Treatment of tropical forages with exogenous fibrolytic enzymes: effects on chemical composition and *in vitro* rumen fermentation

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Summary

The effects of three treatments of fibrolytic enzymes (cellulase from *Trichoderma longibrachiatum* (CEL), xylanase from rumen micro-organisms (XYL) and a 1:1 mixture of CEL and XYL (MIX) on the *in vitro* fermentation of two samples of *Pennisetum clandestinum* (P1 and P2), two samples of *Dichanthium aristatum* (D1 and D2) and one sample of each *Acacia decurrens* and *Acacia mangium* (A1 and A2) were investigated. The first experiment compared the effects of two methods of applying the enzymes to forages, either at the time of incubation or 24 h before, on the *in vitro* gas production. In general, the 24 h pre-treatment resulted in higher values of gas production rate, and this application method was chosen for a second study investigating the effects of enzymes on chemical composition and *in vitro* fermentation of forages. The pre-treatment with CEL for 24 h reduced ($p < 0.05$) the content of neutral detergent fibre (NDF) of P1, P2, D1 and D2, and that of MIX reduced the NDF content of P1 and D1, but XYL had no effect on any forage. The CEL treatment increased ($p < 0.05$) total volatile fatty acid (VFA) production for all forages (ranging from 8.6% to 22.7%), but in general, no effects of MIX and XYL were observed. For both *P. clandestinum* samples, CEL treatment reduced ($p < 0.05$) the molar proportion of acetate and increased ($p < 0.05$) that of butyrate, but only subtle changes in VFA profile were observed for the rest of forages. Under the conditions of the present experiment, the treatment of tropical forages with CEL stimulated their *in vitro* ruminal fermentation, but XYL did not produce any positive effect. These results showed clearly that effectiveness of enzymes varied with the incubated forage and further study is warranted to investigate specific, optimal enzyme-substrate combinations.

Keywords fibrolytic enzymes, tropical forages, ruminal fermentation, batch cultures

Introduction

In the last years, many studies have explored the possibility of improving the nutritive value of forage for ruminants using exogenous fibrolytic enzymes (recently reviewed by Beauchemin and Holtshausen, 2010), but most of them have been conducted with mixed diets for high-producing ruminants or with medium- to high-quality forages, and consequently, there is little published information on how fibrolytic enzyme application can affect the digestion of low-quality forages (Dean et al., 2008; Krueger et al., 2008; Elghandour et al., 2013). However, increasing the digestibility of low-quality forages using exogenous enzymes could lead to significant improvements in ruminant performance in many parts of the world, mainly in the tropics and subtropics. Tropical forages intrinsically have low nutritive value, but often comprise practically the whole diet of ruminants in tropical countries, thus leading to a low productivity of ruminant livestock in these areas.

The effects of exogenous fibrolytic enzymes are influenced by many factors such as type and dose of enzyme, type of diet fed to animals and enzyme application method, among many others (Beauchemin et al., 2003; Beauchemin and Holtshausen, 2010). Regarding the factors related to the diet, the effectiveness of fibrolytic enzymes has been shown to vary with feed (Wallace et al., 2001; Colombatto et al., 2003), enzyme application method (Yang et al., 2000;

Wang et al., 2001) and the component of the diet to which the enzyme is added (Beauchemin et al., 2003).

The objective of this work was to analyse the effects of three enzyme preparations on the *in vitro* ruminal fermentation of six samples of tropical forages. Our hypothesis was that enzyme treatments would improve the degradability of forages and that the extent of the improvement would vary with the chemical composition of forages. The selected enzymes were a cellulase from *Trichoderma longibrachiatum* (CEL), a xylanase from rumen micro-organisms (XYL) and a 1:1 mixture of CEL and XYL (MIX). The cellulase has previously been shown to enhance *in vitro* fermentation of medium-quality forages and forage-based diets (Giraldo et al., 2007a,b, 2008a), but to our knowledge, the xylanase has not been previously tested.

Materials and methods

Substrates, enzymes and characterisation of enzymatic activities

Six samples of tropical forages (four grasses and two legumes) were obtained at different locations of Antioquia (Colombia) at variable regrowth stages to cover a wide range in chemical composition. Two samples of *Pennisetum clandestinum* (P1: harvested at 30-day regrowth; P2: harvested at 60-day regrowth) were harvested at two different locations in Santa Elena (2750 metres above sea level; cold climate), and two samples of *Dichanthium aristatum* (D1: harvested at 42-day regrowth; D2: harvested at 48-day regrowth) were harvested at two different places in Chigorodó (150 metres above sea level; warm climate). In each case, approximately 500 g of forage was harvested by cutting with scissors at 10 cm above ground level. One sample of *Acacia decurrens* (A1) was collected at Santa Elena, and one sample of *Acacia mangium* (A2) was collected at Caucasia (250 metres above sea level; warm climate). For each forage, approximately 500 g of leaves was collected from the low part of the shrubs. All samples were weighed, immediately transported to the laboratory, dried in a forced air oven at 60 °C for 48 h and milled through a 1-mm sieve before chemical analyses and *in vitro* incubations. Chemical composition of forages is given in Table 1.

Three exogenous fibrolytic enzymes were tested: cellulase from *Trichoderma viride* (CEL; Fluka Chemicals, Seelze, Germany), xylanase from ruminal micro-organisms (XYL; Xylanase M6; Megazyme International Ireland Ltd., Wicklow, Ireland) and a 1:1 mixture of both enzymes (MIX). Enzyme preparations were assayed for carboxymethylcellulase, avicelase, xylanase and amylase activities using carboxymethylcellulose, microcrystalline cellulose (Avicel PH-101; Sigma-Aldrich Química S.A., Madrid, Spain), oat spelt xylan and soluble starch as substrates, respectively, and following the procedure described by Giraldo et al. (2008a). All activities were measured at pH 6.5 and 39 °C to resemble optimal ruminal conditions. Analyses were performed in triplicate, and tubes containing only buffer, buffer plus substrate and buffer plus enzyme were also incubated to correct for substrate autolysis and sugars present in enzymes. At pH 6.5 and 39 °C, 1 mg of CEL liberated per min 2.40, 0.385 and 0.040 μmol of glucose from carboxymethylcellulose, soluble starch and Avicel PH-101, respectively, and 1.72 μmol of xylose from oat spelt xylan. Under the same conditions, 1 mg of XYL liberated per min 30.2 μmol of xylose from oat spelt xylan, but no carboxymethylcellulase, avicelase or amylase activities were detected. One enzymatic unit was defined as the amount of enzyme required to release 1 μmol of xylose or glucose per min from the corresponding substrate at 39 °C and pH 6.5. All enzyme treatments were applied at 20 enzymatic units per g substrate DM. For CEL, total enzymatic activity was calculated as the sum of individual activities.

