56
	±7.25^{a}	±4.28^{a}	±5.69^{a}	±9.01^{a}

G1, Control sheep; G2, Enz.1 in a 1:2.5 ratio; G3, Enz.2 in a 1:2.5 ratio; G4, combination of Enz.1 and Enz.2 in equal volume in a 1:2.5 ratio; ADG, average daily gain; DMI, dry matter intake; DM, dry matter; DMD, dry matter digestibility. Values are means±SE; a, b, c means in the same row with different superscripts are significantly different (P < 0.05).

used as substrate for enzyme treatment in this work, and being solely a straw based diet high endoglucanase and xylanase activity obtained are in keeping with earlier observations.

Most commercially available enzymatic products so far tested for ruminants, were not designed specifically for this purpose. Mostly enzymatic preparations containing cellulases and xylanases for use in the food, pulp, paper, textile, fuel and other chemical industries (Beauchemin *et al.* 2003), while those containing fibrolytic enzymes developed originally as silage additives (Feng *et al.* 1996) have been used. Efficiency of microbial synthesis and activity of ß-1, 4-endoglucanase (cellulase) studied by Martins *et al.* (2006) upon supplementation with fibrolytic enzymes in animals fed diets containing corn silage and Tifton 85 hay (*Cynodon* spp.) failed to show effect on the ruminal variable except for greater ß -1, 4-endoglucanase activity which was attributed to the addition of enzymes. According to Morgavi *et al.* (2000a), commercial

Table 3. Changes in the rumen fermentation profile of control and treated group of sheep (40 days trial) at 0 and 4h of feeding straw treated by spraying with lignolytic enzymes

Parameters	Rumen fermentation profile in control and three treatment groups of sheep							
	G1		G2		G3		G4	
	0 h	4h	0h	4h	0 h	4h	0h	4h
pH	6.67±0.22^{a}	6.5±0.26^{a}	6.8±0.20^{a}	6.8±0.14^{a}	7.0±0.09^{a}	6.7±0.23^{a}	6.8±0.33a	6.5±0.08^{a}
TVFA	7.5±1.13 ^{a}	10.1±0.53^{a}	6.9±0.81 ^{a}	8.6±1.05 ^{a}	7.7±0.61 ^{a}	8.4±1.48 ^{a}	10±0.80 ^{ab}	10.1±2.05 ^{a}
NH_3-N	21.7±1.47 ^{a}	21.5±6.05 ^{a}	22.6±1.64 ^{a}	24.0±5.63 ^{a}	25.2±4.06 ^{a}	27.8±6.83 ^{a}	28.7±2.62 ^{a}	28.2±4.72 ^{a}
Protozoa	0 h	4h	0h	4h	0 h	4h	0h	4h
Oligotricha small	0.24±0.003	0.719±0.008	0.11±0.001	0.821±0.009	0.324±0.004	0.584±0.006	0.424±0.005	0.816±0.009
Oligotricha big	0.023±0.000	0.069±0.001	0.004±0.000	0.16±0.002	0.037±0.000	0.122±0.001	0.019±0.000	0.101±0.001
Holotricha Iso	nil	0.006±nil	nil	0.073±0.001	nil	0.027±nil	0.001±0.000	0.009±nil
Holotricha Dasy	nil	0.004±nil	nil	0.003±nil	nil	0.002±nil	nil	nil
Total	0.264±0.003^{a}	0.802±0.009^{a}	0.114±0.001^{a}	1.058±0.012 ^{a}	0.362±0.004^{a}	0.734±0.008 ^{a}	0.444±0.005 ^{a}	0.926±0.001 ^{a}

G1, Control sheep; G2, Enz.1 in a 1:2.5 ratio; G3, Enz.2 in a 1:2.5 ratio; G4, combination of Enz.1 and Enz.2 in equal volume in a 1:2.5 ratio; TVFA, m mol dL-^{1}; NH_3-N-mg dL-^{1}; protozoa counts, 10^5. Values are means±SE; a, b means in the same row with different superscripts are significantly different (P < 0.05).

of the most important factors which influence the relative concentration of different microbes present in the rumen. These microbes produce a wide range of active hydrolytic enzymes, notably cellulases and xylanases, that provide them with the potential to degrade the major structural polysaccharides in plant cell walls. Very high activities of xylanase and CM cellulases were obtained in sheep fed enzyme treated straw as compared to sheep fed untreated straw (G1).

