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Effectiveness of selected fibrolytic enzymes on *in vitro* digestibility of maize (*Zea mays*) stover

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Abstract

An in vitro experiment was conducted to determine the effectiveness of three exogenous fibrolytic enzymes namely cellulase, xylanase and pectinase on dry matter (DM), organic matter (OM) and neutral detergent fibre (NDF) digestibility of maize stover. Cellulase, xylanase and pectinase were added on ground stover individually at a rate of 3g/kg DM maize stover and in combination at a ratio of 50:50. The in vitro dry matter digestibility (IVDMD) of maize stover ranged from 41.6% (control) to 54.8% (cellulase-pectinase combined), a significant increase of up to 24.2%. Combining pectinase with cellulase or xylanase significantly ($p<0.05$) improved the IVDMD by 24.2% and 20.5%, respectively, compared to when pectinase was applied alone (16.3%). The range for in vitro organic matter digestibility (IVOMD)of maize stover was 31.7% (control) to 45.9% (cellulase, xylanase and pectinase combined). Amongst the enzyme treatments, pectinase had a significantly ($p<0.05$) lower IVOM digestibility of 41.5% compared to cellulase-xylanase (45.4%), cellulase-pectinase (45.7%) and cellulase-xylanase-pectinase (45.9%), but it was comparable ($p>0.05$) to xylanase, cellulase and xylanase-pectinase. It was observed that not all the enzymes and enzyme combinations influenced in vitro neutral detergent fibre digestibility (IVNDFD). Improvement ($p<0.05$) in IVNDFD was observed when maize stover was supplemented with cellulase-xylanase and cellulase-pectinase only. Enzymes applied singly did not ($p>0.05$) improve IVNDFD. Compared to the control (26.3%), cellulase-pectinase (31.4%) and cellulase-xylanase (33.2%) had significantly higher IVNDF digestibility ($p<0.05$), resulting in improvements of 16.3% and 20.9%, respectively. The selected exogenous fibrolytic enzymes were effective in degrading maize stover, whether singly applied or in combination, compared to maize stover without enzyme supplementation. Cellulase-pectinase and cellulase-xylanase combinations were highly effective in degrading maize stover.

Keywords: *Exogenous fibrolytic enzymes, in vitro dry matter, in vitro neutral detergent fibre, maize stover*

INTRODUCTION

The shortage of feed supply and utilization of poor-quality roughages in sub-Saharan Africa remains a major constraint in dairy production, especially in smallholder dairy farms. This is exacerbated by a lack of rainfall during the dry season hence prevalent feed scarcity. FAO and Intergovernmental Authority on Development [IGAD] (2019) reported that across the East African Community (EAC), livestock is fed mainly low-quality roughages. These include natural grazing and agro-industrial by-products (AIBPs) such as cereal straws/stovers and sugarcane by-products, amongst many, all of which have a high content of lignocellulosic material (FAO and IGAD, 2019) and lower degradation rates which negatively affect animal performance (Herrero et al., 2013).

Heuzé et al. (2019) estimated that about 900 million tonnes of dry maize stover are produced annually worldwide. In sub-Saharan Africa (SSA), maize is the most important staple cereal crop for more than 300 million people. Annually, it is grown in 25 million hectares, largely in smallholder farms (Badu-Apraku & Fakorede, 2017). In the Eastern and Southern parts of Africa, maize production contributes 20% to the income of agricultural holders (Depetris-Chauvin et al., 2017). In Kenya, maize production in 2019 was 3.8 million metric tonnes (World Data Atlas, 2020). With the assumption

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that a tonne of dry grain produces 0.8 tonnes of dry stover (Lizotte et al., 2015), about 3.04 million metric tonnes of maize stover are available annually in Kenya. Most of the maize stover is simply burnt and a few farmers utilise this energy source for ruminant animal feeding. Maize stover is a potential cereal crop residue to alleviate the feeding challenges faced, especially by smallholder dairy farmers during periods of feed scarcity due to its availability and abundance, however, its nutritive value is relatively low.

