Animal (2015), **9:7**, pp 1153–1162 © The Animal Consortium 2015. Parts of this are a work of the Government of Canada, represented by the Agriculture and Agri-Food Agency of Canada 2015.
doi:10.1017/S1751731115000221

animal

Effect of fibrolytic enzymes added to a *Andropogon gayanus* grass silage-concentrate diet on rumen fermentation in batch cultures and the artificial rumen (Rusitec)

G. O. Ribeiro Jr [1,4], L. C. Gonçalves[1], L. G. R. Pereira[2], A. V. Chaves[3], Y. Wang[4], K. A. Beauchemin[4] and T. A. McAllister[4†]

[1]*Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil;* [2]*Embrapa Gado de Leite – CNPGL, Juiz de Fora, MG 36038-330, Brazil;* [3]*Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia;* [4] *Lethbridge Research Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta T1J 4B1, Canada*

(Received 28 July 2014; Accepted 22 January 2015; First published online 20 February 2015)

In vitro *batch cultures were used to screen four fibrolytic enzyme mixtures at two dosages added to a 60 : 40 silage : concentrate diet containing the* C_4 *tropical grass* Andropogon gayanus *grass ensiled at two maturities – vegetative stage (VS) and flowering stage (FS). Based on these studies, one enzyme mixture was selected to treat the same diets and evaluate its impact on fermentation using an artificial rumen (Rusitec).* In vitro *batch cultures were conducted as a completely randomized design with two runs, four replicates per run and 12 treatments in a factorial arrangement (four enzyme mixtures × three doses). Enzyme additives (E1, E2, E3 and E4) were commercial products and contained a range of endoglucanase, exoglucanase and xylanase activities. Enzymes were added to the complete diet 2 h before incubation at 0, 2 and 4 μl/g of dry matter (DM). Gas production (GP) was measured after 3, 6, 12, 24 and 48 h of incubation. Disappearance of DM (DMD), NDF (NDFD) and ADF (ADFD) were determined after 24 and 48 h. For all four enzyme mixtures, a dosage effect* ($P < 0.05$) *was observed for NDFD and ADFD after 24 h and for DMD, NDFD and ADFD after 48 h of incubation of the VS diet. For the FS diet, a dosage effect was observed for GP and NDFD after 24 h and for GP, DMD, NDFD and ADFD after 48 h of incubation. There was no difference among enzyme mixtures nor was there an enzyme × dose interaction for the studied parameters. Because of the greatest numerical effect on NDF disappearance and the least cost price, enzyme mixture E2 at 4 μl/g of diet DM was selected for the Rusitec experiment. The enzyme did not impact* ($P > 0.05$) *DM, N, NDF or ADF disappearance after 48 h of incubation nor daily ammonia-N, volatile fatty acids or* CH_4 *production. However, enzyme application increased* ($P < 0.05$) *microbial N production in feed particle-associated (loosely-associated) and silage feed particle-bound (firmly associated) fractions. With* A. gayanus *silage diets, degradation may not be limited by microbial colonization, but rather by the ability of fibrolytic enzymes to degrade plant cell walls within this recalcitrant forage.*

Keywords: *Andropogon gayanus*, exogenous fibrolytic enzyme, rumen microbial growth, Rusitec, tropical grass silage

Implications

Fibrolytic enzymes have been included in ruminant diets with the aim of increasing fibre digestibility and consequently growth, milk yield and feed efficiency. The goal of this work was to study the effects of different fibrolytic enzyme mixtures on a tropical grass silage-concentrate diet. An overall increase in fibre degradation after 24 h of incubation in *in vitro* batch cultures was observed for all the enzyme mixtures investigated. However, the same result was not observed using an artificial rumen (Rusitec). Enzyme application increased microbial attachment to the silage, but did not increase fibre degradation in the Rusitec.

† E-mail: tim.mcallister@agr.gc.ca

Introduction

Andropogon gayanus is an important C_4 tropical grass because of its high biomass production and ability to tolerate long dry seasons, as well as the low fertile acidic soils typical of tropical savannas (Centro Internacional de Agricultura Tropical, 1990). Ensiling of tropical grasses is a common management approach to overcoming the scarcity of forage

and avoiding the reduction in forage quality that occurs during the dry season of tropical savannahs. Ribeiro *et al.* (2014) reported CP levels <70 g/kg of dry matter (DM) and NDF levels ranging from 705 to 765 g/kg of DM in *A. gayanus* grass ensiled at increasing stages of maturity in southeastern Brazil. High NDF and ADF levels for *A. gayanus* grass at the vegetative stage (VS) were also reported by Phengvichith and Ledin (2007) in Laos (724 ± 17.9 and 418 ± 30.5 g/kg of DM, respectively) and Ouédraogo-Koné *et al.* (2008) in Burkina Faso (701 ± 19.9 and 385 ± 13.5 g/kg of DM, respectively). These findings suggest that there is merit in using technologies that could enhance the degradability of fibre and increase microbial protein synthesis in ruminants fed *A. gayanus* silage.

Ruminants fed tropical forages obtain their energy mainly from rumen microbial fermentation of structurally complex carbohydrates within plant cell walls. However, this hydrolysis is not complete due to the complexity of cell wall architecture and the limited residence time of chopped forages in the rumen (Wang and McAllister, 2002). Enzymes have been included in ruminant diets with the aim of increasing plant cell wall digestibility, but responses have been variable (Meale *et al.*, 2014). Factors such as formulation, dosage, method of application and the nature of the substrate to which they are applied contribute to this variability (Beauchemin *et al.*, 2003). Some studies have examined the application of enzymes to tropical forages, with little benefit to the feed value of bermudagrass (*Cynodon dactylon*) silages (Mandebvu *et al.*, 1999), bermudagrass, Pensacola bahiagrass (*Paspalum notatum*) (Dean *et al.*, 2008) or Guinea grass (*Panicum maximum* var. Mombasa; Avellaneda *et al.*, 2009). The large variation in nutritional quality among tropical grasses, along with differences in preservation method (fresh forage, hay and silage), suggest that there is a need to further screen fibrolytic enzyme additives for their ability to improve the digestibility of tropical forages.

The objectives of this study were to screen four fibrolytic enzyme additives for their ability to improve the *in vitro* ruminal fermentation of an *A. gayanus* grass silage-concentrate diet using batch cultures. One enzyme mixture was then selected and assessed for its ability to improve ruminal fermentation and microbial protein synthesis of this diet using the rumen simulation technique.