Protease activity elicited an increase in G2 and G3, the groups of sheep fed straw treated with enzymes from individual fungi. Activities of ß glucosidase, acetyl esterase also showed enhanced activities in group of sheep fed treated straws (Table 4). A depression in carboxy methyl cellulase and xylanase activities and increase in amylase activity was observed on a shift from forage based diet to high grain based diet (Hristov *et al.* 2000). Ragi straw was

preparations of enzymatic complexes normally are standardized according to their capacity to degrade cellulose or xylan and particular enzyme activities may, however, be more labile than others (Morgavi *et al.* 2000b). If such an activity were a key component of the mode of action of feed-additive enzymes for ruminants, it would appear likely that enzyme activity might be protected by simple means. The enzyme preparation used by Giraldo *et al.* (2008) was a commercial product from fermentation extracts of *Aspergillus niger* and *T. longibrachiatum.* contained endoglucanase and xylanase activities and was delivered directly into the rumen. Treatment of ragi straw with enzymes from *C. versicolor* and *G. lucidum* in the present study improved digestibility and body weight gain in sheep and caused favorable changes in the rumen enzyme profile. Applying lignolytic enzymes as feed supplements hold promise for achieving enhanced digestibility of crop

Table 4. Changes in the rumen enzymes at 0 and 4h of feeding straw treated by spraying with lignolytic enzymes

Enzymes	G1		G2		G3		G4	
	0 h	4h	0h	4h	0 h	4h	0h	4h
[1]CMC	0.1242±0.03^{c}	0.0213^{a}	0.1457±0.04^{c}	0.0713±0.03^{a}	0.1407±0.03^{c}	0.0516±0.07^{a}	0.1549±0.02^{c}	0.1017 ±0.06^{b}
[1]MCC	0.0471±0.02^{c}	0.0136^{b}	0.0579±0.02^{c}	0.0014^{a}	0.0973±0.04^{cd}	0.0110^{b}	0.1035±0.03^{d}	0.0018^{a}
[1]Amylase	0.9651±0.130^{bc}	0.3903±0.47^{a}	1.0700±0.17^{d}	0.5610±0.23^{a}	0.9969±0.21^{b}	1.0295±0.34^{c}	0.8945±0.14^{b}	0.9443 ±0.40^{b}
[2]Xylanase	59.440±5.25^{b}	22.617±0.02^{a}	62.444±11.17^{b}	36.165±8.82^{a}	65.498±6.25^{bc}	32.444±12.12^{a}	59.457±7.02^{b}	36.724 ±22.01^{a}
[1]FPase	0.0692±0.01^{b}	0.0198^{a}	0.1102±0.04^{c}	0.0572±0.02^{b}	0.0541±0.24^{b}	0.0120^{a}	0.0216±0.02^{b}	0.0195 ±0.02^{a}
[3]Protease	2.146±0.89^{b}	1.507±0.91^{a}	5.668±6.83^{c}	1.280±0.71^{a}	3.709±2.93^{bc}	2.984±1.58^{b}	2.716±1.60^{b}	1.428 ±1.03^{a}
[4]Acetyl Esterase	0.0366±0.002c	0.0998±0.004^{d}	0.0630±0.002^{cd}	0.0831±0.032^{d}	0.0002^{a}	0.0448±0.008^{c}	0.0026^{b}	0.0352 ±0.005^{c}
[4]ß-D-Glucosidase	0.1688±0.02^{c}	0.0833±0.07^{ab}	0.1689±0.08^{c}	0.0464±0.04^{a}	0.3150±0.02^{d}	0.1138±0.08^{b}	0.1797±0.06^{c}	0.0415 ±0.05^{a}
[5]Protein	1.7736±0.24^{ab}	1.2873±0.72^{a}	2.1581±0.27^{b}	1.4187±0.14^{a}	2.4559±0.48^{bc}	1.4032±0.57^{a}	2.1307±0.34^{b}	1.0350 ±0.48^{a}

G1, Control sheep; G2, Enz.1 in a 1:2.5 ratio; G3, Enz.2 in a 1:2.5 ratio; G4, combination of Enz.1 and Enz.2 in equal volume in a 1:2.5 ratio.[1]One unit of enzyme activity expressed as ng of glucose liberated mLmin^{-1}, [2]One unit of enzyme activity expressed as ng of xylose liberated mLmin^{-1}, [3]One unit of enzyme activity expressed as ng of casein hydrolyzed mLmin^{-1},[4]One unit of enzyme activity expressed as microgram of para nitro phenol liberated mLmin^{-1}, [5]Protein expressed as µg min^{-1},Values are means±SE; a, b, c means in the same row with different superscripts are significantly different ($P < 0.05$).

residues for ruminants in the immediate future. Studies with regard to dose and form of enzyme (liquid, powder etc.) to be administered to obtain effective degradation of lignin coupled with enhanced digestibility are further warranted.

From our results it can be concluded that treatment of coarse roughages such as ragi straw with lignolytic enzyme rich media from white rot fungi improves digestibility and performance of sheep. Applying enzymes as feed supplements promises to be a simple technology for achieving enhanced digestibility of crop residues in the near future. More detailed studies using purified enzyme preparations would further enhance our understanding of the mode of action of these enzymes and result in production of nutritionally improved feeds and feed ingredients for ruminants.

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