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Methane production and *in vitro* digestibility of low quality forages treated with a protease or a cellulase

J. M. Cantet[A,C], *D. Colombatto*[A,B] *and G. Jaurena*[A]

[A]Department of Animal Production, School of Agronomy, University of Buenos Aires, Av. San Martín 4453, C1417DSE, Buenos Aires, Argentina.
[B]National Scientific and Technical Research Council (CONICET) of Argentina, Av. Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina.
[C]Corresponding author. Email: jcantet@agro.uba.ar

Abstract. The objective was to assess the impact of application of two enzyme mixtures on the *in vitro* dry matter digestibility, neutral detergent fibre digestibility, net cumulative gas production and methane production after 24 h of incubation of *Milium coloratum* (formely *Panicum coloratum*) and a Patagonian meadow grassland. A protease (Protex 6-L) and a fibrolytic enzyme (Rovabio) were assessed at three application rates (30, 60 and 90 mg/100 mL of distiller water) on the substrates. Meadow samples were higher to Milium ones ($P < 0.05$) for *in vitro* dry matter digestibility and net cumulative gas production at 24 h. Nevertheless, Milium was ~11% higher than meadow ($P < 0.05$) for methane when expressed as a proportion of digested dry matter (g/kg). Rovabio did not induce differences in any variable, but the addition of Protex reduced ($P < 0.05$) *in vitro* dry matter digestibility in both substrates without bringing about differences in methane production. Collectively, the addition of these enzymes did not benefit *in vitro* ruminal fermentation of low quality forages.

Additional keywords: cell wall hydrolysis, enzymes, greenhouses gases, *in vitro* gas production, ruminant.

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Introduction

Currently, as a consequence of climate warming perspectives, and the incorporation (or intensification) of new livestock production lands, there is a renewed interest in improving the knowledge on utilisation of non-traditional forages (e.g. subtropical species, natural grasslands). Among many others, *Milium coloratum* (formerly *Panicum coloratum*, Klein grass) is a C_4-grass with promising perspectives for cattle breeding systems under subtropical conditions (Tomás *et al*. 2009). Similarly, in the Patagonic region of Argentina, natural meadow grasslands are critical resources for sheep and cattle production systems. Sheep and beef cattle production in the southern Argentinean and Chile Patagonian region is characterised by extensive exploitation of grazing natural steppes and meadows, with a marked seasonality during late spring and summer (Jaurena *et al*. 2010). These meadow grasslands (i.e. 'vegas' or 'mallines'; low-land sectors surrounding natural water streams), though representing small areas, account for an important biomass production source, of good nutritional quality (Jaurena *et al*. 2009, 2010). Patagonic meadow grasslands of *Poa pratensis*, *Hordeum pubiflorum* and *Alopecurus* spp. are typical species of this cool southern bioma.

Although these non-traditional forages are highly valued in areas with soil and climatic constraints, they showed nutritional constraints for ruminants (Van Soest 1994). To improve the availability of nutrients, the application of exogenous enzymes may contribute to degradation of cellulose, hemicellulose, and protein in forages (Pinos-Rodríguez *et al*. 2002). Exogenous enzymes are widely used in non-ruminant diets (Bedford 2000), but there has been a long controversy about their use in ruminants as it was presumed that the addition of enzymes would not improve the endogenous fibrolytic activity of the rumen (Beauchemin *et al*. 2003; Colombatto *et al*. 2003*b*) and would excessively degrade the dietary crude protein (CP) reducing the dietary nitrogen (N) utilisation by ruminal microorganisms. However, this latter effect has not been demonstrated in *in vitro* and *in vivo* experiments (Colombatto *et al*. 2003*b*; Eun and Beauchemin 2005). In the past decade, many researchers have shown a renewed interest in exploring their use for ruminants, especially, due to the increasing feeding costs and there is easier access to high quality enzymes (Adesogan *et al*. 2014; He *et al*. 2014).