Material and methods

Preparation of silage and silage diets

A. gayanus grass was produced in the rainy season of 2006–2007 using a previously established pasture in Lagoa Santa County, Minas Gerais, Brazil (19°35'36"S and 43°51'56"W; altitude 747 m). Soil samples were collected for analysis at a depth of 0–20 cm before the start of the experiment. To reduce acidity, limestone was applied (2000 kg/ha) at the beginning of the rainy season. After 30 days of growth, grass was cut 20 cm above the soil level and 250 kg/ha of 08 : 24 : 12 (N : P : K) and 100 kg/ha of 30 : 00 : 20 (N : P : K) were applied (Ribeiro *et al.*, 2014b). *A. gayanus* grass was harvested using a clearing saw (Stihl Ltd, Sao Leopoldo, RS, Brazil) after 56 (VS; 797 growing degree days (GDD)) and 112 (flowering stage (FS); 1604 GDD) days with 10°C as a baseline for GDD. Forages were chopped to a theoretical length of 10–25 mm and ensiled as described previously (Ribeiro *et al.*, 2015). Chopped grass was ensiled in plastic bags within 200 l steel barrels (15 barrels/growth stage), packed by trampling and sealed. Five barrels of each treatment were randomly selected and opened after 150 days of ensiling. Silage (500 g) from five different locations within each barrel were collected and pooled by treatment. Subsamples were then dried at 55°C for 48 h and ground to pass through a 5-mm screen (Arthur Thomas Co., Philadelphia, PA, USA). A second subsample (500 g) of each treatment was pressed using a hydraulic press (2 kgf/cm^3) to obtain ≈100 ml of extract. The pH of the extract was immediately measured (Orion Model 260A; Fisher Scientific, Toronto, ON, Canada) and subsamples were stored at −20°C. Diets were formulated to contain 60% silage and 40% of a corn–soyabean-based concentrate on a DM basis. Both silages and concentrate were ground to pass a 1-mm screen for diets in Experiment 1 and 4 and through 2-mm screens, respectively, for diets in Experiment 2. Silage and concentrate in Experiment 1 were thoroughly mixed for each diet before filling of the filter bags and treatment with enzymes. Chemical composition of silage and concentrate are shown in Table 1.

Experiment 1 Screening enzyme products and determination of the optimal application dosage using batch cultures.

The exogenous fibrolytic enzymes (EFE) were applied to complete diets at dosages of 0 (Control), 2 and 4 μl/g of substrate DM. A series of *in vitro* incubations were carried out in a completely randomized design with two runs and four replicates per run. The runs in each series were conducted on separate days.

The EFE evaluated were as follows: a 75 : 25 mixture of Cellulase Plus and Xylanase Plus (E1; from *Trichoderma longibrachiatum*; Dyadic International, Jupiter, FL, USA); Rovabio Excel LC2 (E2; from *Penicillium funiculosum*; Adisseo France SAS, Antony, France); Rovabio Rips (E3; from *P. funiculosum*; Adisseo France SAS) and Econase RDE (E4; from *T. longibrachiatum*; AB Vista, Marlborough, UK), and were characterized for enzyme activities as previously described (Colombatto and Beauchemin, 2003; Phakachoed *et al.,* 2013). Formulations contained mainly xylanase activity (1804, 1372, 616 and 3034 μmoles of xylose released from oat spelt xylan per min per ml of product for E1, E2, E3 and E4, respectively) and endoglucanase activity (352, 159, 59 and 360 μmoles of glucose released from medium-viscosity carboxymethylcellulose per min per ml for E1, E2, E3 and E4, respectively). Dosages were selected based on previous *in vitro* work with the same enzymes using corn silage (Phakachoed *et al.*, 2013).

Rumen fluid for *in vitro* incubations was obtained from three ruminally cannulated Angus × Hereford cattle fed a

Table 1 *Ingredients and chemical composition of silages and concentrate used to formulate diets*

Item	Vegetative stage			Flowering stage		
	Silage[1]	Concentrate	Diet	Silage[2]	Concentrate	Diet
Chemical composition						
Dry matter (DM) (g/kg)	177	915	908	262	922	911
Organic matter (g/kg DM)	910	960	916	936	944	930
CP (g/kg DM)	69	231	134	54	258	136
Starch (g/kg DM)	5.7	438	179	0.1	403	161
NDF (g/kg DM)	693	106	440	712	107	450
ADF (g/kg DM)	423	36	255	417	38	249
ADL (g/kg DM)	61	–	–	71	–	–
WSC (g/kg DM)	2.2	–	–	2.5	–	–
Ammonia-N (g/kg total N)	112	–	–	50	–	–
pH	5.3	–	–	4.7	–	–
Acetic acid (g/kg DM)	66.1	–	–	8.2	–	–
Propionic acid (g/kg DM)	24.1	–	–	1.1	–	–
Butyric acid (g/kg DM)	61.0	–	–	20.3	–	–
Lactic acid (g/kg DM)	nd	–	–	0.39	–	–
Diet ingredient (g/kg DM)						
Silage	600			600		
Ground corn	257			238		
Soyabean meal	133			152		
Mineral/vitamin premix[3]	10			10		

WSC = water-soluble carbohydrates.
[1]*Adropogon gayanus* grass was ensiled at the vegetative stage (56 days of re-growth).
[2]*A. gayanus* grass was ensiled at the flowering stage (112 days of re-growth).
[3]Per kg of dietary DM: 1.2 g of salt, 4.7 g of calcium carbonate, 3.6 g of dicalcium phosphate, 65 mg of Zn, 28 mg of Mn, 15 mg of Cu, 0.7 mg of I, 0.2 mg of Co, 0.3 mg of Se, 6000 IU of vitamin A, 600 IU of vitamin D and 60 IU of vitamin E.

mixed diet of barley silage, rolled barley grain and a mineral/vitamin supplement (70 : 27 : 3, % DM basis). Cows used as donors of rumen fluid were cared for in accordance with the guidelines of the Canadian Council of Animal Care (1993). Rumen fluid was collected 2 h after the morning feeding, filtered through four layers of cheesecloth, combined in equal portions and transported in a pre-warmed thermos to the laboratory. Rumen fluid and mineral buffer with 0.5 ml of a cysteine sulphide solution (Menke *et al*., 1979) were mixed 1 : 2 under a stream of O_2-free CO_2 at 39°C.

For each *in vitro* incubation, 0.5 g DM of substrate was weighed into an acetone-washed, pre-weighed filter bag (model F57; Ankom Technology Corp., Macedon, NY, USA). Four replicates were prepared for each treatment for each incubation time. The EFE was diluted with distilled water and sprayed (50 μl) directly onto the substrates in filter bags (before sealing) at either 0 (control), 2 or 4 μl/g of DM. Bags were heat-sealed and placed into empty 125-ml amber serum vials and retained at room temperature for 2 h before incubation.

