In vitro gas production kinetics and degradability of a diet for growing lambs: effect of fibrolytic enzyme products at different dose levels

Daniel López-Aguirre\textsuperscript{a,b}, Javier Hernández-Meléndez\textsuperscript{b}, Rolando Rojo\textsuperscript{a}, Fernando Sánchez-Dávila\textsuperscript{c}, Nicolás López-Villalobos\textsuperscript{a,d}, Abdel-Fattah Z. M. Salem\textsuperscript{e}, José Fernando Vázquez-Armijo\textsuperscript{a}, Salomón Ruíz\textsuperscript{b} and Santiago Joaquin\textsuperscript{b}

\textsuperscript{a}Centro Universitario, Autonomous University of the State of Mexico, Temascaltepec, Mexico; \textsuperscript{b}Facultad de Ingeniería y Ciencias, Autonomous University of Tamaulipas, Ciudad Victoria, Mexico; \textsuperscript{c}Facultad de Agronomía, Autonomous University of Nuevo Leon, Gral, Escobedo, Mexico; \textsuperscript{d}Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand; \textsuperscript{e}Facultad de Medicina Veterinaria y Zootecnia, Autonomous University of the State of Mexico, Toluca, Mexico

\textbf{ABSTRACT}

The objective of this study was to evaluate the effects of three fibrolytic enzyme products (cellulase (CEL), xylanase (XYL) and a 1:1 mixture of CEL and XYL (MIX)) at three dose levels (0, 1 and 3 \( \mu \)L/0.5 g DM) on the in vitro fermentation of a diet for growing lambs. Bottles were incubated for 96 h at 39 \(^\circ\)C. A mathematical model was used to estimate the parameters describing the gas production (GP) curve (b, c and L). The results suggest that application of fibrolytic enzymes has the potential to alter asymptotic GP and degradability of ADF and DM of a diet for growing lambs, but most of the results depend on the interaction between enzyme product and dose level. Future studies are required to determine the ideal combination between enzyme product and dose level for optimal degradation of ruminant feeds.

\textbf{Introduction}

Inclusion of higher levels of grain in the diet for ruminants has been associated with declining rates of fibre digestion due to lower fibrolytic activity in the rumen (Martin & Michaelet-Doreau 1995; Noziere et al. 1996) and an apparent microbial preference for non-structural carbohydrates (Mould & Ørskov 1983). This may create conditions in which supplementary fibrolytic exogenous enzymes can have beneficial effects on the degradation of the fibre (Beauchemin et al. 2001; Mendoza et al. 2014). There is a renewed interest in the use of fibrolytic exogenous enzymes for ruminants due to the increase in feeding costs and access to high quality enzymes (Adesogan et al. 2014; He et al. 2014). However, the effectiveness of enzyme products is highly variable (Colombatto et al. 2003a, 2003b; Meale et al. 2014; Mendoza et al. 2014).

Pre-treatment of forages with fibrolytic enzymes can solubilise some fibre and improve the digestibility at short incubation times (Moharry et al. 2009). It appears that effective enzymes work best by removing structural barriers which delay microbial colonisation of digestible fractions (Colombatto et al. 2003a, 2003b) and increase degradation rate of fibre. Exogenous fibrolytic enzymes also seem to work better at close to neutral ruminal pH (Colombatto et al. 2007).

Effect of addition of exogenous enzymes is influenced by factors such as diet composition, type of enzyme preparation, enzyme stability, specific enzyme activities, method of application and dose level (Yang et al. 2000; Morgavi et al. 2001; Wallace et al. 2001; Mendoza et al. 2014). Enzyme mixtures of endoglucanase, xylanase, alfa-amylase and protease activity have been reported to improve in vitro gas production (GP)
kinetics and ruminal fermentation of fibrous feeds (Elghandour et al. 2013) and in vivo nutrient digestibility and ruminal fermentation (Gado et al. 2009). Non-linear effects of dose level have been well established in vivo (Lewis et al. 1999; Kung et al. 2000) and in vitro (Colombatto et al. 2003b), the effect of the fibrolytic enzyme addition is reduced as the dose level increases but varies according to the substrate (Yang et al. 2000). There are also some inconsistencies on the effects of dose levels on ruminal fermentation kinetics. Some researches have shown that efficiency of forage utilisation was increased at the increasing dose levels of exogenous enzymes (Miller et al. 2008) whereas others suggest that exogenous enzymes produced better results at a specific level, rather than showing a dose response (Jalilvand et al. 2008).