Table 1 Chemical composition (g/kg DM) of forages*

	P1	P2	D1	D2	A1	A2
Dry matter content (g/kg fresh matter)	173	187	233	245	428	436
Organic matter	901	906	889	906	962	962
Crude protein	186	133	39	49	193	106
Neutral detergent fibre (NDF)	630	696	736	733	505	488
Hemicellulose†	366	386	336	401	212	170
Acid detergent fibre (ADF)	264	310	400	332	293	318
Cellulose†	258	299	366	306	110	161
Acid detergent lignin (ADL)	5.84	10.6	34.0	25.8	183	157
NDF lignification†	0.93	1.52	4.62	3.52	36.2	32.2

*P1 and P2: samples of *Pennisetum clandestinum*; D1 and D2: samples of *Dichanthium aristatum*, A1: *Acacia decurrens*; A2: *Acacia mangium*.
†Hemicellulose was calculated as the difference between NDF and ADF, cellulose as the difference between ADF and ADL, and NDF lignification as (ADL/NDF) ×100.

Effects of method of application of enzymes on *in vitro* gas production (Experiment 1)

Samples of each substrate (ground through a 1-mm screen) were fermented *in vitro* with buffered rumen fluid to compare the effects of two methods of enzyme application on gas production. Samples of 500 mg of

each forage were accurately weighed into 120-ml serum bottles. Solutions of each enzyme containing 5 enzymatic units per ml were prepared daily in 0.1 M sodium phosphate buffer (pH = 6.5), and 2 ml of the corresponding solution was carefully applied directly onto the substrate inside the bottles either 24 h before starting the incubation (CEL, XYL and MIX) or immediately before the incubation (CEL0, XYL0 and MIX0). Substrates in non-treated bottles (control; CON) received 2 ml of buffer solution without added enzyme. All bottles were kept at room temperature (21–23 °C) for 24 h until incubation with buffered rumen fluid.

Ruminal fluid was obtained from four rumen-cannulated Merino sheep fed medium-quality lucerne hay (158, 472, 301 and 65 g of crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) per kg dry matter (DM), respectively) for *ad libitum* intake. Sheep were managed according to the protocols approved by the León University Institutional Animal Care and Use Committee and had free access to water and mineral/vitamin block during the trial. Ruminal contents of each sheep were obtained immediately before the morning feeding, mixed and strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. Particle-free fluid was mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a proportion 1:4 (vol/vol) at 39 °C under continuous flushing with CO_2. Bottles were pre-warmed (39 °C) prior to the addition of 50 ml of buffered rumen contents under CO_2 flushing. Then, bottles were sealed with rubber stoppers and aluminium caps and incubated at 39 °C. Gas production was measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a calibrated syringe at 3, 6, 9, 12, 18, 24, 36, 48, 72, 96 and 120 h. At each measurement time, the gas in the headspace of the bottles was removed using the syringe until pressure was 0 and released.

After 120 h of incubation, the fermentation was stopped by swirling the bottles in ice, the bottles were opened, and their content was transferred to previously weighed filter crucibles (pore size 100–160 μm) and filtered under vacuum. The residue of incubation was washed with 50 ml of hot distilled water and dried at 50 °C for 48 h, and the degradability of substrate was calculated. The residue was then analysed for ash to calculate the organic matter (OM) apparent disappearance after 120 h of incubation (OMD_{120}). Four incubation runs were performed on different days, so that each treatment was conducted in quadruplicate. In each incubation run, additional bottles without substrate (blanks) but with the corresponding enzyme treatments (2 ml of CON, CEL, XYL and MIX solutions) were included to correct the gas production values for gas release from endogenous substrates and enzyme treatment (Carro et al., 2005).

To estimate the fermentation kinetic parameters, gas production data were fitted using the exponential model (Krishnamoorthy et al., 1991): gas = A $(1 - e^{(-c\ (t-lag))})$, where A is the asymptotic gas production (ml), c is the fractional rate of gas production (h^{-1}), *lag* is the initial delay in the onset of gas production (h), and t is the gas measurement time. The parameters A, c and *lag* were estimated by an iterative least squares procedure using the NLIN procedures of SAS Institute Inc (2012). Halftime of gas production ($T_{1/2}$) was the time (h) when half of the asymptotic gas volume (A) was produced and was calculated as $T_{1/2} = [(\ln 2/c) + lag]$. The average fermentation rate (AFR; ml gas/h) was defined as the average gas production rate between the start of the incubation and $T_{1/2}$ and was calculated as $AFR = A\,c/[2\,(\ln 2 + c\,lag)]$. Finally, the OM effective degradability (OMED;%) was estimated assuming a rumen particulate outflow (Kp) of 0.035 per h, characteristic for sheep fed forages at maintenance level (Ranilla et al., 1998), according to the equation proposed by France et al. (2000): $OMED = [(OMD_{120}\,c)/(c + Kp)]\,e^{(-c\,lag)}$.

Effects of enzymes on chemical composition of substrate

To investigate the effects of enzyme pre-treatments on substrate fibre composition, samples of each forage (250 mg) were weighed into artificial fibre bags (#F57 bags; 50 × 40 mm; 25 ± 10 μm pore size; ANKOM Technology Corporation, Fairport, NY, USA), and 1 ml of buffer solution containing no enzyme or five enzymatic units of the corresponding enzyme solution was added into each bag. Bags were heat sealed and kept at room temperature (21–23 °C) for 24 h before sequential NDF, ADF and acid detergent lignin (ADL) analyses were conducted. For replication, the complete procedure was repeated four times ($n = 4$).

Effects of enzymes on *in vitro* ruminal fermentation (Experiment 2)

The effects of enzymes on *in vitro* ruminal fermentation of forages were investigated in 24 h of incubations. All enzymes were applied 24 h before starting the incubation (treatments CON, CEL, XYL and MIX), and the incubation procedure was as described for the gas production trial, with the exception that no gas

measurements were performed until the end of incubations. After 24 h of incubation, gas production was measured in each bottle using a pressure transducer and a calibrated syringe. Bottles were uncapped, the pH was measured immediately with a pH-metre Basic 20 (Crison Instruments S.A., Alella, Spain), and the fermentation was stopped by swirling the bottles in ice. One millilitre of content was added to 1 ml of deproteinising solution [metaphosphoric acid (100 g/l) and crotonic acid (0.6 g/l)] for volatile fatty acid (VFA) determination, and 1 ml was added to 1 ml 0.5 M-HCl for ammonia-N analysis. Finally, the contents of the bottles were transferred to previously weighed filter crucibles, and the residue of incubation was washed with 50 ml of hot distilled water (50 °C). Crucibles were dried at 50 °C and weighed to calculate DM degradability (DMD). The residue of 24 h of incubation was analysed for NDF to calculate fibre degradability (NDFD).