Inoculum was transferred (80 ml) into vials under a stream of O_2-free CO_2. Vials were sealed with rubber stoppers and incubated at 39°C on an orbital shaker set at 90 oscillations/min for 24 and 48 h. Four blank vials containing empty filter bags and inoculum were incubated to correct for gas production (GP) and DM disappearance. GP from each vial was measured after 3, 6, 12, 24 and 48 h using a water-displacement apparatus (Fedorak and Hrudey, 1983). After 24 and 48 h, four bottles from each treatment were removed from the incubator and placed on ice. Filter bags were removed from the bottles, thoroughly rinsed with cold water and dried at 55°C for 48 h to calculate apparent DM disappearance. The residues in the bags were also analysed for NDF and ADF to estimate NDF and ADF disappearance using the sequential method with ANKOM200 Fibre Analyzer (Ankom Technology Corp.) and reagents, as described by Van Soest *et al.* (1991). Sodium sulphite and α-amylase were used during NDF determination and values were expressed exclusive of ash. Total cumulative gas production (TGP, ml) at 24 and 48 h, corresponding with DM disappearance, was calculated by summing the blank-corrected gas volumes recorded at previous measurement times.

Experiment 2 Determination of EFE on ruminal digestion and rumen microbial protein production using Rusitec.

Experimental design and treatments

As all enzyme preparations had a positive impact on NDF disappearance at 24 h in Experiment 1, E2 (Rovabio Excel LC2) applied at 4 μl/g substrate DM was selected for further assessment in the Rusitec, as it was commercially available at the least cost per unit. The experiment was a completely randomized 2×2 factorial design using two eight-vessel Rusitecs (Czerkawski and Breckenridge, 1977). Diets were as

described in Experiment 1, with the silage either not treated or treated with E2 at 4 μl/g of diet DM. Four replicate vessels were used for each treatment. Silage and concentrate were incubated in separate polyester bags (for concentrate: 50×100 mm; pore size = 50 μm; for silage: 100×200 mm; pore size = 50 μm; ANKOM; Ankom Technology Corp.). For the Rusitec, E2 was applied only to the silage at the same rate as for the complete diet described above, as it was felt that improved 24 h disappearance in the *in vitro* batch culture studies arose mainly from the impact of the additive on silage NDF. As per batch cultures, silage was retained at room temperature for 2 h after treatment with E2 before being placed in the fermenters.

Experimental procedure

To begin the experiment, each fermenter was filled with 180 ml of pre-warmed artificial saliva (McDougall, 1948) modified to contain 0.3 g/l of $(NH_4)_2SO_4$, and 720 ml of filtered rumen fluid. Solid rumen digesta (20 g), silage (6 g) and concentrate (4 g) for each respective treatment were placed in three separate polyester bags within each fermenter. Rumen fluid was obtained from the same three ruminally fistulated cattle, as described in Experiment 1. Rumen solids were obtained from composited samples collected from four locations within the rumen. Fermenters were immersed in a water bath at 39°C and bags within the vessels were moved up and down within the fermentation fluid at eight cycles per min. After 24 h, the nylon bag containing solid rumen digesta was replaced with one bag containing silage and one containing concentrate. Thereafter, polyester bags were replaced daily, resulting in each bag remaining in the fermenter for 48 h.

Artificial saliva (pH 8.2) was infused continuously into each fermenter at 2.9%/h, replacing 70% of the fermenter volume daily. The experiment consisted of 8 days of adaptation (day 1 to 8) and 7 days of sampling (day 9 to 15). Effluent from each fermenter was collected into a 1 l flask, and gas was collected into a re-usable 2 l vinyl collection bag (Curity®; Conviden Ltd, Mansfield, MA, USA) attached to each effluent flask. Daily total GP and effluent volume from each fermenter were recorded at the time of feed-bag exchange and measurement of fluid pH. During feed-bag exchange, fermenters were flushed with O_2-free CO_2.

Sample collection and analysis

From day 9 to 11, the feed bags removed at 48 h were washed under cold tap water until the water was clear. Bags were dried at 55°C to a constant weight. The residue in the silage bags was kept separately for each of the 3 days, whereas the residues in concentrate bags were pooled over 3 days to ensure sufficient sample for chemical analysis. All samples were ground to pass through a 1-mm screen before chemical analysis.

Daily total GP was determined throughout the experiment using a gas metre (Model DM3A, Alexander-Wright, London, England, UK). From day 9 to 14, a 20-ml gas sample was taken from the septum of each collection bag using a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) and transferred to evacuated 6.8-ml exetainers (Labco Ltd, Wycombe, Bucks, UK) for immediate analysis of CH_4.

To determine daily volatile fatty acids (VFA) and NH_3-N production, effluent was collected daily from day 9 to 11 in flasks containing 20 ml of 3.66 M H_2SO_4 (20%, vol/vol; Giraldo *et al.*, 2007b). Subsamples of the fermenter effluent (2.5 ml) were taken directly from the effluent flask at the time of feed-bag exchange and were placed in screw-capped vials containing 0.5 ml of 25% (wt/wt) metaphosphoric acid and stored at −20°C until VFA analysis. At the same time, a 2.5 ml subsample of fermenter effluent was also placed in a screw-capped vial with 0.5 ml of 65% (wt/vol) trichloroacetic acid until analysed for NH_3-N.

To estimate microbial protein synthesis, bacteria in the fermenters were labelled using ^{15}N. On day 8, effluent and feed residue solids were sampled for the determination of background ^{15}N and 0.3 g/l $(NH_4)_2SO_4$ in McDougall's buffer was replaced with 0.3 g/l ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO, USA; minimum ^{15}N enrichment 10.01 atom%) until the end of the experiment. On days 12, 13 and 14, 24 h accumulation of effluent in each flask was preserved with 20% (wt/vol) sodium azide (3 ml) and 50 ml was subsampled for isolation of liquid-associated bacteria.

Feed particle-associated (FPA) and feed particle-bound (FPB) bacterial fractions were prepared from 48-h feed residues on days 12, 13 and 14. Upon removal from the fermenters, solids were squeezed to expel excess liquid from both the silage and concentrate feed bags. Bags were placed together in a plastic bag with 20 ml of McDougall (1948) buffer and processed for 60 s in a Stomacher 400 Laboratory Blender (Seward Medical Ltd, London, England, UK). The processed liquid was squeezed out, poured off and retained. Feed residues were washed twice with 10 ml of McDougall's buffer in each wash. The wash buffer was retained and pooled with the initially expressed fluid to obtain the FPA bacterial fraction, and the total volume was recorded. Washed solid feed residues were considered to represent the FPB bacterial fraction.

To determine ^{15}N concentration, effluent liquid samples were centrifuged ($20\,000 \times \mathbf{g}$, 30 min, 4°C) and the resulting pellets were washed with de-ionized water and centrifuged three times ($20\,000 \times \mathbf{g}$, 30 min, 4°C). The pellet was then re-suspended in distilled water and lyophilized for N and ^{15}N determination. The FPA bacterial samples collected from the stomaching process were centrifuged ($500 \times \mathbf{g}$, 10 min, 4°C). The supernatant was subsequently centrifuged ($20\,000 \times \mathbf{g}$, 30 min, 4°C) and the resulting pellet was washed three times as previously described. The pellet was then re-suspended in distilled water and lyophilized for N and ^{15}N determination. Washed feed residues (FPB fraction) were dried at 55°C for 48 h, weighed for DM determination and ball ground using a mixer mill (MM 400; Retsch Inc., Newtown, PA, USA) before analysis of total N and ^{15}N concentrations.