The objective of this study was to evaluate the effect of three different fibrolytic enzyme products applied at three different dose levels on in vitro GP and fibre degradation of a diet for growing lambs.

Materials and methods

Diet, enzyme product and dose level

A total mixed ration (TMR, 13% CP) was prepared to meet the nutritional requirements (NRC 2007) for growing lambs (Table 1). Samples from the TMR were dried at 65 °C for 48 h in a forced air oven to constant weight, ground in a hammer mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components and in vitro GP.

Two exogenous enzyme products were tested: CEL (Dyadic<sup>®</sup> Cellulase PLUS, Dyadic Int., Jupiter, FL), XYL (Dyadic<sup>®</sup> Xylanase PLUS, Dyadic Int., Jupiter, FL) and 1:1 mixture of both enzymes (MIX; CEL + XYL). Dyadic<sup>®</sup> Cellulase PLUS is a concentrated liquid acid-neutral endo-1,4-β-D-xylanase (E.C. 3.2.1.8) produced by the fermentation of T. longibrachiatum containing 34,000–41,000 U/g of xylanase activity. These activities were provided by the manufacturers and tested in a previous study (Elghandour et al. 2015).

The enzyme treatments (CEL, XYL and MIX) were applied at three doses, namely 0, 1 and 3 µL/0.5 g DM. The enzyme dosages were dissolved in distilled water and applied directly onto the substrate inside the bottles 24 h before adding buffer solution and ruminal fluid. Substrates in non-treated bottles (no enzyme) received 1 ml of distilled water without any added enzyme.

In vitro rumen fermentation

Two factors were studied under a factorial arrangement (three enzyme products × three enzyme doses) generating nine treatments (0, 1 and 3 µL/0.5 g DM of CEL; 0, 1 and 3 µL/0.5 g DM of XYL and 0, 1 and 3 µL/0.5 g DM of MIX). Samples of 500 mg of TMR (substrate) were accurately weighed in triplicate into 120-ml serum bottles with appropriate addition of enzyme products and dose levels.

Rumen inoculum was collected from four growing lambs (Pelibuey, 24 ± 0.3 kg live weight) fitted with permanent rumen cannula and fed ad libitum a total mixed ration made up of 50:50 commercial concentrate (PURINA<sup>®</sup>, Toluca, Mexico) and alfalfa hay formulated to meet all of their nutrient requirements (NRC 2007). Fresh water was available to lambs at all time during the rumen inoculum collection phase. Ruminal content of each animal was obtained immediately before the morning feeding, mixed and strained through four layers of muslin and then kept at 39 °C under a continuous CO₂ stream. Ten millilitres of particle-free ruminal fluid and 40 ml buffer medium (containing micro- and macro-elements, a reducing agent and a reduction indicator of resazurin; Mauricio et al. 1999) were added to each bottle. Negative controls containing buffered rumen fluid with or without enzyme but no substrate, were also included in triplicate for correction of gas produced from small particles present in the ruminal fluid or sugars present in the enzyme products. Cumulative GP (ml/0.5 g DM) was recorded with a pressure transducer fitted with a microprocessor (HD 8804, Delta Ohm, Italy) at 6, 12, 19, 24, 48, 72 and 96 h post incubation at 39 °C. Volume of gas (ml/0.5 g DM) produced after 24 h of

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**Table 1.** Ingredients and chemical composition of diet for growing lambs used in the in vitro test.

| Ingredient                  | g/kg DM |
|-----------------------------|---------|
| Corn grain                  | 302     |
| Sorghum grain               | 280     |
| Soybean meal                | 113     |
| Corn stover                 | 250     |
| Molasses cane               | 30      |
| Mineral premix              | 25      |
| Chemical composition        |         |
| Crude protein, g/kg DM      | 130     |
| Metabolisable energy, Mcal/kg DM | 2.51 |
| Neutral detergent fibre, g/kg DM | 367 |
| Acid detergent fibre, g/kg DM | 139 |
| Hemicellulose, g/kg DM      | 228     |
| Organic matter, g/kg DM     | 935     |

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incubation (GP24) was used as an index of energy feed value.

