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Effect of exogenous fibrolytic enzymes cocktail incorporation on *in vitro* degradation and fermentation characteristics of oats straw based total mixed ration

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ABSTRACT

The study was conducted to evaluate the effects of increasing doses: 0 (control), 0.20, 0.40, 0.60, 0.80 and 1.00 % DM of an exogenous fibrolytic enzymes (EFE) cocktail preparation on *in vitro* gas production (GP), nutrient degradability and fermentation characteristics of oats straw based Total Mixed Ration (TMR) with 60:40 roughage to concentrate ratio using sheep rumen liquor. The chemical composition of all the feed ingredients used for preparation of the experimental TMR (containing 16.63% crude protein and 90.51% organic matter) were within the normal ranges. Increasing the incorporation level of enzyme cocktail linearly as well as quadratically increased net GP, metabolisable energy content, short chain fatty acid concentrations and microbial crude protein production up to 0.60% DM level (L3) with no additional improvement at further higher levels. There were significant improvements in degradability of dry matter, organic matter and neutral detergent fibre up to the enzyme dose of 0.60% DM (L3) with constant values thereafter. Fermentation characteristics response to varying incorporation doses of EFE cocktail also revealed improvements up to 0.60% DM level (L3) with no effect on non-protein nitrogen contents. It is recommended that EFE cocktail incorporation dose of 0.60% DM to be used for efficient utilisation of oats straw based complete feed; however, this requires further testing by *in vivo* studies.

Keywords: Complete feed, Fermentation kinetics, Fibrolytic enzymes, *In vitro* assay, Nutrient degradability

Fibrous crop residues like straws, stovers and other agro-industrial byproducts form bulk and indispensable part are used as source of roughage in ruminant feeding throughout world particularly in developing countries like India (Beigh *et al*. 2017). However, their utilization efficiency in ruminants is limited owing to incomplete ruminal digestion of plant cell walls due to presence of complex links limiting the degradation of nutritional compounds (Kumar *et al*. 2022), therefore it cannot support even the maintenance nutrient requirement of the animals. There is a growing need to improve the feeding value of locally available fodder resources for supplementary feeding. To optimize the nutrient availability from these feedstuffs, incorporation of biologically active enzymes as animal feed additives in ration of farm animals has received considerable attention.

Exogenous Fibrolytic Enzymes (EFE) applications have shown to increase degradation of animal feeds *in vitro* (Eun *et al*. 2007, Wang *et al*. 2012, Abid *et al*. 2022), *in situ* (Salvo *et al*. 2022, Tirado-Gonzalez *et al*. 2017, Bassiouni *et al*. 2011) as well as *in vivo* (Beauchemin *et al*. 2003 Bala *et al*. 2009, Pech-Cervantes *et al*. 2021), although results with EFE addition are variable and somewhat inconsistent (Sujan and Seresinhe 2015), making their biological response difficult to predict. Responses to enzyme addition are non linear, as some studies have shown substantial improvement of nutrient digestibility (Pech-Cervantes *et al*. 2021), while others reported either negative effects (Baloyi 2008) or none at all (Tseu *et al*. 2022). Moreover, the optimum dose level for EFE addition to improve fibre utilization by ruminants is not yet well established. These inconsistent responses to the addition of enzymes indicate that further studies are required to demonstrate the effects of EFE on *in vitro* kinetic parameters. It is important to assess the optimum level of EFE for crop residues based diets before the technology can be used cost effectively in ruminant rations for bringing out consistent improvement in utilization of high roughage based diets. The present investigation was undertaken with the aim to study the effect of EFE cocktail addition at different levels on *in vitro* nutrient degradability and fermentation characteristics of oats straw based total mixed ration (TMR) using rumen liquor of sheep.

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MATERIALS AND METHODS

Experimental feed additive and basal diet: Prior to use in the current study, the EFE cocktail preparation (ALLENZIMIX-EP, supplied by Mitushi Pharma, Ahmedabad, Gujarat, India) was assayed for its enzyme activities according to the methods of Valdes *et al*. (2015). Analysis showed its enzyme activity as: 800000 IU/g cellulase, 400000 IU/g phytase, 450000 IU/g β-glucanase, 400000 IU/g xylanase and 350000 IU/g pectinase obtained through an anaerobic fermentation process.