Protozoa counts were determined daily from day 9 to 11 using pooled fluid samples collected daily from both the 48 h silage and concentrate feed bags from each fermenter.

Bags were pressed to expel fermentation fluid and a 2.5 ml subsample was obtained and preserved using 2.5 ml of methyl green formalin-saline solution. Protozoa samples were stored in the dark at room temperature until enumerated by light microscopy with a Levy–Hausser counting chamber (Hausser Scientific, Horsham, PA, USA).

Feed and fermentation residues were analysed for DM (Association of Official Analytical Chemists (AOAC), 2006; method 930.15) and ash (AOAC, 2006; method 942.05). The NDF and ADF contents were determined by the sequential method with the ANKOM200 Fibre Analyzer using reagents as described by Van Soest *et al.* (1991) and expressed exclusive of ash. Sodium sulphite and α-amylase were used during NDF determination. Lignin analyses were performed on ADF residues, using the direct sulphuric acid method (Robertson and Van Soest, 1981). Total N (AOAC, 2006; method 990.03) and atom per cent excess (APE) of ^{15}N were analysed using a MS (NA 1500; Carlo Erba Instruments, Rodano, Italy). Starch was determined in concentrate and silage samples as described by Karkalas (1985), and water-soluble carbohydrates were determined in silage according to Bailey (1967).

Silage extract was analysed for NH_3-N using the Kjeldahl method, with the volatile N fraction distilled by heating extracts at pH > 7 with ~2 g of MgO, and for VFA using a GC-17A Gas Chromatograph (Shimadzu Corp., Kyoto, Japan), equipped with a flame ionization detector and fitted with a Nukol fused silica capillary column (15 m; 0.53 mm i.d.; 0.5 µm film thickness; Supelco, Bellefonte, PA, USA), as described by Playne (1985). The gas chromatograph was operated isothermally at 200°C (column), with the inlet and detectors maintained at 225°C.

Concentrations of VFA and NH_3-N in the liquid effluent were analysed by gas chromatography (Wang *et al.*, 2001) and the modified Berthelot method (Rhine *et al.*, 1998), respectively. Methane concentration in gas was determined using a Varian gas chromatograph equipped with GS-CarbonPLOT 30 m × 0.32 mm × 3 µm column and thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, ON, Canada) at an isothermal oven temperature of 35°C with helium as the carrier gas (27 cm/s).

Calculations

Total daily effluent microbial N (MN) production (mg/day) was calculated based on N concentrations in the bacterial pellet from the effluent and sample size (weight and volume). The MN production (mg/day) from the FPA fraction obtained from the stomacher extraction process was estimated as described for the effluent microbial fraction. The MN production (mg/day) from the FPB fraction was estimated using the following equation:

$$MN = \frac{APEinRN}{APEinMN} \times RN$$

where APE in residue nitrogen (RN) is the per cent excess of ^{15}N in the solid residue, APE in MN is the per cent excess of ^{15}N in the microbial fraction of the effluent and RN is the total N in the residues (in mg; Wang *et al.*, 2000). Total daily MN production (mg/day) was calculated as the sum of microbial production in the effluent, FPA, FPB of silage residues and FPB of concentrate residues.

True DM disappearance was calculated by subtracting the microbial mass from feed residues. Microbial mass in feed residues was calculated by multiplying MN production (mg) in feed residues by the microbial mass per mg of MN (g of DM of microbial pellet/mg of MN). Microbial mass per mg of MN was determined in FPA bacterial pellets.

Ammonia-N and daily VFA production were calculated by multiplying the concentration of the fermentation end product in the effluent by the daily production of effluent.

Statistical analysis

Data were analysed using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA). In Experiment 1, data obtained from the two diets were analysed separately as completely randomized design with enzyme additive, dose and enzyme × dose interactions included in the model as fixed effects according to the model below:

$$Y_{ijkl} = \mu + E_i + D_j + ED_{ij} + R_k + e_{ijkl}$$

where Y_{ijkl} is the observation, μ the overall mean effect, E_i the effect of enzyme additive (E1, E2, E3 or E4; d.f. = 3), D_j the effect of dose (0, 2 or 4 µl/g DM; d.f. = 2), ED_{ij} the effect of enzyme × dose interactions (d.f. = 6), R_k the random effect of run (1–2) and e_{ijkl} the residual error. When the main effect of dose was significant ($P < 0.05$), orthogonal polynomial contrasts were performed to test for linear or quadratic dose responses. For the Rusitec (Experiment 2), the MIXED procedure model included the fixed effects of diet, enzyme, day of sampling, diet × enzyme, diet × day, enzyme × day and diet × enzyme × day, with the day of sampling from each fermenter treated as a repeated measure accordingly to the following model:

$$Y_{ijklm} = \mu + E_i + F_j + D_k + EF_{ij} + ED_{ik} + FD_{jk} + EFD_{ijk} + R_l + e_{ijklm}$$

where Y_{ijklm} is the observation, μ the overall mean effect, E_i the effect of enzyme (enzyme or no enzyme; d.f. = 1), F_j the effect of diet (VS or FS; d.f. = 1), D_k the effect of day of sampling, EF_{ij} the effect of enzyme × diet interactions (d.f. = 1), ED_{ik} the effect of enzyme × day of sampling interactions, FD_{jk} the effect of diet × day of sampling interactions, EFD_{ijk} the effect of enzyme × diet × day of sampling interactions, R_l the random effect of fermenter (1–4) and e_{ijklm} the residual error. Therefore, the individual fermenter was used as the experimental unit for statistical analysis. The minimum values of Akaike's Information Criterion were used to select the covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured and banded for each parameter. Means were compared using the least square mean linear hypothesis. Significance was declared at $P \leqslant 0.05$. There were no diet × day, enzyme × day and diet × enzyme × day effects for all the parameters

evaluated, and as a result neither day nor interactions involving day are reported in the tables.

Results

Enzyme screening

There was no enzyme or enzyme × dose interaction ($P > 0.05$) for DM or NDF disappearances or TGP after 24 and 48 h of incubation for either the FS or VS diets (Table 2). There was also no enzyme or enzyme × dose interaction ($P > 0.05$) for ADF disappearance after 24 h of incubation for either diet and after 48 h for the FS diet. An enzyme effect ($P < 0.05$) for ADF disappearance after 48 h of incubation for the VS diet was observed, with lower ($P < 0.05$) ADF disappearance for E4 compared with the other enzyme products. However, dosage did affect ($P < 0.05$) NDF or ADF disappearance after 24 h or DM, NDF and ADF disappearance after 48 h when the diet contained VS silage. For the diet containing grass ensiled at the FS, a dosage effect ($P < 0.05$) was observed for NDF and TGP disappearance at 24 h and for TGP and disappearances of DM, NDF and ADF after 48 h of incubation.