At the end of incubation (96 h) bottles were uncapped, pH was measured using a pH metre (Conductronic pH 15, Puebla, Mexico) and contents of each serum bottle were filtered using filter bags (ANKOM F-57, ANKOM, Macedon, NY) under vacuum. Fermentation residues in the bags were dried at 105 °C overnight to estimate the DM disappearance.

**Analytical procedures and degradability determination**

Samples of TMR were analysed for dry matter (DM), ash and N content according to the AOAC methods (AOAC 1999). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) content (Van Soest et al. 1991) were analysed using the ANKOM<sup>200</sup> Fibre Analyser unit (ANKOM Technology, Macedon, NY). For NDF analysis, samples were treated with α-amylase (Sigma A-3403 Sigma-Aldrich<sup>®</sup> Co., St. Louis, MO), and the neutral detergent solution contained sodium sulphite and the residues were not corrected for residual ash. Hemicellulose content was calculated from the difference between NDF and ADF. The residue of 48 h of incubation was analysed for NDF and ADF to calculate fibre degradability (NDFD and ADFD, respectively).

**Calculations**

Estimates of kinetics parameters of GP (ml/0.5 g DM) were obtained using the NLIN procedure (version 9.2; SAS Institute, Cary, NC) according to France et al. (2000) using the following model:

\[
GP_t = b \times [1 - e^{-c(t-L)}]
\]

where GP<sub>t</sub> is the volume of GP at time t; b is the asymptotic GP (ml/0.5 g DM); c is the rate of GP (/h) and L (h) is the discrete lag time prior GP.

Metabolisable energy value of the TMR at 24 h (ME, MJ/kg DM) was estimated according to Menke and Steingass (1988) as:

\[
ME = 2.20 + 0.136 \times GP24 + 0.057CP
\]

where GP24 is the volume of gas produced up to 24 h expressed as ml/0.5 g DM and CP is the percentage of crude protein on DM basis.

Short chain fatty acid (SCFA, mmol) concentrations were calculated according to Getachew et al. (2002) as:

\[
SCFA = -0.00425 + 0.0222 \times GP24
\]

where GP24 is the 24-h net GP (ml/200 mg DM).

**Statistical analysis**

Data for *in vitro* ruminal fermentation were analysed using a randomised complete design with three enzyme products (CEL, XYL and MIX) x three dose levels (0, 1 and 3 ml/0.5 g DM) in a factorial arrangement with three repetitions (version 9.2; SAS Institute, Cary, NC). The linear model was:

\[
y_{ijk} = \mu + E_i + D_j + ED_{ij} + e_{ijk}
\]

where \( y \) = the dependent variable; \( \mu \) = overall mean; \( E \) = effect of i-enzyme product; \( D \) = effect of j-dose level; \( ED \) = interaction between the i-enzyme product and j-dose level; and \( e \) = the residual error.

Significant interactions at \( p < 0.05 \) were detected for *in vitro* ruminal parameters. Linear and quadratic polynomial contrasts were used to determine effect of dose level on all dependent variables.

**Results**

Curves of cumulative GP modelled with the kinetic model of France et al. (2000) for each of the enzyme product and dose level are shown in Figure 1. Estimated parameters of this model are presented in Table 2. Least squares and standard errors of GP at different times are also shown in Table 2. Curves of cumulative GP modelled with the kinetic model for enzymes products are shown in Figure 2.

A significant enzyme product by dose level interaction (\( p < 0.05 \)) was found for cumulative GP up to 72 and 96 h of incubation. The asymptotic GP was affected (\( p < 0.02 \)) by enzyme product and dose level, with a significant linear response (\( p < 0.05 \)). The rate of GP (parameter c) and cumulative GP up to 6, 12, 19, 24 and 48 h after incubation was affected (\( p < 0.05 \)) by dose level, but lag time (parameter L) was not affected. Dose level affected (\( p < 0.05 \)) the rate of GP with a quadratic response (\( p < 0.01 \)). Dose level also affected (\( p < 0.05 \)) cumulative GP up to 6h, 12h, 19h, 24h and 48h after addition of the enzyme product with a quadratic effect.