The basal TMR for *in vitro* assay was based on oats straw (*Avena sativa*)-40 parts, mixed grass hay-20 parts and concentrate mixture-40 parts formulated with roughage: concentrate ratio of 60:40 to meet nutrient requirements (ICAR 2013b) recommended for growing sheep. Mixed grass hay comprised of clover (*Trifolium repens*) and rye (*Lolium perenne*) in equal proportions (1:1 ratio). The basal TMR was added with EFE cocktail at five different levels of 0.20 (L_1), 0.40 (L_2), 0.60 (L_3), 0.80 (L_4) and 1.00 (L_5) per cent substrate dry matter (DM), while the basal feed without additive (L_0) served as control.

In vitro assay: The study was taken up to determine the dose response effect of incorporating EFE cocktail as feed additive on nutrient degradability and fermentation pattern to select the effective dose that could be recommended for *in vivo* applicability. Around 250 mL of rumen liquor was collected in early morning before feeding through a stomach tube (FG-20) from each of the three adult donor Corriedale sheep fed *ad lib.* on a diet of oats straw and concentrate mixture in a ratio of 60:40 with free access to fresh water. The collected rumen liquor were mixed on a volume basis, and filtered through four layers of cheesecloth. The incubation inoculum was prepared by diluting the rumen liquor in a 1:4 (v/v) ratio as per Tilley and Terry (1963) method with a buffer medium (consisted per liter of 0.45 g dipotassium phosphate, 0.45 g potassium dihydrogen phosphate, 0.9 g ammonium sulfate, 0.12 g calcium chloride dihydrate, 0.9 g sodium chloride, 0.19 g magnesium sulfate heptahydrate, 1.0 g trypticase peptone, 1.0 g yeast extract, and 0.6 g L-cysteine hydrochloride, which was autoclaved for 15 min at 121°C while adjusting its *p*H to 6.9 using 10 N sodium hydroxide solution). Mixed inoculum was stirred in a water bath at 39°C with purging CO_2 until its use (10-15 min later). In 100 mL sterile pre-warmed incubation flasks fitted with calibrated syringes, 500 mg (<1 mm ground) of each sample was placed without (L0) or with 1 (L1), 2 (L2), 3 (L3), 4 (L4) and 5 (L5) mg EFE cocktail to which 40 mL of the incubation inoculum was added. The flasks were stoppered with Bunsen valves and incubated for 48 h at 39°C. Flasks were gently swirled every 8 h by hand. Each sample was incubated in four replicates.

At the end of the 48 h of the incubation period, flask contents were acidified using 6 M HCL to reach a final *p*H of 1.3-1.5. After a few seconds, when the foam subsided, pepsin powder was added to a final concentration of 0.2% (w/v). The flasks were re-incubated for an additional 48 h period at the end of which the fermentation process was stopped by swirling the flasks in ice. The flasks were then uncapped, contents were transferred into tubes and centrifuged at 2500 rpm for 15 min to obtain supernatant for estimation of fermentation parameters and pellet as non-fermented residue for determination of degraded substrate.

The *p*H was measured from supernatant immediately after centrifugation using a portable digital *p*H meter (ECOPHTEST 1, EuTech Instruments, India). The pellets were dried in a forced air oven at 65°C overnight to determine the residual DM weights. Then, to determine ash content, the residues were kept at 600°C for 1 h to estimate organic matter (OM). Besides, NDF content of the residue left after 48 h incubation was estimated. *In vitro* DM, OM and NDF degradability were calculated, respectively as the DM, OM and NDF which disappeared from the initial weight inserted into the respective flask. *In vitro* gas production was measured at 24 h post-incubation by syringes fitted to the incubation flasks.