Efficient utilisation of maize stover as an energy source for ruminants is hindered by low voluntary intake emanating from the low crude protein content and high fibre content. Maize stover is poorly digestible attributed to the polymers of hemicellulose, cellulose and lignocellulose which make it recalcitrant to enzyme saccharification and microbial digestion, making its utilisation poor (Zhao et al., 2018). Maize stover has high lignin (12.2%) and low crude protein (4.8%) content (Montañez-valdez et al., 2015), with the contents of neutral detergent fibre (NDF) and acid detergent fibre (ADF) ranging from 55.9 – 85.5 % DM and 25.7 – 54.7 % DM, respectively (Montañez-valdez et al., 2015; Abera et al., 2018). Consequently, maize stover alone does not meet the nutritional requirements of livestock thus a need for strategies to improve the digestibility of this feed resource for efficient utilisation.

Many strategies thus far have been studied for the enhancement of low-quality forages for animal feeding. These include mechanical processing, steam treatment, genetic improvement, alkali and acid treatment, and biological treatments, as reviewed by Adesogan et al. (2019). Until recently, the use of enzymes in ruminant diets had been abandoned due to the inconsistency of results from the studies conducted, the high costs of feed enzymes, and the use of other technologies to potentially improve animal performance (Yang et al., 1999). The renewed interest in the use of feed enzymes for ruminants is due to the high costs of livestock production, coupled with the availability of enzyme preparations (Beauchemin et al., 2003). Fibrolytic enzymes, also known as fibre-degrading enzymes, are produced naturally by ruminal bacteria, fungi, and protozoa, and these are responsible for the digestion of cellulose and hemicellulose in feedstuffs (Knowlton et al., 2002). These fibrolytic enzymes are mainly cellulases and xylanases, which, in previous studies, have demonstrated capability in improving feed utilization and animal performance (Arriola et al., 2011; Holtshausen et al., 2011; El-Bordeny et al., 2015) while other studies reported no effect of enzyme supplementation on dry matter intake (Bernard et al., 2010; Dean et al., 2013; Peters et al., 2015) and milk yield (Bernard et al., 2010; Azzaz et al., 2013).

The results of the use of exogenous fibrolytic enzymes (EFE) in ruminant livestock have been inconsistent across studies, which have been attributed to several factors affecting the efficacy of enzymes such as enzyme activity, application rate and composition, the type of animal, animal differences and the portion of the diet to which EFE are applied (Beauchemin et al., 2004). As yet, very limited studies have been conducted on the *in vitro* digestibility of maize stover treated with exogenous fibrolytic enzymes (Carreón et al., 2010; Gallardo et al., 2010; Bhasker et al., 2013; Fon and Nsahlai, 2013). This study, therefore, aimed at determining the effectiveness of fibrolytic enzymes (cellulase, xylanase and pectinase), singly or in combination, on *in vitro* dry matter, organic matter and neutral detergent fibre digestibility of maize stover.

MATERIALS AND METHODS

Experimental site and material

This study was conducted in the Animal Nutrition laboratory of Egerton University, Njoro Main Campus, Nakuru, Kenya. The same variety (H6213) of maize stover was collected after harvesting of maize cobs from the same field in Ngongongeri farm of Egerton University. It was chaffed to a length of 1-2 cm with a chaff cutter and stored in bags in a dry, clean and aerated place in Tatton Agriculture Park (TAP), Egerton University. The maize stover consisted of stalks, leaves, and husks. The fibrolytic enzymes under investigation were xylanase, cellulase, and pectinase, which were obtained from Beijing Smile Feed Sci. and Tech. Co., LTD, Beijing, China. The specifications of the enzymes as per the manufacturer's report are presented in Table 1.

Table 1: Enzyme specification

Item	Enzyme product name		
	Smizyme Xylanase	**Smizyme Cellulase**	**Smizyme Pectinase**
Enzyme concentration (U/g)	10,000	5,000	10,000
Enzyme activity (U/g)	11,850	6,352	10,936
Source organism	*Aspergillus oryzea*	*Trichoderma longibrachiatum*	*Aspergillus oryzea*
Optimum temperature (^oC)	37	37.5	37.5
Optimum pH	5 - 6.6	5	5.5 - 6
Recommended dosage	100g/ton complete feed	100g/ton complete feed	100g/ton complete feed