For the VS diet, a higher dose quadratically influenced ($P < 0.05$) NDF and ADF disappearance after 24 h of incubation. With 48 h of incubation, increasing enzyme dose also resulted in a quadratic effect ($P < 0.05$) on DM, NDF and ADF disappearances of the VS diet, with a lower disappearance being observed for 2 μl/g of substrate DM. Increasing enzyme dose linearly increased ($P < 0.05$) NDF disappearance and TGP in the FS diet after 24 h and ADF disappearance after 48 h of incubation. Increasing enzyme dose for FS diet also quadratically impacted ($P < 0.05$) DM and NDF disappearances and TGP after 48 h of incubation.

In general, an increase in NDF disappearance after 24 h was observed with all enzyme mixtures investigated for both VS

Table 2 *Effect of enzyme (E) and dose (D) on the disappearance of dry matter (DM), NDF, ADF and total cumulative gas production (TGP) from* Andropogon gayanus *grass silage-concentrate diet after 24 and 48 h of incubation in ruminal fluid (*n = 8*)*

	Enzyme[3]				Dose (μl/g DM)					*P*-value		
	E1	E2	E3	E4	0	2	4	Contrast	RMSE	Enzyme	Dose	Enzyme × Dose
Vegetative stage[1]												
24 h												
Disappearance (g/kg)												
DM	449	457	448	454	446	457	453	ns	25	0.58	0.28	0.92
NDF	80	80	79	83	65[b]	94[a]	83[a]	L, Q	35	0.99	0.01	0.77
ADF	63	66	75	64	52[b]	80[a]	70[a]	L, Q	31	0.66	0.01	0.06
TGP (ml/g DM)	81	80	81	79	80	80	80	ns	7	0.48	0.91	0.58
48 h												
Disappearance (g/kg)												
DM	577	576	578	562	577[a]	561[b]	581[a]	Q	27	0.19	0.02	0.09
NDF	284	286	291	258	286[a]	257[b]	296[a]	Q	49	0.14	0.01	0.21
ADF	263[a]	264[a]	271[a]	230[b]	268[a]	232[b]	271[a]	Q	50	0.04	0.01	0.14
TGP (ml/g DM)	118	118	120	115	120	115	118	ns	8	0.33	0.08	0.49
Flowering stage[2]												
24 h												
Disappearance (g/kg)												
DM	476	475	474	474	469	474	481	ns	21	0.98	0.10	0.99
NDF	85	86	81	85	75[b]	83[ab]	94[a]	L	29	0.94	0.04	0.99
ADF	87	84	77	84	78	85	87	ns	33	0.78	0.53	0.99
TGP (ml/g DM)	90	89	89	90	86[b]	90[a]	92[a]	L	9	0.94	0.01	0.81
48 h												
Disappearance (g/kg)												
DM	600	598	600	604	590[b]	608[a]	604[a]	L, Q	25	0.87	0.01	0.45
NDF	299	295	299	308	277[b]	317[a]	307[a]	L, Q	48	0.79	0.01	0.13
ADF	291	279	290	297	268[b]	303[a]	297[a]	L	52	0.69	0.02	0.14
TGP (ml/g DM)	127	124	126	127	121[b]	129[a]	127[a]	L, Q	9	0.79	0.01	0.33

L, Q: within a row, the main effect of dose is linear or quadratic, respectively ($P < 0.05$). ns: within a row, the main effect of dose is not linear or quadratic ($P > 0.05$).
[a,b]Means within a row within the main effect of enzyme or dose having different superscript letters are different at $P < 0.05$.
[1]*Andropogon gayanus* grass was ensiled at the vegetative stage (56 days of re-growth) and was mixed with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).
[2]*A. gayanus* grass was ensiled at the flowering stage (112 days of re-growth) and was mixed with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).
[3]E1: 75 : 25 combination of Cellulase Plus and Xylanase Plus (source organism *Trichoderma longibrachiatum*; Dyadic International, FL, USA); E2: Rovabio Excel LC2 (source organism *Penicillium funiculosum*; Adisseo France SAS, Antony, France); E3: Rovabio Rips (source organism *P. funiculosum*; Adisseo France SAS) and E4: Econase RDE (*T. longibrachiatum*; AB Vista, Marlborough, UK).

and FS diets. Of the enzymes screened, E2 was selected for the Rusitec experiment, because it increased NDF disappearance after 24 h incubation of the VS and the FS diet by 10% and 33%, respectively, and was commercially available at the least cost price. The significant quadratic effect of dose suggested that maximal response was achieved at 4 μl/g of diet DM.

Rusitec
There were no enzyme or diet × enzyme interactions ($P > 0.05$) on total (silage + concentrate), silage or concentrate true DM and N disappearances (Table 3). Similarly, no enzyme or diet × enzyme interactions ($P > 0.05$) were observed for silage NDF or ADF disappearance. There was also no effect of diet ($P > 0.05$) on total, silage or concentrate true DM disappearance. Higher ($P < 0.05$) silage N disappearance was observed for the FS diet compared with the VS diet, but disappearance of concentrate N was higher ($P < 0.05$) for the VS diet. As a result, total N disappearance was higher ($P < 0.05$) for the VS diet than for the FS diet. No difference was observed ($P > 0.05$) between diets for silage NDF or ADF disappearance.

There were no enzyme or diet × enzyme interactions ($P > 0.05$) for pH, daily NH_3-N and VFA production or acetate : propionate ratio (C2 : C3) (Table 4). The pH, daily production of total VFA, acetate, propionate, butyrate, isobutyrate and caproate or C2 : C3 ratio also did not differ ($P > 0.05$) among diets. Daily production of ammonia-N and isovalerate were higher ($P < 0.05$) for the VS diet compared with the FS diet. However, valerate production was higher ($P < 0.05$) for the FS diet than for the VS diet.

No significant effect ($P > 0.05$) of diet, enzyme or diet × enzyme was observed for GP (ml/day and ml/g DM fermented) or CH_4 production (as % of total gas, mg/day and mg/g of DM incubated or fermented).

Diet and diet × enzyme interactions were not observed ($P > 0.05$) for total, silage FPB fraction, FPA or effluent MN production, but there was a diet × enzyme interaction ($P < 0.05$) for the concentrate FPB MN fraction. Enzyme application reduced ($P < 0.05$) MN associated with the concentrate FPB fraction of the VS diet, but increased ($P < 0.05$) its association with the FS diet. Enzyme application also increased ($P < 0.05$) the MN associated with the FPA and silage FPB fractions in both diets. No enzyme effect ($P > 0.05$) on MN production was observed in the other fractions (effluent and total). There were no effects ($P > 0.05$) of diet, enzyme or diet × enzyme on the efficiency of microbial synthesis or numbers of protozoa.