Sample analysis and calculations: Samples of feeds and basal diets were dried, ground and subjected to different chemical analysis in triplicate according to AOAC (2000). Also, the non-fermented residues left after 48 h of incubation were analysed for DM, OM and NDF contents. Nitrogen content was estimated by Kjeldahl method (AOAC 2000). Concentrations of cellulose were determined as per Crampton and Maynard (1938), while neutral detergent fibre (NDF) and acid detergent fibre (ADF) as per Van Soest *et al*. (1991).

Metabolizable energy (ME, MJ/kg DM) content of the diets was estimated according to Menke *et al*. (1979). The partitioning factor at 24 h of incubation (PF_{24}) and microbial CP biomass production (MCP) were calculated according to Blummel *et al*. (1997). Net gas production (NGP) for the incubated dietary samples was determined by subtracting the average blank gas volumes from the cumulative gas volumes of each sample. Gas yield (GY_{24}) was calculated as the volume of gas (mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g) as per Elghandour *et al*. (2013). Short chain fatty acid concentration (SCFA) was calculated according to Getachew *et al*. (2002).

The supernatant samples left after separation of non-fermented residues from incubation media were preserved after adding few drops of saturated solution of mercuric chloride and kept in labeled polypropylene bottles at -20°C for further analysis. The samples were analysed for total volatile fatty acids (TVFA) according to the method of Barnett and Reid (1957) using Markham steam distillation apparatus, total nitrogen (Total-N) and tricarboxylic acid-precipitable nitrogen (TCA-ppt-N) by micro-Kjeldahl method as per AOAC (2000), and ammonia nitrogen (NH_3-N) as per Weatherburn (1967). Non-protein-N (NPN) was calculated by subtracting the TCA-ppt-N from total-N contents.

Statistical analysis: The *in vitro* experiment was

completed in one run, using single ruminal inoculum (a pool from three animals) for all the levels of EFE cocktail. Each level (statistical treatment) was incubated in quadruplet (statistical replicates) which added upto 24 flasks for all the levels (4 flasks × 6 TMR's). The orthogonal contrasts were designed to test linear and quadratic responses of the degradability parameters and fermentation characteristics to gradual increasing levels of EFE using statistical software program SPSS (version 20.0) for Windows (SPSS Inc., Chicago, Illinois, USA). Any p value less than 0.05 ($p<0.05$) was taken as statistically significant.

RESULTS AND DISCUSSION

Chemical composition: The chemical composition (on % DM basis) of experimental basal TMR along with few individual feed ingredients and the feed additive are presented in Table 1. The chemical composition of oats straw was comparable to those of the earlier reports by Afzal *et al*. (2009). The basal TMR contained 16.63% crude protein and 90.51% OM which is comparable with the findings of Sachan *et al*. (2014). The crude protein and OM content in EFE cocktail were 16.84 and 100.00%, respectively which are in agreement with the reports of Ganai *et al*. (2011). The chemical composition of all the feed ingredients used for preparation of the experimental TMR under present study were within the normal range as prescribed for Indian feeds and fodders (ICAR 2013a) with slight variations which might be due to differences in geographical location, stage of maturity, nutrient profile of soils where they were grown and plant part (i.e. twigs, leaves, soft stem) sampled, and method of harvesting. Inconsistencies in chemical composition could also be due to sampling site and climatic influences on species growth and plant nutrient accumulation.

Fermentation kinetics: Increasing EFE dose level improved ($p<0.01$) NGP, ME content, SCFA and MCP production linearly as well as quadratically up to 0.60% incorporation level (L_3); however, PF_{24} and GY_{24} were not affected by EFE additions (Table 2). EFE in ruminant diets increases the rate of fermentation and probably degradation of feedstuffs which is evidenced by increase in gas production at 24 h incubation. Gas production response to varying levels of enzyme addition differed in agreement to the findings of Gado *et al*. (2017) who attributed GP response to enzyme level×feed type interactions to different nutrient contents. In the present study, EFE addition to TMR produced more gas at 0.60% DM than the lower incorporation levels, which is expected because the greater the enzyme level, the higher the enzymatic activity (Elghandour *et al*. 2016). However, further higher enzyme level had no additional effect because higher enzyme level may have prevented binding of all enzymes to substrate receptors, which reduced proportional attachment by ruminal microorganisms to fibre (Togtokhbayar *et al*. 2015). Colombatto *et al*. (2003) concluded that increasing the level of enzyme from 1× to 5× increased the rate and extent of GP, but that addition at the 10× levels was not effective. Increased *in vitro* GP with EFE addition may