Nutritional content of maize stover

Samples of chopped maize stover were dried in triplicate in a forced-air oven at 55 ^oC for 72 hours to determine as-fed moisture content. Chopped maize stover was also sampled, dried at 65 ^oC to a constant weight, ground to pass through a 1-mm sieve using a hammer mill, and stored in sampling bottles for further analysis. Samples were analysed for dry matter (DM) by drying samples at 105 ^oC overnight, organic matter (OM) using the AOAC Official method 942.05 revisited (Thiex et al., 2012), crude protein (CP) using the

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AOAC Official method 2001.11 (AOAC, 2002), ether extract (EE) using AOAC Official method 920.39 (AOAC, 2002), and gross energy using a bomb calorimeter (C 7000 Isoperibolic, Janke and Kunkel IKA – Analysentechnik, Staufen, Germany) according to the International Organisation for Standardisation (ISO 9831, 1998), crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) following methods outlined by Van Soest et al., (1991), Ca and P were analysed using atomic absorption spectrophotometry. Metabolisable energy, total digestible nutrients, NFC, NFE, cellulose, and hemicellulose were calculated as follows:

Metabolisable energy (ME_MJ/kg DM) = 14.78-0.0147*ADF (Menke & Steingass, 1988)

TDN of all forages = 88.9 – (0.779 x %ADF)

Non-Fibre Carbohydrates (NFC) = 100 – (%NDF + %CP + %EE + %Ash) (Iqbal et al., 2009)

Nitrogen free extracts (NFE) = %DM – (%EE + %CP + %Ash + %CF) (Forejtová et al., 2005)

Cellulose = ADF - ADL; Hemicellulose = NDF – ADF

Experimental procedure

***In vitro* procedure**

In vitro digestibility of enzyme-treated maize stover was studied following the two-staged technique outlined by Tilley and Terry (1963). Three rumen liquor donor bucks, with *ad libitum* access to fresh water and mineral lick, were adapted for 4 weeks to a 65% forage: 35% concentrate diet to ensure that the microbial population changed little from day to day and that the microbial population in the rumen fluid was composed of fibrolytic as well as amylolytic and proteolytic bacteria (Soliva & Hess, 2007). Maize stover was the sole roughage. Samples of 0.5 g DM ground stover were weighed into 80-90 ml glass tubes. The experimental enzymes (cellulase, xylanase and pectinase) were then accurately weighed and added into the tubes in powder form. Each enzyme was applied at 3 g/kg DM (Gallardo et al., 2010) of maize stover and at a ratio of 50:50 to make up 3 g/kg DM in enzyme combinations, each treatment was replicated six (6) times. A control (maize stover without enzyme supplementation), a buffer blank, and a blank with the respective enzyme (s) were prepared. The enzymes were not solubilised in water before the addition.

Using a stomach tube, rumen content was collected just before the morning feeding (Yáñez-Ruiz et al., 2016) from the three bucks and put into pre-warmed (38 ^{o}C) thermos flasks. The rumen content was pooled at a ratio of 50:50 and transported immediately to the laboratory, flushed with CO_2, then kept at 38 ^{o}C. The rumen content was squeezed through four layers of cheesecloth while gassing with CO_2 and the rumen fluid collected into a pre-warmed (38 ^{o}C) CO_2-filled thermos flask and flushed with CO_2. A buffer solution was prepared according to McDougall (1948) and was mixed with the strained rumen liquor at a ratio of 4:1. The mixture (1 rumen liquor: 4 buffer solution – v/v) was stirred, gassed with CO_2, then 50 ml was pipetted into the digestion tubes containing maize stover samples and enzymes kept in a water bath maintained at 38 ^{o}C. The remaining space in each tube was thoroughly flushed with CO_2, and the tube was then sealed with a rubber cork fitted with a sealed rubber tube. The 4 mm slit in the rubber tube on the valve was cut with a sharp knife; the slit normally remained closed, opening only to release gas from inside the tube. After sealing, the tubes were incubated at 38 ^{o}C in the dark for 48 hours, being shaken 4 times a day by hand.