Analytical procedures, calculations and statistical analyses

Dry matter, ash and N content of forages were determined according to the Association of Official Analytical Chemists (AOAC), (1999). Neutral detergent fibre, ADF and acid detergent lignin analyses were carried out according to Van Soest et al. (1991) using an ANKOM^{220} Fibre Analyzer unit (ANKOM Technology Corporation). Sodium sulphite was used in the analysis, and both NDF and ADF were expressed inclusive of residual ash. Hemicellulose content was calculated as the difference between NDF and ADF. Concentrations of VFA and ammonia-N in rumen fluid were analysed as described by Carro and Miller (1999).

The amounts of VFA produced in each bottle were obtained by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. When data from all forages were analysed together within Experiment 1 (6 substrates × 3 enzyme treatments × 2 methods of application) and within Experiment 2 (6 substrates × 3 enzyme treatments), significant interactions ($p < 0.05$) were detected for most parameters in both Experiments, and therefore, data were analysed separately for each forage within each Experiment. Data from Experiment 1 were analysed by ANOVA according to the following model:

$$Y_{ab} = \mu + i_a + E_b + e_{ab},$$

where Y_{ab} = the dependent variable; μ = the overall mean; i = the random effect of incubation day (a = 1–4); E = the fixed effect of enzyme treatment (b = 1–7; CON, CEL0, CEL, XYL0, XYL, MIX0 and MIX); and e = the residual error. Fermentation data from Experiment 2 were analysed with the same model, but there were only four enzyme treatments (b = 1–4; CON, CEL, XYL and MIX). Significance was declared at $p < 0.05$, whereas $p < 0.10$ values were considered as trends and discussed. When a significant effect of enzyme treatment was detected, differences between means were assessed by Tukey' test. All statistical analyses were conducted using the PROC MIXED of SAS Institute Inc, (2012). Within each forage and enzyme treatment, there were four values for each of the measured variables.

Results

Effects of method of application of enzymes on *in vitro* gas production (Experiment 1)

As shown in Table 2, no enzyme treatment increased potential gas production of any forage compared with controls, with the exception of CEL0, CEL, MIX0 and MIX treatments, which increased *A* parameter for A1 and A2 forages ($p < 0.05$). Compared with buffer-treated substrates, the treatment with CEL0 and CEL increased ($p < 0.05$) *c* and AFR and reduced $T_{1/2}$ for P1, D1, D2 and A2 samples, but for P2 and A1 forages, these effects were only observed for CEL treatment. The OMED of all forages was increased by CEL treatment ($p < 0.05$), but CEL0 was effective only for D2 forage. For all forages, the pre-treatment of forages with cellulase for 24 h increased *c*, AFR and OMED and decreased $T_{1/2}$ compared with the application of the cellulase immediately before incubation. The XYL0 treatment had no effect on any parameter for P1 and A2 forages, but reduced parameter *c* when was applied to P2, D2 and A1 samples ($p < 0.05$). The pre-treatment of the two *P. clandestinum* and *Acacia* samples with xylanase for 24 h before incubation increased *c* and AFR ($p < 0.05$) and decreased $T_{1/2}$ ($p < 0.05$) compared with controls, but had no effects for *D. aristatum* samples ($p > 0.05$). For all forages, MIX treatment increased *c*, AFR and OMED and reduced $T_{1/2}$ compared with controls ($p < 0.05$), although no effects were observed when the mixture of enzymes was applied immediately before incubation for P2 and A1 samples, and only subtle effects were noticed for the rest of forages.

The effects of the 24 h pre-treatment with enzymes on NDF, ADF and cellulose content of substrates are shown in Table 3. Compared with buffer-treated substrate, CEL treatment reduced NDF content of P1, P2, D1 and D2 samples ($p < 0.05$) and hemicellulose

Table 2 Parameters of gas production kinetics (*A*, *c*, AFR and $T_{1/2}$) and organic matter effective degradability (OMED) of forages incubated in batch cultures of rumen micro-organisms and supplemented with enzymes at the time of incubation (CEL0, XYL0 and MIX0) or 24 h before incubation (CEL, XYL and MIX)

		Enzyme treatment*								
Forage†	Item‡	CON	CEL0	CEL	XYL0	XYL	MIX0	MIX	SEM	p value
P1	*A*	149^{ab}	155^{b}	155^{b}	146^{a}	150^{ab}	152^{b}	155^{b}	1.8	0.027
	c	0.028^{a}	0.030^{b}	0.034^{c}	0.028^{a}	0.029^{b}	0.030^{b}	0.033^{c}	0.0005	<0.001
	AFR	2.06^{a}	2.36^{b}	2.62^{c}	2.02^{a}	2.22^{b}	2.26^{b}	2.54^{c}	0.05	<0.001
	$T_{1/2}$	25.2^{c}	23.0^{b}	20.6^{a}	25.2^{c}	23.6^{b}	23.4^{b}	21.3^{a}	0.33	<0.001
	OMED	33.1^{a}	34.7^{ab}	37.4^{c}	33.0^{a}	34.7^{ab}	33.7^{a}	36.2^{bc}	0.55	<0.001
P2	*A*	155^{ab}	160^{b}	150^{a}	155^{ab}	152^{a}	156^{ab}	150^{a}	1.9	0.036
	c	0.023^{b}	0.024^{bc}	0.027^{d}	0.021^{a}	0.025^{c}	0.023^{b}	0.027^{d}	0.0005	<0.001
	AFR	1.81^{b}	1.88^{b}	2.05^{d}	1.64^{a}	1.89^{bc}	1.82^{b}	2.01^{bc}	0.04	<0.001
	$T_{1/2}$	30.4^{d}	29.8^{cd}	25.6^{a}	33.1^{e}	28.1^{bc}	30.2^{cd}	26.2^{ab}	0.65	<0.001
	OMED	27.1^{b}	27.5^{b}	29.6^{c}	25.6^{a}	27.8^{b}	27.1^{b}	29.5^{c}	0.37	<0.001
D1	*A*	138^{ab}	144^{c}	133^{a}	139^{ab}	130^{a}	140^{b}	132^{a}	1.4	<0.001
	c	0.025^{b}	0.029^{c}	0.034^{d}	0.023^{a}	0.025^{ab}	0.027^{c}	0.033^{d}	0.0006	<0.001
	AFR	1.75^{a}	2.09^{c}	2.28^{d}	1.63^{a}	1.59^{a}	1.90^{b}	2.17^{cd}	0.05	<0.001
	$T_{1/2}$	28.2^{de}	24.2^{bc}	20.5^{a}	30.1^{e}	28.9^{e}	25.9^{cd}	21.4^{ab}	0.92	<0.001
	OMED	24.5^{ab}	26.1^{b}	28.5^{c}	23.3^{a}	23.6^{a}	25.5^{b}	28.7^{c}	0.50	<0.001
D2	*A*	155^{ab}	160^{ab}	157^{ab}	154^{ab}	148^{a}	165^{b}	154^{ab}	4.5	0.310
	c	0.029^{b}	0.033^{d}	0.040^{f}	0.027^{a}	0.029^{b}	0.031^{c}	0.038^{e}	0.0004	<0.001
	AFR	2.24^{a}	2.61^{b}	3.13^{c}	2.12^{a}	2.16^{a}	2.51^{b}	2.94^{c}	0.06	<0.001
	$T_{1/2}$	24.2^{cd}	21.5^{b}	17.5^{a}	25.4^{d}	24.1^{cd}	22.9^{c}	18.3^{a}	0.45	<0.001
	OMED	28.3^{ab}	30.4^{c}	34.2^{d}	27.8^{a}	28.2^{ab}	29.5^{bc}	33.4^{d}	0.44	<0.001
A1	*A*	47.2^{a}	54.1^{bc}	55.4^{c}	47.4^{a}	46.8^{a}	52.1^{b}	51.2^{b}	0.92	<0.001
	c	0.048^{b}	0.048^{b}	0.062^{d}	0.044^{a}	0.054^{c}	0.047^{ab}	0.060^{d}	0.0009	<0.001
	AFR	1.14^{ab}	1.30^{c}	1.72^{e}	1.05^{a}	1.27^{c}	1.22^{bc}	1.47^{d}	0.02	<0.001
	$T_{1/2}$	14.9^{c}	14.9^{c}	11.3^{a}	16.2^{c}	13.1^{b}	15.4^{c}	11.8^{ab}	0.51	<0.001
	OMED	17.1^{ab}	16.7^{ab}	18.4^{c}	16.0^{a}	17.7^{bc}	16.8^{ab}	18.2^{c}	0.33	0.002
A2	*A*	42.8^{ab}	50.2^{cd}	52.7^{d}	40.8^{a}	44.4^{b}	47.3^{c}	49.2^{c}	0.91	<0.001
	c	0.048^{a}	0.052^{bc}	0.060^{d}	0.047^{a}	0.054^{c}	0.049^{ab}	0.058^{d}	0.0009	<0.001
	AFR	1.03^{a}	1.31^{bc}	1.61^{d}	0.97^{a}	1.21^{b}	1.18^{b}	1.44^{c}	0.05	<0.001
	$T_{1/2}$	15.4^{e}	13.6^{bcd}	11.7^{a}	15.0^{de}	13.1^{abc}	14.5^{cde}	12.1^{ab}	0.47	<0.001
	OMED	18.2^{ab}	18.9^{ab}	20.4^{c}	17.9^{a}	19.4^{bc}	18.1^{ab}	20.4^{c}	0.69	0.108