Table 1. Ingredients and chemical composition (on % dry matter basis) of the experimental diets

Physical composition					
Ingredients					Parts
Oats straw					40.00
Mixed grass hay					20.00
Crushed maize					11.00
Wheat bran					5.00
De-oiled rice bran					4.00
Mustard oil cake					6.00
Soybean					11.40
Molasses					1.00
Mineral mixture‡					0.60
Common salt					0.40
Urea					0.60
Chemical composition					
Attribute	Oats straw	Mixed grass hay	Concentrate mixture	EFE cocktail	Basal TMR
OM	92.08	91.45	89.52	100.00	90.51
CP	6.78	15.97	26.47	16.84	16.63
NDF	69.20	39.20	22.20	-	44.80
ADF	48.10	25.80	11.20	-	28.40
Hemicellulose	21.10	13.40	11.00	-	16.40
Cellulose	31.90	18.50	5.40	-	17.90

‡ Mineral mixture: 18 Calcium (g/100 g), 12 Phosphorous (g/100 g), 2 Magnesium (g/100 g), 2.3 Sulphur (g/100 g), 210 Zinc (mg/100 g), 55 Iron (mg/100 g), 10 Iodine (mg/100 g), 60 Copper (mg/100 g), 210 Manganese (mg/100 g), 8 Cobalt (mg/100 g), 30 Fluorine (mg/100 g). ADF, Acid Detergent Fibre; CP, Crude Protein; EFE, Exogenous Fibrolytic Enzymes cocktail; NDF, Neutral Detergent Fibre, OM, Organic Matter; TMR, Total Mixed Ration.

Table 2. *In vitro* fermentation kinetics of basal TMR incorporated with gradual increasing levels of EFE cocktail at 48h of incubation

Attribute	EFE level‡						SEM	*p*-value	
	L_0	L_1	L_2	L_3	L_4	L_5		L	Q
NGP (mL/500 mg)	58.17[a]	60.17[b]	63.93[c]	67.90[d]	68.13[d]	68.03[d]	0.975	<0.001	<0.001
ME (MJ/kg DM)	6.31[a]	6.42[b]	6.62[c]	6.84[d]	6.85[d]	6.85[d]	0.053	<0.001	<0.001
PF_{24} (mg/mL)	4.28	4.43	4.23	4.27	4.31	4.34	0.018	0.930	0.180
GY_{24} (mL/g DM)	233.92	225.81	236.65	234.56	231.88	230.63	0.967	0.976	0.151
SCFA (mmol/g DM)	2.56[a]	2.65[b]	2.82[c]	3.00[d]	3.01[d]	3.00[d]	0.044	<0.001	<0.001
MCP (mg/g DM)	241.40[a]	268.27[bc]	259.03[ab]	280.24[cd]	287.88[d]	290.65[d]	4.319	<0.001	0.092

‡Levels of incorporation of EFE in basal TMR comprising of : L_0, Control (basal diet without EFE); L_1, basal diet incorporated with 0.20% DM of EFE; L_2, basal diet incorporated with 0.40% DM of EFE; L_3, basal diet incorporated with 0.60% DM of EFE; L_4, basal diet incorporated with 0.80% DM of EFE, L_5 , basal diet incorporated with 1.00% DM of EFE. EFE, Exogenous Fibrolytic Enzymes cocktail; GY_{24}, Gas Yield at 24 h of incubation; MCP, Microbial CP Biomass Production; ME, metabolizable energy; NGP, net gas production; PF_{24}, partitioning factor at 24 h of incubation; SCFA: Short Chain Fatty Acid concentrations; SEM: Standard Error Mean; L: Linear Response; Q: Quadratic Response. Means with different lowercase superscripts in a row differ significantly among the levels.

increase the net energy density of the diets and stimulate MCP production which is positively correlated with high activities of the rumen microbes (Oba and Allen 2000). Enzyme incorporation stimulates rumen microbial growth and activity causing improvement in MCP, SCFA production and ME concentration in the diet (Vallejo *et al*. 2016). These results are in conformity with those of Elghandour *et al*. (2016) who observed that the addition of EFE to feeds improved fermentation kinetics, ME, SCFA and MCP.