After 48 hours of incubation, the tubes were stored at 4 ^{o}C for 2 h to stop fermentation. Then pre-weighed glass fibre filter crucibles (porosity 1 crucibles, 70 mL capacity) were used to separate supernatants from residual plant particles and adherent microbial biomass by vacuum filtration. The particles were washed with 100 mL of distilled water and then oven-dried at 65 ^{o}C to a constant weight to determine the potential DM disappearance at 48 hours. Thereafter, three of the dried residues per treatment were used for the determination of organic matter digestibility by ashing at 550 ^{o}C overnight while the remaining three were used for *in vitro* NDF digestibility, in which the residues were subjected to fibre analyses following procedures of Van Soest et al. (1991).

In vitro DM digestibility was calculated using the formula of Tilley and Terry (1963):

In vitro DM digestibility (%) = [A – (B – C)/A] * 100

where:
A = dry weight of the sample
B = dry weight of residue after digestion
C = dry weight of reagent blank
In vitro organic matter digestibility (%) was calculated as:
In vitro organic matter digestibility (%) = [(A x (%OM/100)) – (B – C) x 100] / A

where:
A = Dry weight of sample
B = weight of ash of sample residue
C = weight of ash of blank

In vitro neutral detergent fibre digestibility (IVNDFD) was calculated as follows:

$NDFD\ (\%) = [(NDF_{feed} - NDF_{residue})/NDF_{feed}] \times 100$

where:
NDF_{feed} is the content of NDF (g/kg DM) of feed incubated
$NDF_{residue}$ is the amount of NDF (g/kg DM) in the residue

Statistical analysis

The experiment was undertaken in a completely randomized design (CRD). The data were normally distributed as per the normality test done using Box plot and Shapiro-Wilk test and a homogeneity test done using Leven's test. The data were subjected to analysis of variance (ANOVA) of Statistical Analysis Software (Version 9.4 SAS Institute Inc., Cary, NC, USA) using the following model:

$Y_{ij} = \mu + T_i + e_{ij}$

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where: Y_{ij} response variable, μ = overall mean, T_i = treatment effect, e_{ij} = random error

Significance was declared at $p<0.05$, and mean separation was done using Duncan's multiple range test (Duncan, 1955).

RESULTS

Chemical composition and nutritive value of maize stover

The chemical composition of the maize stover used in this study is presented in Table 2. The maize stover had a high dry matter (DM) and neutral detergent fibre content of 96.2% and 60.6 %, respectively. Presented in Table 3 is the nutritive value of maize stover used in this study as determined by digestibility and metabolizable energy. Maize stover had a metabolizable energy and total digestible nutrients of 14.2% and 55.7%, respectively.

Table 2: Composition of maize stover used in the study (n=3)

Parameter	Composition (%DM unless stated otherwise)	SEM
As-fed dry matter (%)	89.5	0.060
Dry matter (%)	96.2	0.093
Moisture (%)	3.76	0.093
Chemical composition		
Organic matter (OM)	93.7	0.414
Crude protein (CP)	4.67	0.221
Ether extract (EE)	1.73	0.045
Crude fibre (CF)	36.6	0.158
Ash	6.32	0.414
Non-fibre carbohydrates	26.7	0.386
*Nitrogen-free extracts (NFE)	46.9	0.675
Minerals		
Calcium (g/kg DM)	11.5	1.17
Phosphorus (g/kg DM)	2.03	0.039
Cell wall constituents		
Neutral detergent fibre (NDF)	60.6	0.478
Acid detergent fibre (ADF)	42.7	0.415
Acid detergent lignin (ADL)	2.70	0.003
Cellulose	40.0	0.413
Hemicellulose	17.9	0.875
Calorific/Gross energy (MJ/kg DM)	17.5	0.129

*NFE represents the soluble CHO of the feed, such as starch and sugar.

Table 3: Nutritive value of maize stover to ruminants (n=3)

Parameter	Composition (% or as stated	SEM
Dry matter digestibility at 48 hours	40.7	0.468
Organic matter digestibility at 48 hours	32.8	1.34
NDF digestibility at 48 hours	29.0	1.008
Total digestible nutrients (TDN)	55.7	0.323
Metabolisable energy (ME_MJ/kg DM)	14.2	0.006