*CON: control (no enzyme); CEL: cellulase from *Trichoderma longibrachiatum*; XYL: xylanase from ruminal micro-organisms; MYX: 1:1 mixture of CEL and XYL. All treatments were applied at 20 enzymatic units/g of substrate DM 24 h before incubation.

†P1 and P2: samples of *Pennisetum clandestinum*; D1 and D2: samples of *Dichanthium aristatum*, A1: *Acacia decurrens*; A2: *Acacia mangium*.

‡*A*: asymptotic gas production (ml/500 mg DM); *c*: fractional rate of fermentation (h^{-1}); AFR: average fermentation rate (ml/h); $T_{1/2}$: half time of gas production (h); OMED: organic matter effective degradability (%) for a fractional passage rate of 0.035 h^{-1}.

[a, b, c, d, e, f]Within a row, means without a common superscript letter differ ($p < 0.05$; Tukey's test).

content of D1 and D2 samples ($p < 0.05$), but had no effect on A1 and A2 fibre composition. In contrast, no effects of XYL were observed for any forage, and a reduction in NDF by MIX treatment was only observed for P1 and D1. In general, no effects of enzymes on ADF and cellulose content were detected, with the exception of CEL treatment which reduced ADF content in P1 ($p < 0.05$).

Effects of enzymes on *in vitro* ruminal fermentation (Experiment 2)

The effects of enzyme treatment on *in vitro* ruminal fermentation of *P. clandestinum*, *D. aristatum* and *Acacia* samples are shown in Tables 4, 5 and 6 respectively. No effects of XYL on fermentation parameters were observed for any forage. There were no effects of enzyme treatments on final pH (results not shown) or NH_3-N concentrations for any forage, with the exception of CEL and MIX that increased NH_3-N concentrations for P1 ($p < 0.05$).

Compared with controls, CEL treatment increased the production of gas, total VFA, propionate, butyrate and other minor VFA for both P1 and P2 forages ($p < 0.05$). The treatment of P1 with CEL increased both DMD and NDFD ($p < 0.05$), and no effect was observed for P2. The treatment of both *P. clandestinum* samples with MIX increased propionate and butyrate

Table 3 Effect of the 24 h pre-treatment with exogenous fibrolytic enzymes on neutral detergent fibre (NDF), hemicellulose, acid detergent fibre (ADF) and cellulose content [g/kg dry matter (DM)] of forages (n = 4)

		Enzyme treatment*					p
Forage†	Item‡	CON	CEL	XYL	MIX	SEM	value
P1	NDF	630^{c}	613^{a}	625^{bc}	619^{ab}	3.3	0.012
	Hemicellulose	364	355	362	359	3.3	0.194
	ADF	264^{b}	258	263^{b}	260^{ab}	1.3	0.024
	Cellulose	258	253	257	259	1.9	0.202
P2	NDF	696^{bc}	683^{a}	700^{c}	687^{ab}	3.7	0.026
	Hemicellulose	386	375	390	380	3.1	0.111
	ADF	310	308	310	307	1.3	0.255
	Cellulose	299	296	298	296	1.5	0.370
D1	NDF	736^{b}	710^{a}	738^{b}	719^{a}	6.0	0.010
	Hemicellulose	336^{bc}	316^{a}	340^{c}	329^{ab}	4.3	0.010
	ADF	400	394	398	390	3.7	0.250
	Cellulose	366	365	363	356	4.2	0.343
D2	NDF	733^{b}	709^{a}	731^{b}	719^{ab}	4.8	0.013
	Hemicellulose	401^{b}	381^{a}	403^{b}	393^{ab}	4.1	0.010
	ADF	332	328	328	326	2.9	0.570
	Cellulose	305	301	302	299	2.1	0.378
A1	NDF	505	507	510	509	6.0	0.753
	Hemicellulose	212	212	209	210	4.7	0.897
	ADF	293	295	301	299	4.1	0.184
	Cellulose	110	111	109	114	1.8	0.089
A2	NDF	489	493	494	491	3.1	0.731
	Hemicellulose	171	179	174	172	4.2	0.572
	ADF	318	314	320	319	4.0	0.768
	Cellulose	161	165	167	163	2.7	0.493

*CON: control (no enzyme); CEL: cellulase from *Trichoderma longibrachiatum*; XYL: xylanase from ruminal micro-organisms; MYX: 1:1 mixture of CEL and XYL. All treatments were applied at 20 enzymatic units/g of substrate DM 24 h before incubation.