Discussion

Enzyme screening
It has been suggested that a major limitation to the widespread commercial use of fibrolytic enzymes for ruminants is the variability of responses for a given product depending upon the diet and feeding conditions (Beauchemin *et al.*, 2003). Phakachoed *et al.* (2013) tested the same four EFE as used in this study with corn silage, and reported that all enzymes resulted in an overall increase in DM, NDF and ADF disappearance after 24 and 48 h of *in vitro* ruminal incubation. An overall increase in fibre (NDF and ADF) degradation was also observed for all enzyme mixtures investigated in our *in vitro* batch cultures. The C_4 grasses are the main forage for livestock farming in tropical and subtropical regions of the world, and their nutritive value is often intrinsically low. Therefore, the performance of ruminants fed tropical grasses is often suboptimal (Facchini *et al.*, 2012). Increasing the digestibility and consequently the intake of metabolizable energy by ruminants fed tropical forages may have a huge impact on livestock production, contributing to sustainable food production and increased profitability in the agricultural sector of many developing nations.

According to Colombatto *et al.* (2003a and 2003b), EFE have their greatest impact on fibre digestion within the first

Table 3 *Effect of exogenous fibrolytic enzymes[1] (EFE) on true DM, nitrogen (N), NDF and ADF disappearance of* Andropogon gayanus *grass silage-concentrate diet differing in grass maturity at ensiling in the rumen simulation technique (*n *= 4)*

	Stage		Enzyme[1]			*P*-value		
Item	Vegetative[2]	Flowering[3]	− EFE	+EFE	RMSE	Diet	EFE	Diet × EFE
Total true DM disappearance	674	694	688	680	42	0.15	0.60	0.16
Silage true DM disappearance	552	574	561	565	34	0.09	0.72	0.32
Concentrate true DM disappearance	856	858	864	850	66	0.91	0.50	0.35
Total N disappearance	769	632	708	693	77	0.001	0.6	0.46
Silage N disappearance	455	487	460	482	47	0.02	0.11	0.16
Concentrate N disappearance	898	674	797	775	70	0.001	0.52	0.35
Silage NDF disappearance	517	534	528	523	42	0.17	0.68	0.73
Silage ADF disappearance	534	562	550	547	49	0.06	0.84	0.80

[1]Rovabio Excel LC2 (source organism *Penicillium funiculosum*; Adisseo France SAS, Antony, France) at 4 μl/g of substrate DM. −EFE = diet without enzyme; +EFE = diet with enzyme.
[2]*Andropogon gayanus* grass was ensiled at the vegetative stage (56 days of re-growth) and was incubated with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).
[3]*A. gayanus* grass was ensiled at the flowering stage (112 days of re-growth) and was incubated with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).

Table 4 *Effect of exogenous fibrolytic enzyme[1] (EFE) on pH, ammonia-N (mg/day), volatile fatty acid (VFA, mmol/day), gas, methane (CH_4) and microbial N production, efficiency of microbial synthesis (EMS) and protozoa numbers of* Andropogon gayanus *grass silage-concentrate diet differing in grass maturity at ensiling in the rumen simulation technique (*n *= 4)*

	Stage		Enzyme[1]			*P*-value		
Item	Vegetative[2]	Flowering[3]	−EFE	+EFE	RMSE	Diet	EFE	Diet × EFE
pH	6.78	6.80	6.79	6.78	0.06	0.14	0.80	0.22
Ammonia-N	81.0	56.4	70.6	66.9	11.4	0.001	0.30	0.07
Total VFA	49.9	48.4	49.4	48.9	7.3	0.48	0.82	0.29
Acetate (C2)	28.1	27.1	27.9	27.2	4.2	0.40	0.59	0.36
Propionate (C3)	14.0	13.0	13.4	13.5	2.0	0.10	0.94	0.29
Butyrate	5.1	5.5	5.2	5.3	0.7	0.07	0.76	0.18
Valerate	1.2	1.8	1.4	1.5	0.2	0.001	0.22	0.02
Isovalerate	0.71	0.45	0.58	0.57	0.12	0.001	0.81	0.59
Isobutyrate	0.55	0.41	0.46	0.49	0.26	0.08	0.70	0.98
Caproate	0.27	0.30	0.28	0.29	0.06	0.07	0.58	0.89
C2/C3	2.02	2.09	2.08	2.03	0.09	0.11	0.31	0.73
Gas (ml/day)	1906	1603	1765	1743	373	0.10	0.90	0.60
Gas (ml/g DM fermented)	333	279	301	311	65	0.10	0.73	0.60
CH_4 (%)	5.01	4.88	5.1	4.79	1.23	0.65	0.25	0.45
CH_4 (mg/day)	75.2	59.4	67.4	67.2	23.0	0.09	0.99	0.99
CH_4 (mg/g DM incubated)	8.03	6.34	7.19	7.18	2.45	0.09	0.99	0.99
CH_4 (mg/g DM fermented)	11.91	9.63	10.78	10.76	3.41	0.10	0.99	0.84
Production of microbial N (mg/day)								
Total	81.4	85.0	83.0	83.4	6.9	0.08	0.85	0.42
Feed particle-bound (FPB) Concentrate	5.5	5.0	5.3	5.2	0.9	0.29	0.84	0.02
FPB Silage	27.7	27.9	26.7	28.9	2.6	0.88	0.05	0.95
Feed particle-associated	6.7	7.0	6.4	7.3	1.4	0.52	0.04	0.49
Effluent	41.5	44.8	44.4	42.0	6.4	0.08	0.21	0.26
EMS (g microbial N/kg organic matter truly fermented)	13.5	13.8	13.6	13.8	1.3	0.48	0.66	0.77
Protozoa ($\times 10^4$/ml)	3.01	2.84	2.96	2.88	1.05	0.68	0.85	0.61

−EFE = diet without enzyme; +EFE = diet with enzyme.
[1]Rovabio Excel LC2 (source organism *Penicillium funiculosum*; Adisseo France SAS, Antony, France) at 4 μl/g of substrate DM.
[2]*Andropogon gayanus* grass was ensiled at the vegetative stage (56 days of re-growth) and was incubated with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).
[3]*A. gayanus* grass was ensiled at the flowering stage (112 days of re-growth) and was incubated with a corn–soyabean-based concentrate at the ratio of 60 : 40 (silage : concentrate, DM basis).

6 to 12 h of incubation, with little impact on total GP or the extent of fibre digestion after 24 to 96 h. Eun and Beauchemin (2007) observed increases in GP and apparent organic matter degradability after 18 h of incubation when EFE were applied to alfalfa hay. Short-term, rather than long-term (e.g., 48 h), incubations have been used to assess the effect of fibrolytic enzymes, because long-term incubations are not representative of the residence time of fibre in the rumen of high producing dairy cows (Colombatto *et al.*, 2003b; Eun and Beauchemin, 2007). However, in beef cattle fed high tropical forage (C_4) diets (Wylie *et al.*, 2000), ruminal residence times can be substantially longer, possibly making a longer incubation period (48 h) more relevant to studying responses to EFE. In the present study, the DM disappearance of the FS diet after 48 h of incubation was increased with EFE application.