***In vitro* dry matter digestibility (IVDMD)**

Fibrolytic enzymes significantly ($p<0.05$) improved the *in vitro* dry matter digestibility (IVDMD) of maize stover compared to the control (Table 4). The IVDMD of maize stover ranged from 41.6% (control) to 54.8% (cellulase-pectinase combined), a significant increase of up to 24.2%. The IVDMD of maize stover was lower when the enzymes were applied singly compared to when they were applied in combination. However, combining all three enzymes (cellulase-xylanase-pectinase) was not significantly ($p>0.05$) different from xylanase-pectinase treatment and when the enzymes were applied individually. The cellulase-pectinase combination had the highest ($p<0.05$) DM digestibility of 54.8% followed by the cellulase-xylanase combination (53.9%). Combining pectinase with cellulase or xylanase significantly ($p<0.05$) improved the IVDMD by 24.2% and 20.5%, respectively, compared to when pectinase was applied alone (16.3%). An increase ($p<0.05$) of 19.0% in IVDMD of maize stover was observed with the use of cellulase-xylanase-pectinase combination.

Table 4: Digestibility of maize stover treated with fibrolytic enzymes

Treatment	DMD[1] (%)	OMD[2] (%)	NDFD[2] (%)
Control	41.6^{e}	31.7^{c}	26.3^{c}
Cellulase	51.3^{cd}	44.9^{ab}	31.1^{abc}
Xylanase	50.4^{cd}	43.7^{ab}	29.4^{abc}
Pectinase	49.7^{d}	41.5^{b}	27.7^{bc}
Cellulase-Xylanase	53.9^{ab}	45.4^{a}	33.2^{a}
Cellulase-Pectinase	54.8^{a}	45.7^{a}	31.4^{ab}

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Xylanase-Pectinase	52.3[bc]	45.1[ab]	30.5[abc]
Cellulase-Xylanase-Pectinase	51.3[cd]	45.9[a]	31.1[abc]
SEM	0.432	0.789	1.04
p	<0.0001	<0.0001	<0.0001

[1] Sample size (N) = 6; [2] Sample size (N) = 3

[abcde] Means in the same column with different superscripts differ significantly ($p<0.05$)

***In vitro* organic matter digestibility (IVOMD)**

A similar trend was seen in the IVOM digestibility of maize stover where a significant ($p<0.05$) increase in organic matter (OM) digestibility of maize stover was observed with the use of enzymes compared to the control (Table 4). The range for IVOM digestibility of maize stover was 31.7% (control) to 45.9% (cellulase, xylanase and pectinase combined), a significant improvement of up to 31%. Amongst the enzyme treatments, pectinase had a significantly ($p<0.05$) lower IVOM digestibility of 41.53% compared to cellulase-xylanase (45.4%), cellulase-pectinase (45.7%) and cellulase-xylanase-pectinase (45.9%), but it was comparable ($p>0.05$) to xylanase, cellulase and xylanase-pectinase.

***In vitro* neutral detergent fibre digestibility (IVNDFD)**

Contrary to the observed improvements in IVDMD and IVOMD by enzymes, it was observed that not all the enzymes and enzyme combinations influenced IVNDF digestibility. Improvement ($p<0.05$) in neutral detergent fibre (NDF) digestibility (Table 4) was observed when maize stover was supplemented with cellulase-xylanase and cellulase-pectinase only. Enzymes applied singly did not ($p>0.05$) improve NDF digestibility. Compared to the control (26.3%), cellulase-pectinase (31.4%) and cellulase-xylanase (33.2%) had significantly higher IVNDF digestibility ($p<0.05$), resulting in improvements of 16.3% and 20.9%, respectively. The control had a similar ($p>0.05$) IVNDF digestibility with most of the enzyme treatments.

DISCUSSION

The structural organization of the plant cell wall matrix of forages influences its degradation through the accessibility of rumen microbes to the plant cell wall components for digestion and thus the availability of polysaccharides for rumen microbial digestion (Hatfield, 1993). In this study, improvement ($p<0.05$) in *in vitro* dry matter (DM), NDF and OM digestibility of maize stover was observed with the addition of cellulase, xylanase and pectinase and their combinations compared to the control. These findings are in line with previous studies that reported similar observations with the use of exogenous fibrolytic enzymes. For instance, Bhasker et al. (2013) reported an improvement ($p<0.01$) in *in vitro* DM digestibility of maize stover with cellulase and xylanase supplementation compared to the control.