†P1 and P2: samples of *Pennisetum clandestinum*; D1 and D2: samples of *Dichanthium aristatum*, A1: *Acacia decurrens*; A2: *Acacia mangium*.

‡NDF and ADF are inclusive of residual ash and were assayed without amylase. Cellulose was calculated as ADF – acid detergent lignin.

[a, b, c]Within a row, means without a common superscript letter differ ($p < 0.05$; Tukey's test).

production ($p < 0.05$), but positive effects on DMD and NDFD were only observed for P1. For both *D. aristatum* samples, CEL treatment resulted in increased production of gas, total VFA, acetate, propionate and other minor VFA, and DMD ($p < 0.05$). There were no effects of MIX on fermentation of D1, but MIX increased total VFA, acetate and other VFA productions for D2.

The treatment of both A1 and A2 samples with CEL increased the production of gas, total VFA (by 4 and 6% for A1 and A2, respectively), acetate, propionate and other minor VFA ($p < 0.05$), but had no effects on DMD or NDFD. Treating A2 with MIX also increased gas, total VFA, acetate, butyrate and other minor VFA ($p < 0.05$), although no effects of MIX were observed on fermentation of A1.

Discussion

The gas production technique was chosen for the first study because it is a simple screening tool to evaluate substrate degradation, as gas production rate is assumed to be directly proportional to rate of substrate degradation (Menke and Steingass, 1988). This study was designed to compare the efficacy of two methods of enzyme application, as a previous work (Yang et al., 2000; Wang et al., 2001) has shown that a pre-treatment of feed with enzymes before feeding or incubation with ruminal fluid enhanced the beneficial effects of enzymes on ruminal fermentation. In contrast, no differences between application methods have been reported (Hong et al., 2003), suggesting that the response to enzyme pre-treatment may be influenced by the type of feed, by type of enzymes or even by ruminal conditions (Hong et al., 2003). In our study, the pre-treatment of forages with XYL for 24 h increased c, AFR, OMED and reduced $T_{1/2}$ compared with the addition of XYL immediately before incubation for P1, P2, A1 and A2 forages, but no differences between application methods were observed for D1 and D2 forages, confirming that effects of enzyme pre-treatment may be influenced by the nature of the substrate to which enzymes are applied. Compared with CEL0 and MIX0 treatments, the pre-treatment of forages with CEL and MIX for 24 h resulted in increased c, AFR, OMED and reduced $T_{1/2}$ for all forages, which indicates a stimulation of forage degradation rate. In contrast, in general, there were no effects of enzymes on asymptotic gas production, supporting the general agreement that enzymes increase the rate rather than the extent of feed degradation in the rumen (Beauchemin and Holtshausen, 2010).

Some authors have suggested that the pre-treatment of feed with enzymes could create a stable enzyme–feed complex (Kung et al., 2000), but others have indicated an alteration in the fibre structure which would stimulate microbial colonisation (Newbold, 1997; Nsereko et al., 2000). Giraldo et al. (2007a,b) showed clearly that the treatment of grass hay with fibrolytic enzymes stimulated the initial phases of microbial colonisation in Rusitec fermenters, resulting in greater colonisation of feed particles even after 48 h of incubation, and similar results were reported by Wang et al. (2001) after treating a 50:50 lucerne hay:barley grain substrate with exogenous xylanase in Rusitec fermenters. The results of our first

Table 4 Effect of the 24 h pre-treatment with exogenous fibrolytic enzymes on final ammonia-N concentration, gas and volatile fatty acid (VFA) production, acetate/propionate ratio (Ac/Pr), and degradability of dry matter (DMD) and neutral detergent fibre (NDFD) after 24-h *in vitro* fermentation of two samples (500 mg DM) of *Pennisetum clandestinum* with mixed rumen micro-organisms (n = 4)

		Enzyme treatment*					
Forage†	Item	CON	CEL	XYL	MIX	SEM	p value
P1	Gas (ml)	58.1^{a}	68.2^{b}	59.5^{a}	65.6^{b}	1.01	<0.001
	NH_3-N (mg/l)	262^{a}	289^{b}	269^{a}	286^{b}	6.5	0.049
	VFA (μmol)						
	Total	2018^{ab}	2214^{b}	1909^{a}	2137^{ab}	75.9	0.801
	Acetate	1428	1531	1334	1490	58.0	0.160
	Propionate	403^{a}	457^{b}	388^{a}	436^{b}	13.7	0.023
	Butyrate	119^{a}	146^{b}	118^{a}	139^{b}	4.0	0.002
	Others‡	68.5^{a}	79.6^{b}	68.7^{a}	72.5^{ab}	2.56	0.044
	Ac/Pr (mol/mol)	3.54^{b}	3.35^{a}	3.44^{ab}	3.42^{ab}	0.044	0.049
	DMD (%)	48.7^{a}	51.5^{b}	48.7^{a}	50.7^{b}	0.45	0.003
	NDFD (%)	41.3^{a}	46.1^{c}	41.9^{a}	44.4^{b}	0.47	<0.001
P2	Gas (ml)	53.7^{a}	60.2^{b}	53.4^{a}	57.5^{ab}	1.29	0.014
	NH_3-N (mg/l)	247	259	264	259	8.0	0.542
	VFA (μmol)						
	Total	1829^{a}	2071^{b}	1829^{a}	1932^{ab}	62.8	0.049
	Acetate	1306	1448	1290	1390	46.4	0.120
	Propionate	357^{a}	417^{b}	364^{a}	373^{b}	11.3	0.018
	Butyrate	111^{a}	139^{c}	115^{ab}	121^{b}	2.9	<0.001
	Others‡	54.2^{a}	66.7^{b}	53.9^{a}	55.3^{a}	2.03	0.004
	Ac/Pr (mol/mol)	3.66^{bc}	3.47^{a}	3.54^{ab}	3.73^{c}	0.052	0.038
	DMD (%)	41.1	42.1	42.0	42.4	0.70	0.636
	NDFD (%)	34.8	35.4	36.3	36.8	1.04	0.496

*CON: control (no enzyme); CEL: cellulase from *Trichoderma longibrachiatum*; XYL: xylanase from ruminal micro-organisms; MYX: 1:1 mixture of CEL and XYL. All treatments were applied at 20 enzymatic units/g of substrate DM 24 h before incubation.

†P1 and P2: samples of *Pennisetum clandestinum* obtained at different locations.

‡Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

$^{a, b, c}$Within a row, means without a common superscript letter differ ($p < 0.05$; Tukey's test).

study clearly showed that the tested enzymes were more effective when a 24 h pre-treatment of forages was allowed, and consequently, this method of enzyme application was selected for the second study.