Rusitec

The higher total N disappearance and levels of silage ammonia-N in the VS diet resulted in higher NH_3-N in the fermenter fluid. Supplementing FS silage with concentrate promoted silage DM disappearance, resulting in values that were similar to the diets containing the VS silage. In a previous metabolism study, conducted with rams receiving these same silages but with no concentrate, grass ensiled at the FS exhibited a lower apparent DM digestibility than grass ensiled at the VS (380 and 526 g/kg DM, respectively; Ribeiro *et al.*, 2015). The CP content in the grass ensiled at the FS was <70 g/kg DM and most probably limited microbial growth in the rumen of the rams, resulting in reduced diet digestibility.

Only minor differences in VFA profile were noted across diets, with higher isovalerate production from the VS than the FS diet, a result that is likely reflective of the higher protein content leading to increased deamination of leucine. However, no differences in daily acetate, propionate, total VFA production or C2 : C3 ratio were observed. The similarities between the VS and FS diets observed for gas (ml/day and ml/g DM fermented) and CH_4 production (mg/day and mg/g of DM incubated or fermented) are consistent with the

total true DM disappearance, VFA production and C2 : C3 ratio, which did not differ between these two diets.

Application of EFE 2 h before feeding did not impact DM, N, NDF or ADF disappearance after 48 h of incubation in the Rusitec. Daily NH_3-N, VFA and CH_4 productions were also not affected by EFE in this study. Wang *et al.* (2001) reported that applying an enzyme preparation to the feed 24 h before feeding increased barley grain DM disappearance after 48 h of incubation, but had no effect when enzymes were directly infused into the fermenters. Although Giraldo *et al.* (2007a) have shown an increase in 48 h DM, NDF and ADF disappearance in the Rusitec with enzyme application to a forage-based diet 24 h before feeding, additional work by this same group observed these responses after 6 and 24 h of incubation, but not after 48 h (Giraldo *et al.*, 2007b). In addition, Wang *et al.* (2001) did not observe higher DM or NDF disappearance of alfalfa hay after 48 h of incubation when enzymes were applied 24 h before feeding. According to Nsereko *et al.* (2000), most of the release of reducing sugars from high DM feeds as a result of the addition of EFE occurs within 2 h after application. The enzyme-mediated increase in NDF and ADF disappearance after 24 h of incubation in our batch culture study also supports the contention that applying enzymes 2 h before feeding provides sufficient time for the hydrolysis of sugars from plant cell walls. Supplementing ruminant diets with EFE has been reported to increase the rate, but seldom the extent of feed digestion (Meale *et al.*, 2014). The lack of an effect of EFE on daily NH_3-N, VFA and CH_4 production in this study agrees with the lack of an effect of this additive on DM and fibre digestibility. The results from this Rusitec study are in agreement with previous *in situ* work carried out with bermudagrass (*C. dactylon*) silages (Mandebvu *et al.*, 1999) and with bermudagrass hay and Pensacola bahiagrass (*P. notatum*) hay (Dean *et al.*, 2008) and *in vivo* with Guinea grass (*P. maximum* var. Mombasa) hay (Avellaneda *et al.*, 2009), studies which did not find any effects of fibrolytic enzymes on the digestion of these high-fibre tropical grasses.

A synergistic relationship between EFE and rumen microbiota and an increase in bacterial attachment are proposed modes of action, whereby exogenous enzymes may improve feed digestion in the rumen (Morgavi *et al.*, 2000). However, the exact mechanism that stimulates microbial attachment is unknown. According to Cheng and McAllister (1997), metabolic products of primary colonizers of feedstuffs are thought to attract secondary colonizers to feed surfaces and stimulate attachment. Wang *et al.* (2001) proposed that products released as a result of hydrolysis by EFE accumulate on the surface of the feed particles and serve as chemoattractants to ruminal microbes. Increased ^{15}N incorporation into FPA and FPB fraction by EFE in the Rusitec has been previously observed (Wang *et al.*, 2001; Giraldo *et al.*, 2007a). Applying an EFE to tropical silage 2 h before feeding in this study increased microbial colonization of silage, as shown by increased MN production in FPA and silage FPB fractions; however, DM and fibre degradation remained unaffected. These results suggest that enzyme application 2 h before feeding was sufficient to promote interaction between the enzyme and substrate, but in these tropical silage diets degradation may not be limited by microbial colonization, but rather by the ability of fibrolytic enzymes to degrade these recalcitrant substrates. Other factors such as organization of forage plant tissues (Wilson, 1993), anatomical limitations to digestion of thick-walled fibre particles (Wilson and Mertens, 1995) and lignin cross-linkages (Jung and Allen, 1995) may all hinder the degradation of plant cell walls. In the Rusitec, silage samples were ground to pass through a 4-mm screen compared with the batch cultures where samples were ground to pass through a 1-mm screen. The increase in surface area by finer grinding may have facilitated enzyme access to substrates, resulting in an increase in fibre disappearance observed with EFE application. Care must be taken when recommending enzyme additives for ruminants based on batch culture results, as the substrate presented for fermentation often differs in particle size from that which is normally consumed.

In conclusion, all EFE products tested increased NDF disappearance after 24 h of incubation in batch culture. However, applying EFE enzymes to *A. gayanus* grass silage diets under continuous fermentation conditions in the Rusitec did not affect diet DM, NDF and ADF degradation but increased microbial attachment to the silage. Further studies are needed to evaluate different enzyme products, application methods, pre-treatments and dosages of enzymes for tropical forage diets.

Acknowledgements

This study was conducted with funding from FAPEMIG – Minas Gerais State Research Foundation (process CAG – 2653/06) and Alberta Meat and Livestock Foundation (ALMA). The first author gratefully acknowledges CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the Doctorate Sandwich Scholarship. The authors also thank K. Munns, W. Smart, M. L. He and D. Vedres (Agriculture and Agri-food Canada – Lethbridge Research Centre) for their technical assistance and Dyadic International Ltd (Florida, USA), AB Vista (Marlborough, UK) and Adisseo France SAS (Antony, France) for providing samples of their commercial enzymes.

References

Association of Official Analytical Chemists (AOAC) 2006. Official methods of analysis, 18th edition. AOAC, Arlington, VA, USA.

Avellaneda JH, Pinos-Rodríguez JM, González SS, Bárcena R, Hernández A, Cobos M, Hernández D and Montañez O 2009. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestion of Guinea grass hay. Animal Feed Science and Technology 149, 70–77.

Bailey RW 1967. Quantitative studies of ruminant digestion of ingested plant carbohydrates from the reticulo-rumen. New Zealand Journal of Agricultural Research 10, 15–32.

Beauchemin KA, Colombatto D, Morgavi DP and Yang W 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. Journal of Animal Science 81, E37–E47.

Canadian Council on Animal Care 1993. Guide to the care and use of experimental animals. Canadian Council on Animal Care, Ottawa, Ontario, Canada.