The effect of proteolytic enzymes in addition to fibrolytic enzymes has also been studied, hypothesising that such enzymes would act over the cell wall protein, enhancing the accessibility of the ruminal microorganisms to the most digestible substrates (Colombatto and Beauchemin 2009). With enzyme mixtures of endoglucanase, xylanase, alfa-amylase and protease activity, there have been reported improvements of digestibility and fermentation parameters in *in vitro* (Elghandour *et al*. 2013) and *in vivo* (Gado *et al*. 2009) studies. Although studies on the

effects of exogenous enzymes on ruminant productivity have shown different results, there exists enough evidence that enzyme action on forage cell wall may improve its ruminal digestion (Feng *et al*. 1996; Eun and Beauchemin 2007).

Furthermore, it is well known that ruminal fermentation of structural carbohydrates contributes to methane production (CH_4p, Moe and Tyrrell 1979). CH_4 is not only an important greenhouse gas with a heating power ~25 times greater than CO_2, but it also constitutes an important feedstuff energy loss during ruminal fermentation (Johnson and Johnson 1995). Previous reports have shown that the addition of enzymes to low quality forages can improve their digestibility and could even reduce their CH_4p (Beauchemin *et al*. 2008; Grainger and Beauchemin 2011), but results have been inconsistent. For example, in an *in vivo* screening, a protease enzyme failed to reduce CH_4p (McGinn *et al*. 2004). Moreover, Giraldo *et al*. (2007*a*) reported that the addition of enzymes to grass hay diets increased the *in vitro* CH_4p at early stages of fermentation (up to 24 h), with no effects thereafter.

In this first approximation, the primary objective of this study was to assess the impact of application of two enzymes (i.e. one protease and one cellulase-based mixture) on two forages of great importance in South America (i.e. *Milium coloratum* and a Patagonian meadow grassland) through *in vitro* dry matter and fibre digestibility (ivDMD and NDFD, respectively), and CH_4p. The underlying hypothesis was that both enzymes may improve ivDMD and NDFD (concurrently reducing CH_4p) due to the improvement of the ruminal availability of the cell wall carbohydrates; whereas the cellulase would hydrolyse the cellulose itself, the protease is expected to improve the accessibility of cell wall carbohydrates to a variety of cellulose and hemicellulose enzymes.

Material and methods

Experimental treatments

A protease [Protex 6-L (PROTEX); Genencor Int., Inc., CA, USA] and a fibrolytic enzyme [Rovabio, (R), Adisseo, Alpharetta, GA, USA] were assessed at three doses (30, 60 and 90 mg/100 mL of distiller water) on two different substrates: *Milium coloratum* (Milium) and a Patagonian mixed meadow grass (Meadow). The concentration chosen for the tested enzymes were based on previous work (Colombatto *et al*. 2003*a*, 2003*b*; Colombatto and Beauchemin 2009), which used a dose of 1.5 mg/kg of DM consumed, this dose being equivalent to 30 mg/100 mL (considering an average of 0.2 g DM incubated in each bottle in this study). Simultaneously, Control samples (both substrates without addition of enzymes), blanks for enzymes (containing buffer carbonate-bicarbonate medium, ruminal liquor and the respective concentration of the enzyme, i.e. PROTEX and R at 30, 60 and 90 mg/100 mL), and blanks for ruminal liquor (ruminal liquor + buffer medium) were tested. The same procedure was repeated during three different weeks (i.e. three runs to obtain three repetitions) with the ruminal liquor, which was obtained from two different animals each time.

Two factors were studied under a factorial arrangement (i.e. 2 Enzymes × 3 Doses) generating six enzyme treatments (30, 60 and 90 mg/100 mL of PROTEX, P30, P60 and P90, respectively; and 30, 60 and 90 mg/100 mL of R, R30, R60 and R90, respectively), on two substrates: Milium and Meadow. Treatment results were corrected by the respective controls and blank samples (Table 1). Based upon previous evidence indicating that the addition of enzymes to pure cellulose and xylan samples 20 h before *in vitro* incubation increased the release of sugars (Colombatto *et al*. 2003*c*), enzymatic preparations were added to the incubation medium (i.e. 1 mL of enzymatic preparation + 38 mL of buffer medium) the night before to the incubation, in the bottles which had been hermetically sealed and kept at 4°C.