Similarly, compared to the control, exogenous fibrolytic enzymes were reported to have significantly improved the *in vitro* DM digestibility of rice straw silage by 15.6% (Ding et al., 2022). Yang et al. (2024) concluded that the inclusion of exogenous fibrolytic enzymes in dairy cows' diets enhances DM and OM digestibility. The observed improvements in IVDM and IVOM digestibility may be credited to the fibrolytic enzymes being capable of hydrolysing the polysaccharides into simple substances thus facilitating microbial growth. This may be due to the colonisation and penetration of cellulolytic microbes and their hydrolytic enzymes onto the exposed surfaces of the feed particles (Beauchemin et al., 2004). Moreover, exogenous fibrolytic enzymes and ruminal enzymes work in synergy and increase bacterial numbers (Yang et al., 2000) which might have aided the digestion of maize stover. This may also be the reason behind the observed lower IVDMD when the enzymes were applied singly compared to when they were applied in combination.

When cellulase and xylanase were used in combination with pectinase, an improvement ($p<0.05$) in the IVDMD was observed compared to the control and a slight improvement ($p>0.05$) in the same was observed compared to when cellulase and xylanase were used singly. The slight improvement may be ascribed to the breakdown of the pectin substance of maize stover by pectinase (Garg et al., 2016). Medie et al. (2012) reported that a complex combination of enzymes (cellulases, hemicellulases and pectinases) is produced by a group of microorganisms called cellulolytic microorganisms, and they work synergically to break down cellulose and the other polymers associated with it. The cellulase-pectinase combination resulted in higher ($p<0.05$) IVDMD of maize stover compared cellulase-xylanase-pectinase combination. This observation may be attributed to the competition amongst the enzymes with the ruminal microbes for binding sites on the feed particles.

In the current study it was observed that the IVNDF digestibility of maize stover was not significantly ($p>0.05$) improved when the fibrolytic enzymes were applied singly. Adding fibrolytic enzyme combinations, cellulase-xylanase and cellulase-pectinase, significantly ($p<0.05$) improved IVNDF digestibility compared to the control. This observation is consistent with the findings of Ding et al. (2022) who reported a significant ($p<0.001$) improvement of 16.5% in *in vitro* NDF digestibility with fibrolytic enzyme treatment on rice straw silage compared to the control. The fibrolytic enzyme used by Ding et al. (2022) was a mixture of cellulase and hemicellulose (*w/w*, 1:1). A similar improvement (16.3%) was observed in this study with the use of a cellulase-pectinase combination. Also reported in this study is a 20.9% improvement in IVNDF digestibility with the use of a cellulase-xylanase combination. Cellulases are responsible for cleaving the beta 1,4-D-glucan linkages of cellulose (Sukumaran et al., 2005); hence, the improvement in IVNDF digestibility can be attributed to the attachment and colonisation of lignocellulose surfaces by rumen microbes as a result of the loosening between cellulose and hemicellulose

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caused by enzyme degradation of lignocellulose (Ding et al., 2022).

CONCLUSION

The selected fibrolytic enzymes were effective in degrading maize stover, whether singly applied or in combination, compared to maize stover without enzyme supplementation. Maize stover supplemented with pectinase had the least digestibility of DM and OM; however, when combined with cellulase or xylanase, pectinase slightly increased the digestibility of maize stover compared to when these enzymes were used singly. Cellulase-pectinase and cellulase-xylanase combinations were highly effective in degrading maize stover. There was no advantage of combining all three enzymes (cellulase-xylanase-pectinase) compared to using them singly to improve the DM and OM digestibility of maize stover. However, the best treatments were cellulase-pectinase and cellulase-xylanase as these combinations further improved NDF digestibility of maize stover compared to the control.

RECOMMENDATION

This study recommends the use of exogenous fibrolytic enzymes as they improve the digestibility of maize stover. However, further studies are required to investigate the effect of different inclusion levels and combinations of these enzymes for optimum digestibility of maize stover.

ETHICAL CONSIDERATION

Procedures involving the handling of animals during the experiment were approved by the Egerton University Institutional Scientific and Ethics Review Committee, approval number *EUISERC/APP/204/2022*. A research license was obtained from the National Commission for Science, Technology and Innovation (NACOSTI), license number NACOSTI/P/22/21751.

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