*In vitro* rumen fermentation profile (pH, ME and SCFA), DMD, NDFD and ADFD of the diet for growing lambs are shown in Table 3. Enzyme product affected (\( p < 0.05 \)) pH, ADFD and DMD. Dose level affected ME and SCFA with a linear (\( p < 0.05 \)) and quadratic (\( p < 0.01 \)) response. The enzyme product by dose level interaction was significant (\( p < 0.05 \)) for pH, DMD and ADFD.

**Discussion**

Gas production *in vitro* appears related to the chemical composition of the feed, in particular to the fibre...
content and its structural polysaccharides (Jalilvand et al. 2008). Musco et al. (2016) reported negative significant correlations between content of NDF in grass and in vitro organic matter degradability ($r = -0.70$) and fermentation rate of GP ($r = -0.70$). The fermentation of high concentrate diets, in ruminal fluid is generally enzyme-limited. In the present study, the addition of fibrolytic enzymes to the diet for growing lambs increased GP during the final period (GP72 and GP96) of fermentation, which may be a reflection of an increase in bacterial numbers, and hence, hydrolytic capacity of the ruminal fluid. This view is similar to previous hypotheses that exogenous enzymes increase fibrolytic activity due to the increased number of ruminal microbes (Colombatto et al. 2003b), and increased bacterial attachment and synergistic effects with

### Table 2. In vitro rumen gas kinetics and cumulative gas production of a diet for growing lambs with different dose levels (0, 1 and 3 μL/0.5 g DM) of different exogenous liquid enzyme products.

| Enzyme | Dose | b     | c     | L     | GP6  | GP12 | GP19 | GP24 | GP48 | GP72 | GP96 |
|--------|------|-------|-------|-------|------|------|------|------|------|------|------|
| CEL    | 0    | 217   | 0.041 | 3.22  | 27.5 | 54.2 | 105.6| 131.8| 175.8| 207.6| 211.8 |
|        | 1    | 233   | 0.044 | 3.19  | 30.2 | 65.1 | 119.2| 146.7| 192.8| 225.7| 229.9 |
|        | 3    | 244   | 0.033 | 3.14  | 25.7 | 52.5 | 103.3| 131.0| 181.2| 225.0| 232.8 |
| XYL    | 0    | 217   | 0.041 | 3.22  | 27.5 | 54.2 | 105.6| 131.8| 175.8| 207.6| 211.8 |
|        | 1    | 229   | 0.042 | 3.21  | 28.7 | 60.0 | 112.4| 139.1| 184.4| 218.3| 223.4 |
|        | 3    | 218   | 0.036 | 3.24  | 25.2 | 48.2 | 97.3 | 123.1| 168.4| 204.3| 209.7 |
| MIX    | 0    | 217   | 0.041 | 3.22  | 27.5 | 54.2 | 105.6| 131.8| 175.8| 206.5| 211.8 |
|        | 1    | 217   | 0.042 | 3.22  | 28.4 | 54.2 | 106.9| 133.4| 177.4| 208.1| 212.0 |
|        | 3    | 224   | 0.037 | 3.29  | 26.7 | 49.7 | 101.6| 128.3| 175.2| 218.0| 216.0 |
| SEM    |      | 4.8   | 0.002 | 0.056 |     | 0.452| 1.18 | 1.40 | 1.49 | 1.52 | 1.78 |

**p** Values

- Enzyme: 0.0150; 0.9393; 0.3322; 0.8124; 0.0986; 0.0788; 0.0120; 0.0028; 0.0047
- Dose: 0.0200; 0.0029; 0.9299; 0.0199; 0.0009; 0.0002; 0.0004; 0.0011; 0.0102; 0.0181
- Interaction: 0.0602; 0.7136; 0.6412; 0.8364; 0.2783; 0.1871; 0.1715; 0.0571; 0.0227; 0.0322
- Linear: 0.0317; 0.0037; 0.9042; 0.0630; 0.0911; 0.0766; 0.1510; 0.7869; 0.1701; 0.0146
- Quadratic: 0.3945; 0.0057; 0.7133; 0.0071; 0.0008; 0.0002; 0.0004; 0.0021; 0.0629; 0.1328

*Means in the same column with different superscripts are significantly different ($p < 0.05$).

**a** Asymptotic gas production (ml/0.5 g DM); **b** rate of gas production (/h); **L** initial delay before gas production begins (h).