Van de Vyver (2011) treated two tropical forages (kikuyu and weeping love grass) with a mixture of fibrolytic enzymes and observed a reduction in their metaxylem cell wall thickness at 12 h of incubation with ruminal fluid compared with distilled water treated forages, confirming the direct actions of fibrolytic enzymes on cell wall structure as part of the mode of action of exogenous enzymes. A reduction in NDF or ADF content of forages following treatment with fibrolytic enzymes has also been observed for a wide range of substrates (Giraldo et al., 2007b, 2008a; Dean et al., 2008; Krueger et al., 2008). In our study, **CEL** decreased NDF content of P1, P2, D1 and D2 forages and hemicellulose content of D1 and D2 forages and MIX decreased NDF content of P1 and D1, but cell wall composition of A1 and A2 was not affected by any treatment. These results clearly show that enzymes had different effects on cell wall components of forages, and therefore, forage characteristics influenced the response. However, other mechanisms of action of enzymes than direct effects on cell wall should be involved, because CEL was effective in increasing degradation rate of A1 and A2 forages but had no effect on their NDF, ADF or cellulose content; similarly, MIX increased degradation rate of P2, D2, A1 and A2 forages without affecting their fibre fractions content. It is possible that enzymes altered or weakened the cell wall structure, thus allowing earlier access of ruminal micro-organisms to cell contents and increasing degradation rate, but these modifications were not reflected in changes in NDF, ADF or cellulose content of forages.

Volatile fatty acids constitute the main source of energy for the host animal, and any increase in their production *in vitro* should reflect an increase in the amount of organic matter fermented, as batch cultures are closed systems. The CEL treatment increased VFA production by 9.7, 13.2, 21.9, 10.5, 8.6 and 22.7% for P1, P2, D1, D2, A1 and A2 forages respectively. The response was highly variable and could not be easily related to chemical composition of forages. Thus, D1 and D2 had similar NDF content (736 and 733 g/kg

Table 5 Effect of the 24 h pre-treatment with exogenous fibrolytic enzymes on final ammonia-N concentration, gas and volatile fatty acid (VFA) production, acetate/propionate ratio (Ac/Pr), and degradability of dry matter (DMD) and neutral detergent fibre (NDFD) after 24-h *in vitro* fermentation of two samples (500 mg DM) of *Dichantium aristatum Benth* with mixed rumen micro-organisms ($n = 4$)

		Enzyme treatment*					
Forage†	Item	CON	CEL	XYL	MIX	SEM	p value
D1	Gas (ml)	58.8[a]	67.8[b]	59.7[a]	60.9[a]	1.32	0.004
	NH_3-N (mg/l)	157	171	167	163	5.6	0.353
	VFA (μmol)						
	Total	1887[a]	2301[b]	1756[a]	2018[ab]	101.4	0.023
	Acetate	1366[a]	1643[b]	1268[a]	1456[ab]	74.8	0.034
	Propionate	347[a]	442[b]	325[a]	379[a]	19.5	0.010
	Butyrate	134[a]	159[b]	124[a]	139[ab]	6.8	0.033
	Others‡	40.7[a]	57.2[b]	39.2[a]	43.8[a]	3.72	0.028
	Ac/Pr (mol/mol)	3.94[b]	3.72[a]	3.90[b]	3.84[ab]	0.048	0.028
	DMD (%)	37.6[a]	40.9[b]	37.5[a]	38.6[ab]	0.55	0.005
	NDFD (%)	35.2[a]	38.0[c]	34.3[a]	36.8[ab]	0.72	0.002
D2	Gas (ml)	67.4[a]	76.9[b]	68.2[a]	71.6[a]	1.31	0.002
	NH_3-N (mg/l)	157	164	160	165	5.4	0.699
	VFA (μmol)						
	Total	2104[a]	2325[b]	2064[a]	2178[b]	36.6	0.003
	Acetate	1482[a]	1640[b]	1456[a]	1522[a]	24.9	0.003
	Propionate	399[ab]	452[c]	389[a]	425[bc]	8.8	0.003
	Butyrate	176	178	174	179	5.1	0.925
	Others‡	46.8[a]	54.7[b]	44.4[a]	51.6[b]	1.23	<0.001
	Ac/Pr (mol/mol)	3.71	3.63	3.74	3.68	0.051	0.257
	DMD (%)	42.1[a]	46.3[b]	43.8[a]	44.6[ab]	0.81	0.031
	NDFD (%)	40.8	45.7	42.7	43.7	1.16	0.082

*CON: control (no enzyme); CEL: cellulase from *Trichoderma longibrachiatum*; XYL: xylanase from ruminal micro-organisms; MYX: 1:1 mixture of CEL and XYL. All treatments were applied at 20 enzymatic units/g of substrate DM 24 h before incubation.

†D1 and D2: samples of *Dichantium aristatum Benth* obtained at different locations.

‡Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

[a, b, c]Within a row, means without a common superscript letter differ ($p < 0.05$; Tukey's test).

DM, respectively) and cell wall lignification degree (4.6 and 3.5% of NDF), but the increase in VFA production above the levels in controls was rather different (414 and 221 μmol per culture for D1 and D2, respectively). Likewise, NDF and cell wall lignification degree were similar in A1 and A2 samples (508 g/kg DM and 36% for A1, and 488 g/kg DM and 32% for A2), but the observed increase in VFA production above the values in control cultures was 2.3 times greater in A2 than in A1 (277 and 121 μmol per culture, respectively). The cross-linking of lignin to cell wall carbohydrates limits the action of enzymes and cell wall digestion, but conventional fibre analyses do not account for the complexity of lignin–carbohydrate complexes in tropical forages.

Several *in vivo* (Beauchemin et al., 2000; Giraldo et al., 2008b) and *in vitro* studies (Dong et al., 1999; Wang et al., 2001; Krueger and Adesogan, 2008) have reported that treating different feeds with fibrolytic enzymes produced changes in molar proportions of VFA, but shifts in pattern of VFA seem to be influenced by the type of feed and enzyme preparations. In the present study, treating P1 and P2 with CEL and MIX increased the production of propionate and butyrate without affecting that of acetate, thus reducing the acetate:propionate ratio and indicating a change in fermentation pattern. Similar changes have been reported by others (Yang et al., 2002; Krueger and Adesogan, 2008), indicating that fibrolytic enzymes can make the fermentation more gluconeogenic and hence improve the energetic efficiency of the fermentation (Krueger and Adesogan, 2008). These changes in VFA profile have been attributed to fermentation of sugars released by enzymatic cell wall hydrolysis, but it has been also suggested that VFA changes may reflect a shift in the species profile of colonising bacteria in response to pre-treatment of feed with fibrolytic enzymes (Wang et al., 2001). However, effects of enzymes on VFA profile varied with the forage incubated, as CEL and MIX did not affect the acetate:propionate ratio for D2, A1 and A2 forages, thus indicating that production of both VFA was modified in the same proportion.