In vitro nutrient degradability: The results of dietary EFE incorporation effects on *in vitro* nutrient degradability are summarised in Table 3. Addition of EFE cocktail was effective in improving ($p<0.01$) DM, OM and NDF degradability *in vitro* linearly as well as quadratically; however, the effect was significant only upto 0.60% DM incorporation level (L3). Enzyme application to the substrate enhances the attachment and colonization of rumen microorganisms to the feed particles (Wang *et al*. 2001), increase fibre digestion and alter ruminal fermentation (Nsereko *et al*. 2002), and/or act synergistically with endogenous ruminal enzymes to increase hydrolytic capacity of the rumen (Morgavi *et al*. 2001). Other workers (Togtokhbayar *et al*. 2015, Vallejo *et al*. 2016) also reported improvement in nutrient degradability pattern either *in vitro* or *in sacco* by application of exogenous enzymes. At higher EFE incorporation levels (>0.60% incorporation level), no further additional improvement in nutrient degradability was evident which might be due to the fact that beneficial disruption of the feed area may get diminished because the excess exogenous enzyme attached to feed may have restricted microbial attachment and limited digestion of feed.

Dietary application with EFE improves fermentation of feeds through fibre hydrolysis and/or solubilization which causes enhancement in fibre degradation (Elsiddig 2019). Increased NDF degradability enhances the energy density of diets as depicted by higher ME contents, and stimulates the microbial production as reflected by higher MCP production at corresponding levels in the present study. Besides, higher nutrient (OM) degradability due to enzyme addition suggest a stimulated rate of fermentation which is closely correlated with gas production, as reported by Diaz *et al*. (2013) who evaluated *in vitro* commercial fibrolytic enzymes for improving the nutritive value of low-quality forages.

Fermentation characteristics: EFE incorporated at gradual increasing levels altered all the fermentation metabolites except NPN (Table 4). Increased TVFA with increased EFE level was opposite of the *p*H response, but was consistent with the increase of SCFA concentrations, nutrient degradability and ME contents; however, the prominent ($p<0.01$) effect was observed only up to 0.60% DM incorporation level (L3). This might be due

Table 3. *In vitro* nutrient degradability (% DM) of basal TMR incorporated with gradual increasing levels of EFE cocktail at 48h of incubation

Attribute	EFE level‡						SEM	*p*-value	
	L_0	L_1	L_2	L_3	L_4	L_5		L	Q
Dry matter	49.95[a]	52.62[ab]	54.22[b]	57.85[c]	58.75[c]	58.92[c]	0.717	<0.001	<0.001
Organic matter	51.32[a]	53.13[a]	56.54[b]	63.30[c]	63.87[c]	63.92[c]	1.591	<0.001	0.009
Neutral detergent fibre	31.41[a]	33.36[b]	35.80[c]	39.63[d]	39.87[d]	40.44[d]	1.052	<0.001	0.001

‡Levels of incorporation of EFE in basal TMR comprising of : L_0: Control (basal diet without EFE) ; L_1, basal diet incorporated with 0.20% DM of EFE; L_2, basal diet incorporated with 0.40% DM of EFE; L_3, basal diet incorporated with 0.60% DM of EFE; L_4, basal diet incorporated with 0.80% DM of EFE; L_5 basal diet incorporated with 1.00% DM of EFE. EFE, Exogenous Fibrolytic Enzymes cocktail; SEM, Standard Error Mean; L, Linear Response; Q, Quadratic Response. Means with different lowercase superscripts in a row differ significantly among the levels.