**Enzyme products of Dyadic**: cellulase (CEL cellulase), xylanase (XYL xylanase) and mixture 1:1 of both products (MIX CEL + XYL).
Enzymes produced by a variety of microbes are capable of degrading lignocellulosic materials to SCFA, but require substantive rumen retention time (Kumar et al. 2009). In our study, lag time of the GP curve was not affected by the enzyme product and also by the dose level. However, Colombatto et al. (2003a, 2003b) mentioned that enzymes could degrade complex substrates to simpler forms at early stages of fermentation to allow faster ruminal microbial colonisation and fermentation. Some authors have suggested that pre-treatment of feed with enzymes could create a stable enzyme–feed complex (Kung et al. 2000), but others have indicated an alteration in fibre structure, which would stimulate microbial colonisation (Nsereko et al. 2000).

The major fibrolytic enzymes are cellulases and xylanases which degrade cellulose and hemicellulose, respectively. Some authors mention that the combination of products with different enzymatic activities (Eun & Beauchemin 2007) or the use of multi-enzyme (Gado et al. 2011; López et al. 2013) products may be more effective in degrading forage compared with enzymes of single activity. In our study the GP kinetics was better for CEL compared with XYL and MIX. These results clearly show that enzymes had different effects on diet components, and therefore, diet characteristics should be involved, because CEL was effective in increasing degradation rate of substrate. It is possible that enzymes altered or weakened the cell wall structure, thus allowing earlier access of ruminal microorganisms to cell contents and increasing the degradation rate of the diet (Nsereko et al. 2002; Morgavi et al. 2004; Giraldo et al. 2008), but these modifications were not reflected in changes with MIX and XYL. In contrast, Diaz et al. (2015) reported that the use of

### Table 3. *In vitro* rumen fermentation profile, dry matter and fibre degradability of a diet for growing lambs with different enzyme products

| Enzyme | Dose | pH | ME | SCFA | NDFD | ADFD | DMD |
|--------|------|----|----|------|------|------|-----|
| CEL    | 0    | 6.56 | 10.5 | 1.227 | 763.3 | 485.6 | 754.2 |
|       | 1    | 6.62 | 11.2 | 1.333 | 786.5 | 627.1 | 858.8 |
|       | 3    | 6.59 | 10.4 | 1.203 | 746.5 | 497.0 | 751.7 |
| XYL   | 0    | 6.56 | 10.5 | 1.227 | 763.3 | 485.6 | 754.2 |
|       | 1    | 6.55 | 10.8 | 1.276 | 758.8 | 482.9 | 742.3 |
|       | 3    | 6.51 | 9.8  | 1.120 | 784.6 | 505.3 | 757.4 |
| MIX   | 0    | 6.56 | 10.5 | 1.227 | 763.3 | 485.6 | 754.2 |
|       | 1    | 6.50 | 10.5 | 1.120 | 774.5 | 469.9 | 744.7 |
|       | 3    | 6.48 | 10.1 | 1.117 | 775.0 | 509.3 | 765.0 |
| SEM   |     | 0.009 | 0.086 | 0.014 | 4.33  | 10.37 | 6.82 |

**p Values**

| Enzyme | 0.0005 | 0.1047 | 0.9670 | 0.8727 | 0.0148 | <0.0001 |
|--------|--------|--------|--------|--------|--------|---------|
| Dose   | 0.0926 | 0.0008 | 0.0008 | 0.6377 | 0.0694 | <0.0001 |
| Interaction | 0.0434 | 0.2467 | 0.2482 | 0.2049 | 0.0018 | <0.0001 |
| Linear | 0.1837 | 0.0322 | 0.0262 | 0.6077 | 0.4737 | 0.8150 |
| Quadratic | 0.4701 | 0.0016 | 0.0017 | 0.4389 | 0.1548 | 0.0798 |

ME: metabolisable energy (MJ/kg DM); SCFA: short chain fatty acids (mmol/g DM); NDFD: neutral detergent fibre degradability (mg/g NDF); ADFD: acid detergent degradability (mg/g ADF); DMD: dry matter degradability (mg/g DM).

* Means in the same column with different superscripts are significantly different (p < 0.05).