The lack of effect of XYL on fermentation parameters found in Experiment 2 is apparently in contrast to the increased AFR and reduced $T_{1/2}$ observed for P1,

Table 6 Effect of the 24 h pre-treatment with exogenous fibrolytic enzymes on final ammonia-N concentration, gas and volatile fatty acid (VFA) production, acetate/propionate ratio (Ac/Pr), and degradability of dry matter (DMD) and neutral detergent fibre (NDFD) after 24-h *in vitro* fermentation of samples (500 mg DM) of *Acacia decurrens* (A1) and *Acacia mangium* (A2) with mixed rumen micro-organisms ($n = 4$)

		Enzyme treatment*					
Forage	Item	CON	CEL	XYL	MIX	SEM	p value
A1	Gas (ml)	27.4^{ab}	33.3^{c}	25.2^{a}	30.2^{bc}	1.33	0.010
	NH_3-N (mg/l)	218	227	225	222	4.1	0.504
	VFA (μmol)						
	Total	1413^{a}	1534^{b}	1397^{a}	1412^{a}	26.2	0.011
	Acetate	1007^{a}	1084^{b}	997^{a}	1002^{a}	20.6	0.034
	Propionate	287^{a}	314^{b}	282^{a}	284^{a}	4.8	0.001
	Butyrate	82.0^{a}	92.5^{b}	81.3^{a}	85.4^{a}	1.65	0.003
	Others†	38.0^{a}	43.7^{b}	36.4^{a}	39.7^{a}	0.84	<0.001
	Ac/Pr (mol/mol)	3.51	3.45	3.54	3.53	0.050	0.258
	DMD (%)	26.5	27.8	26.3	25.8	0.74	0.325
	NDFD (%)	18.3	20.3	17.3	17.9	0.93	0.184
A2	Gas (ml)	26.8^{a}	32.0^{b}	27.0^{a}	29.6^{bc}	0.78	0.003
	NH_3-N (mg/l)	192	209	196	199	6.6	0.371
	VFA (μmol)						
	Total	1220^{a}	1497^{c}	1261^{ab}	1427^{bc}	56.8	0.021
	Acetate	872^{a}	1059^{bc}	905^{ab}	1019^{bc}	42.0	0.034
	Propionate	237^{a}	296^{b}	239^{a}	278^{b}	19.5	0.020
	Butyrate	79.8^{a}	100^{c}	83.0^{ab}	91.3^{bc}	2.92	0.003
	Others†	31.8^{a}	42.2^{c}	34.1^{a}	38.8^{bc}	1.85	0.013
	Ac/Pr (mol/mol)	3.68	3.58	3.79	3.67	0.078	0.112
	DMD (%)	26.0	27.0	26.2	26.1	0.33	0.198
	NDFD (%)	22.7	23.4	22.3	22.1	0.40	0.179

*CON: control (no enzyme); CEL: cellulase from *Trichoderma longibrachiatum*; XYL: xylanase from ruminal micro-organisms; MYX: 1:1 mixture of CEL and XYL. All treatments were applied at 20 enzymatic units/g of substrate DM 24 h before incubation.

†Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

[a, b, c]Within a row, means without a common superscript letter differ ($p < 0.05$; Tukey's test).

P2, A1 and A2 forages in Experiment 1. However, it has to be taken into account that these gas production parameters reflect what occurs at initial stages of fermentation, whereas fermentation parameters were measured after 24 h of incubation. Giraldo et al. (2008a) analysed the effects of three fibrolytic enzymes on *in vitro* fermentation of different substrates and observed that all enzymes increased gas and VFA production at 8 h of incubation, but some of these effects were not detected after 24 h of incubation. Other studies (Colombatto et al., 2003; Giraldo et al., 2007a,b; Ranilla et al., 2008) have also reported short-term effects of enzymes on substrate fermentation with limited or null effects later during the incubation, and there is a general agreement that enzymes can increase the rate, but not the extent of feed degradation in the rumen (Beauchemin and Holtshausen, 2010). Our results indicate that XYL could have stimulated initial fermentation of some forages (P1, P2, A1 and A2), but effects disappear after 24 h of incubation. In agreement with this hypothesis, in the first study, there were no effects of XYL on OMED of any forage despite the increased gas production rates observed for P1, P2, A1 and A2 forages, and the lack of effects on OMED is consistent with the absence of effects on DMD and NDFD observed in Experiment 2. Additionally, it has been noticed that feed enzymes compete with enzymes produced by fibrolytic bacteria in the rumen for available binding sites on feed (Morgavi et al., 2004), and this competition may also provide an explanation for the lack of effect of XYL, because this enzyme was produced by ruminal micro-organisms.

In the present study, we decided to consider all determined enzyme activities to calculate the amount of enzyme applied to substrates, although they should reflect the activity on different chemical fractions of forages. Although all enzymes were applied at the same rate, the amounts of each individual enzymatic activity were variable, because CEL presented mainly carboxymethylcellulase and xylanase activity, with low amylase and avicelase activities, but XYL presented only xylanase activity. The CEL was the most effective treatment of all forages, which would indicate that it presented the most appropriate combination of enzymatic activities for the tested forages. However, the magnitude of effects varied with the forage, indicating that limiting enzymatic activity is likely

substrate dependent (Eun and Beauchemin, 2007). Because only one sample per forage and harvest date was tested in this study, no specific conclusions about the effectiveness of the tested enzymes to improve the degradation of each forage can be drawn. As pointed out by Wallace et al. (2001), a precise identification of the enzymatic activity causing a positive response in ruminal fermentation might make it possible to develop more effective fibrolytic enzyme products.

In conclusion, the results indicate that the treatment of tropical forages with a fibrolytic enzyme produced by *Trichoderma longibrachiatum* had a stimulatory effect on rumen fermentative activity, because VFA production and substrate degradability were significantly increased, but the extent of this stimulation varied with the forage used. In contrast, the use of a xylanase from ruminal micro-organisms produced only subtle effects on *in vitro* gas production of certain forages, suggesting that it contributed little, if any, to ruminal fibrolytic activity. Future work is required to find the ideal combination of highly active enzymes for optimising the degradation of tropical forages, and the challenge is to identify the key enzyme activities and dose rates required to better match the complexity of these forages, thus ensuring cost-effective use of enzymes in ruminant feeding in the tropics and subtropics.

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References

Association of Official Analytical Chemists (AOAC), 1999: *Official Methods of Analysis*, 16th edn, 5th revision. AOAC International, Gaithersburg, MD, USA.

Beauchemin, K. A.; Holtshausen, L., 2010: Developments in enzyme usage in ruminants. In: M. R. Bedford, G. G. Partridge (eds), *Enzymes in Farm Animal Nutrition*, 2nd edn, CABI, Oxford, United Kingdom, pp. 206–230.