Centro Internacional de Agricultura Tropical 1990. *Andropogon gayanus* Kunth: a grass for tropical acid soils. Centro Internacional de Agricultura Tropical, Cali, Colombia.

Cheng K-J and McAllister TA 1997. Compartmentation in the rumen. In The rumen microbial ecosystem (ed. PN Hobson and CS Stewart), pp. 492–552. Blackie Academic & Press, London, UK.

Colombatto D and Beauchemin KA 2003. A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. Canadian Journal of Animal Science 83, 559–568.

Colombatto D, Mould FL, Bhat MK and Owen E 2003a. Use of fibrolytic enzymes to improve the nutritive value of ruminant diets: a biochemical and *in vitro* rumen degradation assessment. Animal Feed Science and Technology 107, 201–209.

Colombatto D, Mould FL, Bhat MK, Morgavi DP, Beauchemin KA and Owen E 2003b. Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms *in vitro*. Journal of Animal Science 81, 1040–1050.

Czerkawski JW and Breckenridge G 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371–384.

Dean DB, Adesogan AT, Krueger NA and Littell RC 2008. Effects of treatment with ammonia or fibrolytic enzymes on chemical composition and ruminal degradability of hays produced from tropical grasses. Animal Feed Science and Technology 145, 68–83.

Eun JS and Beauchemin KA 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. Animal Feed Science and Technology 132, 298–315.

Facchini FDA, Reis VRA, Roth AP, Magalhães KA, Peixoto-Nogueira SC, Casagrande DR, Reis RA and MDLTM Polizeli 2012. Effects of *Aspergillus* spp. exogenous fibrolytic enzymes on *in vitro* fermentation of tropical forages. Journal of Science Food and Agriculture 92, 2569–2573.

Fedorak PM and Hrudey SE 1983. A simple apparatus for measuring gas-production by methanogenic cultures in serum bottles. Environmental Technology Letters 4, 425–432.

Giraldo LA, Ranilla MJ, Tejido ML and Carro MD 2007a. Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. British Journal of Nutrition 98, 753–761.

Giraldo LA, Tejido ML, Ranilla MJ and Carro MD 2007b. Effects of exogenous cellulase supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. Journal of Animal Science 85, 1962–1970.

Jung HG and Allen MS 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. Journal of Animal Science 73, 2774–2790.

Karkalas J 1985. An improved enzymatic method for determination of native or modified starch. Journal of Science Food and Agriculture 36, 1019–1027.

Mandebvu P, West JW, Froetschel MA, Hatfield RD, Gates RN and Hill GM 1999. Effect of enzyme or microbial treatment of bermudagrass forages before ensiling on cell wall composition, end products of silage fermentation and *in situ* digestion kinetics. Animal Feed Science and Technology 77, 317–329.

McDougall EI 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43, 99–109.

Meale SJ, Beauchemin KA, Hristov AN, Chaves AV and McAllister TA 2014. BOARD-INVITED REVIEW: opportunities and challenges in using exogenous enzymes to improve ruminant production. Journal of Animal Science 92, 427–442.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D and Schneider W 1979. Estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas-production when they are incubated with rumen liquor *in vitro*. Journal of Agricultural Science 93, 217–222.

Morgavi DP, Beauchemin KA, Nsereko VL, Rode LM, Iwaasa AD, Yang WZ, McAllister TA and Wang Y 2000. Synergy between ruminal fibrolytic enzymes and enzymes from *Trichoderma longibrachiatum*. Journal of Dairy Science 83, 1310–1321.

Nsereko VL, Morgavi DP, Rode LM, Beauchemin KA and McAllister TA 2000. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay fiber by mixed rumen microorganisms *in vitro*. Animal Feed Science and Technology 88, 153–170.

Ouédraogo-Koné S, Kaboré-Zoungrana CY and Ledin I 2008. Intake and digestibility in sheep and chemical composition during different seasons of some West African browse species. Tropical Animal Health and Production 40, 155–164.

Phakachoed N, Suksombat W, Colombatto D and Beauchemin KA 2013. Use of fibrolytic enzymes additives to enhance *in vitro* ruminal fermentation of corn silage. Livestock Science 157, 100–112.

Phengvichith V and Ledin I 2007. Effect of feeding different levels of wilted cassava foliage (*Manihot esculenta*, Crantz) on the performance of growing goats. Small Ruminant Research 71, 109–116.

Playne MJ 1985. Determination of ethanol, volatile fatty acids, lactic and succinic acids in fermentation liquids by gas chromatography. Journal of Science Food and Agriculture 36, 638–644.

Rhine ED, Mulvaney RL, Pratt EJ and Sims GK 1998. Improving the berthelot reaction for determining ammonium in soil extracts and water. Soil Science Society of America Journal 62, 473–480.

Ribeiro GO Jr, Teixeira AM, Velasco FO, Faria WG Jr, Pereira LGR, Chaves AV, Gonçalves LC and McAllister TA 2014. Production, nutritional quality and *in vitro* methane production from *Andropogon gayanus* grass harvested at different maturities and preserved as hay or silage. Asian Australasian Journal of Animal Science 27, 330–341.

Ribeiro GO Jr, Teixeira AM, Velasco FO, Faria WG Jr, Jayme DJ, Maurício RM, Gonçalves LC and McAllister TA 2015. Methane production and energy partitioning in sheep fed *Andropogon gayanus* grass ensiled at three regrowth stages. Canadian Journal of Animal Science 95, 103–110.

Robertson JB and Van Soest PJ 1981. The detergent system of analysis and its application to human foods. In The analysis of dietary fiber in food (ed. WPT James and O Theander), pp. 123–158. Marcel Dekker, New York, NY, USA.

Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysachharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Wang Y and McAllister TA 2002. Rumen microbes, enzymes and feed digestion – a review. Asian Australasian Journal of Animal Science 15, 1659–1676.

Wang YX, McAllister TA, Yanke LJ, Xu ZJ, Cheeke PR and Cheng K-J 2000. In vitro effects of steroidal saponins from Yucca Schidigera extract on rumen microbial protein synthesis and ruminal fermentation. Journal of Science Food and Agriculture 80, 2114–2122.

Wang Y, McAllister TA, Rode LM, Beauchemin KA, Morgavi DP, Nsereko VL, Iwaasa AD and Yang W 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the Rumen Simulation Technique (Rusitec). British Journal of Nutrition 85, 325–332.

Wilson JR 1993. Organization of forage plant tissues. In Forage cell wall structure and digestibility (ed. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 1–32. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA.

Wilson JR and Mertens DR 1995. Cell wall accessibility and cell structure limitations to microbial digestion of forage. Crop Science 35, 251–259.

Wylie MJ, Ellis WC, Matis JH, Bailey EM, James WD and Beever DE 2000. The flow of forage particles and solutes through segments of the digestive tracts of cattle. British Journal of Nutrition 83, 295–306.