Enzyme products of Dyadic*: cellulase (CEL cellulase), xylanase (XYL xylanase) and mixture 1:1 of both products (MIX CEL + XYL).

Calculated according to Menke and Steingass (1988) based on gas production.

Calculated according to Getachew et al. (2002).

hydrolysis of ruminal microorganisms. Another report (Nsereko et al. 2002) showed that the addition of a fibrolytic enzyme preparation increased the number of celluliose-utilising, xylanolytic and amyloolytic bacteria, but had no effect on the number of cellululolytic bacteria; the population density of *Ruminobacter amylophilus* was increased also by the addition of the fibrolytic enzyme. Moreover, Chung et al. (2012) reported that *Selenomonas ruminantium* tended to increase linearly with increasing dose levels of a fibrolytic enzyme.

In this study, dose level had a significant effect on ME, SCFA and GP up to 6, 12, 19, 24 and 48 h of incubation after adding the enzyme. Togtokhbayar et al. (2015) demonstrated that the addition of low and moderate dose levels (1.0–1.5 μL/g) of a commercial enzyme product with xylanase activity increases the GP at all the incubation times, using wheat straw as a substrate. However, in our study the highest dose level of xylanase (3 μL/0.5 g of growing lamb diet) may have prevented binding of enzymes to substrate receptors, which might have reduced proportional attachment by ruminal microorganism to fibre (Beauchemin et al. 2001). Colombatto et al. (2003b) suggest that increasing the dose level of an enzyme from 1x to 5x increased the rate of GP, but dose levels of 10x were ineffective.

Figure 2. Cumulative gas production (ml 0.5/g DM) from *in vitro* fermentation of a diet for growing lambs with different exogenous liquid enzyme products. CEL: cellulase; XYL: xylanase; MIX: mixture 1:1 of both products.
xylanase product from ruminal microorganism produced only subtle effects on GP of certain forages.

The interaction effect between enzyme product and dose level was significant for DMD and ADFD suggesting there is an optimal combination between enzyme product and dose level for a maximum value of DMD and ADFD. Addition of 1 μl/0.5 g DM resulted in the highest values of DMD and ADFD. Direct-fed enzymes have been shown to enhance microbial colonisation of feeds by increasing the numbers of ruminal fibrolytic microbes (Nsereko et al. 2002; Morgavi et al. 2004) resulting in an increased degradation rate of ruminal fibre (Giraldo et al. 2008), increased synthesis of ruminal microbial protein (Yang et al. 1999; Nsereko et al. 2002) and increased total tract digestibility (Gado et al. 2009, 2011). Responses in grain-fed sheep to enzyme supplementation have been variable with no changes in DM intake (Rojo et al. 2005) or total tract digestibility (McAllister et al. 1999), with no improvements in sheep live weight or feed conversion efficiency (McAllister et al. 2000; Mora-Jaimes et al. 2002).

Mendoza et al. (2014) suggested that the response to exogenous enzymes depends on the quality of feed, particularly the proportion of the NDF that is potentially digestible in the rumen. They indicated that NDF contains three fractions classified according to the kinetics of digestion of the rumen: digestible, potentially digestible and indigestible. The potentially digestible fraction disappears from the rumen by passage and digestion. The indigestible fraction does not supply nutrients to the ruminant, passing undigested to appear in the faeces. These authors hypothesised that the addition of exogenous enzymes will improve the digestibility of forages that have high proportion of potentially digestible fraction. This hypothesis may explain well the observed positive effect of the enzyme product on the degradability of DM and ADF evaluated in this study, but apparently contradicts the not observed positive effect on the degradability of NDF. However, the diet for growing lambs formulated in this study had a low level of NDF (36.7%) with a low level corn stover (250 g/kg DM), suggesting that this diet had low level of potentially digestible NDF and therefore the effect of enzyme product on the degradability of NDF was not significant.

Conclusions

In conclusion, the effect of adding exogenous enzymes is influenced by factors such as type of enzyme product and dose level. The results found in this study suggest that application of exogenous cellulases has the potential to alter asymptotic GP and degradability of DM and ADF of the diet, but most of the results depend on enzyme product by dose level interaction. Future studies are required to find the ideal combination between enzyme product and dose level for optimising the degradation of ruminant feeds.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

ORCID

Nicola López-Villalobos https://orcid.org/0000-0001-6611-907X

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