Beauchemin, K. A.; Ivan, M.; McAllister, T. A.; Rode, L. M.; 2000 : New feed additives as alternatives to sub-therapeutic levels of antimicrobials in beef cattle diets. In: T. A. McAllister, K. Jakober, J. Hawkins (eds), *National Beef Science Seminar. New Science for a New Century*, Lethbridge, Alberta, Canada, pp. 1–7.

Beauchemin, K. A.; Colombatto, D.; Morgavi, D. P.; Yang, W. Z., 2003: Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science* **81**, E37–E47.

Carro, M. D.; Miller, E. L., 1999: Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semicontinuous culture system (RUSITEC). *British Journal of Nutrition* **82**, 149–157.

Carro, M. D.; Ranilla, M. J.; Tejido, M. L., 2005: Using the *in vitro* gas production technique to test feed additives: effects of correcting values for different blanks. *Animal Feed Science and Technology* **123**, 173–184.

Colombatto, D.; Morgavi, D. P.; Furtado, A. F.; Beauchemin, K. A., 2003: Screening of exogenous enzymes for ruminant diets: relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal of Animal Science* **81**, 2628–2638.

Dean, D. B.; Adesogan, A. T.; Krueger, N. A.; Littell, R. C., 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.

Dong, Y.; Bae, H. D.; McAllister, T. A.; Mathison, G. W.; Cheng, K. J., 1999: Effects of exogenous fibrolytic enzymes, α-bromoethanesulfonate and monensin on fermentation in a rumen simulation (Rusitec) system. *Canadian Journal of Animal Science* **79**, 491–498.

Elghandour, M. M. Y.; Salem, A. Z. M.; Gonzalez-Ronquillo, M.; Bórquez, J. L.; Gado, H. M.; Odongo, N. E.; Peñuelas, C. G., 2013: Effects of exogenous enzymes on *in vitro* gas production kinetics and ruminal fermentation of four fibrous feeds. *Animal Feed Science and Technology* **179**, 46–53.

Eun, J. S.; Beauchemin, K. A., 2007: Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology* **132**, 298–315.

France, J.; Dijkstra, J.; Dhanoa, M. S.; López, S.; Bannink, A., 2000: Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. *British Journal of Nutrition* **83**, 131–142.

Giraldo, L. A.; Ranilla, M. J.; Tejido, M. L.; Carro, M. D., 2007a: Influence of exogenous fibrolytic enzyme and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. *British Journal of Nutrition* **98**, 753–761.

Giraldo, L. A.; Tejido, M. L.; Ranilla, M. J.; Carro, M. D., 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.

Giraldo, L. A.; Tejido, M. L.; Ranilla, M. J.; Carro, M. D., 2008a: Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. *Animal Feed Science and Technology* **141**, 306–325.

Giraldo, L. A.; Tejido, M. L.; Ranilla, M. J.; Ramos, S.; Carro, M. D., 2008b: Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in

sheep fed a grass hay based diet. *Journal of Animal Science* **86**, 1617–1623.

Hong, S. H.; Ong, S. H.; Lee, B. K.; Choin, N. J.; Lee, S. S.; Yun, S. G.; Ha, J. K., 2003: Effects of enzyme application method and levels and pre-treatment times on rumen fermentation, nutrient degradation and digestion in goats and steers. *Asian-Australasian Journal of Animal Science* **16**, 389–393.

Krishnamoorthy, U.; Soller, H.; Steingass, H.; Menke, K. H., 1991: A comparative study on rumen fermentation of energy supplements *in vitro*. *Journal of Animal Physiology and Animal Nutrition* **65**, 28–35.

Krueger, N. A.; Adesogan, A. T., 2008: Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Animal Feed Science and Technology* **145**, 84–94.

Krueger, N. A.; Adesogan, A. T.; Staples, C. R.; Krueger, W. K.; Deana, D. B.; Littell, R. C., 2008: The potential to increase digestibility of tropical grasses with a fungal, ferulic acid esterase enzyme preparation. *Animal Feed Science and Technology* **145**, 95–108.

Kung, L. Jr; Treacher, R. J.; Nauman, G. A.; Smagala, A. M.; Endres, K. M.; Cohen, M. A., 2000: The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science* **83**, 115–122.

Menke, K. H.; Steingass, H., 1988: Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* **28**, 7–55.

Morgavi, D. P.; Beauchemin, K. A.; Nsereko, V. L.; Rode, L. M.; McAllister, T. A.; Wang, Y., 2004: *Trichoderma* enzymes promote *Fibrobacter succinogenes* S85 adhesion to, and degradation of, complex substrates but not pure cellulose. *Journal of the Science of Food and Agriculture* **84**, 1083–1090.

Newbold, J.; 1997: Proposed mechanisms for enzymes as modifiers of ruminal fermentation. In: C. R. Staple (ed.) *Proceedings of the 8th Annual Florida Ruminant Nutrition Symposium*, Gainesville, University of Florida, Florida, USA, pp. 146–159.

Nsereko, V. L.; Morgavi, D. P.; Rode, L. M.; Beauchemin, K. A.; McAllister, T. A., 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.

Ranilla, M. J.; López, S.; Giráldez, F. J.; Valdés, C.; Carro, M. D., 1998: Comparative digestibility and digesta flow kinetics in two breeds of sheep. *Animal Science* **66**, 389–396.

Ranilla, M. J.; Tejido, M. L.; Giraldo, L. A.; Tricçarico, J. M.; Carro, M. D., 2008: Effects of an exogenous fibrolytic enzyme preparation on *in vitro* ruminal fermentation of three forages and their isolated cell walls. *Animal Feed Science and Technology* **145**, 109–121.

SAS Institute Inc, 2012. SAS/STAT® 12.2 User's guide. SAS Inst Inc, Cary, NC, USA.

Van de Vyver, W. F. J.; 2011: Fibrolytic enzymes in ruminant nutrition and their effect on forage cell wall integrity. Doctoral Thesis. Stellenbosch University. South Africa.

Van Soest, P. J.; Robertson, J. B.; Lewis, B. A., 1991: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**, 3583–3597.

Wallace, R. J.; Wallace, S. J. A.; McKain, N.; Nsereko, V. L.; Hartnell, G. F., 2001: Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms *in vitro*. *Journal of Animal Science* **79**, 1905–1916.

Wang, Y.; McAllister, T. A.; Rode, L. M.; Beauchemin, K. A.; Morgavi, D. P.; Nsereko, V. L.; Iwaasa, A. D.; 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.

Yang, W. Z.; Beauchemin, K. A.; Rode, L. M., 2000: A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science* **83**, 2512–2520.

Yang, W. Z.; Beauchemin, K. A.; Vedres, D. D., 2002: Effects of pH and fibrolytic enzymes on digestibility, bacterial protein synthesis, and fermentation in continuous culture. *Animal Feed Science and Technology* **102**, 137–150.