Table 4. *In vitro* fermentation characteristics of basal TMR incorporated with gradual increasing levels of EFE cocktail at 48 h of incubation

Attribute	EFE level‡						SEM	*p*-value	
	L_0	L_1	L_2	L_3	L_4	L_5		L	Q
*p*H	6.92^{c}	6.88^{c}	6.83^{b}	6.79^{ab}	6.80^{ab}	6.77^{a}	0.014	<0.001	0.014
TVFA (mmol/L)	82.67^{a}	84.51^{ab}	87.02^{b}	90.50^{c}	91.31^{c}	91.40^{c}	0.862	<0.001	0.028
Nitrogen fractions (mg/dL)									
Ammonia-N	27.33^{a}	27.24^{a}	27.51^{ab}	28.00^{b}	28.04^{b}	28.03^{b}	0.094	<0.001	0.675
Total-N	87.73^{a}	90.94^{b}	95.16^{c}	98.93^{d}	99.47^{d}	100.03^{d}	1.139	<0.001	<0.001
TCA- precipitable-N	30.80^{a}	31.93^{a}	35.16^{b}	41.07^{c}	41.87^{c}	41.65^{c}	1.141	<0.001	0.007
Non protein-N	56.93	59.00	60.00	57.87	57.61	58.38	0.315	0.851	0.055

‡Levels of incorporation of EFE in basal TMR comprising of : L_0, Control (basal diet without EFE) ; L_1, basal diet incorporated with 0.20% DM of EFE; L_2, basal diet incorporated with 0.40% DM of EFE; L_3, basal diet incorporated with 0.60% DM of EFE; L_4, basal diet incorporated with 0.80% DM of EFE; L_5, basal diet incorporated with 1.00% DM of EFE. EFE, Exogenous Fibrolytic Enzymes cocktail; TVFA, Total Volatile Fatty Acids; SEM: Standard Error Mean, L, Linear Response; Q, Quadratic Response. Means with different lowercase superscripts in a row differ significantly among the levels.

to greater enzymatic hydrolysis of feeds into readily fermentable substrates that depressed *p*H and raised the TVFA concentration when fermented with addition of EFE (Vallejo *et al*. 2016). In the present study, this was also confirmed by increased nutrient degradability with gradual increasing levels of EFE incorporation up to L3 level. Elghandour *et al*. (2013) reported decreased ruminal *p*H values when incubated various fibrous feeds with different levels of exogenous fibrolytic enzymes. These results are in conformity with the findings of Almaraz *et al.* (2010) who also reported an increase in TVFA concentration by exogenous enzymes application *in vitro*. Also, Omar *et al*. (2009) concluded that supplementation of enzymes to steer rations improved digestibility and rumen SCFA concentrations.

In nitrogen fractions, increased ($p<0.01$) content of ammonia-N at higher EFE incorporation levels compared to control (L0) and lowest level (L1) represents the faster rate of nutrient degradation and/or fermentation by EFE addition. Ammonia-N content at all the levels was sufficient for optimum microbial growth which is reported to be 5-7 mg/dL (Satter and Slyter 1974), suggesting sufficient protein content of the feed as growth ration for sheep. Increased ($p<0.01$) concentration of total-N as well as TCA-precipitable-N with increasing levels of EFE addition has been observed. Enzyme addition might have stimulated and/or increased total rumen microbial numbers, which is reflected by the increase in TCA-precipitable-N and thus in total-N contents as an indicator of enhanced rumen microbial protein synthesis, which is also confirmed by increased levels of MCP to the corresponding EFE incorporation levels. These results corroborates well with the findings of Vallejo *et al*. (2016) who also observed increased microbial protein production by enzyme application *in vitro*.

Increasing the exogenous fibrolytic enzymes cocktail incorporation dose in TMR linearly increased gas production, fermentation rate, microbial CP production, nutrient degradability and fermentation characteristics up to the level of 0.60% DM with no further additional improvement at higher levels, suggesting the EFE cocktail dose of 0.60% DM to be used for efficient utilisation of oats straw based complete feeds, which although needs further testing by *in vivo* studies.

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