Substrate and enzyme products

Milium coloratum (DM = 911 g/kg fresh weight) harvested at vegetative state from Rafaela (Province of Santa Fe, Argentina, Lat. 31°10′S – Long. 61°28′O – Alt. 91 m a.s.l.) was kindly provided by the 'Program for the Improvement of Forage and Feedstuffs Assessment (PROMEFA)' of the 'Animal Nutrition Research and Services Center (CISNA)' – School of Agriculture, University of Buenos Aires (Table 2). The Patagonian mixed meadow grass was harvested in Santa Cruz province (Argentina, Lat. 51°37′S – Long. 69°15′O – Alt. 6 m a.s.l.), during the spring season (dominated by *Poa pratensis*, *Hordeum pubiflorum* y *Alopecurus* spp., DM = 860 g/kg fresh weight). PROTEX activity extracted from *Bacillus licheniformis* [at neutral pH, 7.5 equivalents of a standard (protease from *Streptomyces griseus*) per millilitre of undiluted enzyme product] has already been characterised by Colombatto and Beauchemin (2009). R (extracted from *Penicillium funiculosum*) has xylanase activity

Table 1. Arrangement of the experimental treatments

Dose[A]	Corrected by[B]	
	Protex	Rovabio
Control	Bl-R	Bl-R
30	Bl-E (Protex 30)	Bl-E (Rovabio 30)
60	Bl-E (Protex 60)	Bl-E (Rovabio 60)
90	Bl-E (Protex 90)	Bl-E (Rovabio 90)

[A]Control (no enzyme addition); 30, 60 and 90 (enzyme doses at 30, 60 and 90 mg/100 mL).

[B]Bl-R, blanks for ruminal liquor (ruminal liquor and buffer medium); Bl-E, blank for enzymes (ruminal liquor, buffer medium and corresponding enzyme).

Table 2. Chemical composition of *Milium coloratum* and Patagonian mixed meadow grass substrates

Chemical fraction[A] (g/kg DM)	Milium	Meadow
Organic matter	910	880
$aNDF_{OM}$	704	635
ADF_{OM}	377	320
ADL_{SA}	52	27
Crude protein	71	158
Ether extract	22	17
Hemicellulose	327	314
Cellulose	325	293

[A]$aNDF_{OM}$, neutral detergent fibre (ash-free) with alfa-amilase; ADF_{OM}, acid detergent fibre (ash-free); ADL_{SA}, acid detergent lignin with sulfuric acid.

(555 μmol of glucose/mL added enzyme), and endo-glucanase activity (181 μmol glucose/mL added enzyme). These activities were provided by the manufacturers.

Chemical and nutritional characterisation

All procedures were adjusted to the standardised protocols proposed by PROMEFA (Table 2). In short, samples were prepared by drying (65°C, 48 h) and milling (1 mm; Wiley mill) before further characterisation. All results were reported on a DM basis (after 105°C during 4 h). Ash content was measured after combustion at 550°C for 4 h (AOAC International 1990). CP (= total N × 6.25) was determined by Kjeldahl (AOAC International 1990) with a Pro-Nitro (Selecta J.P., Barcelona, Spain) and ether extract was assessed with a Soxhlet extractor by using petroleum ether (AOAC International 1990). Neutral detergent fibre with α-amylase ($aNDF_{OM}$, Goering and Van Soest 1970) and acid detergent fibre (ADF_{OM}) were reported as ash-free (AOAC International 1990) by using an ANKOM fibre analyser (Model 220, Ankom Corp., Macedon, NY, USA).

Samples were also characterised by *in vitro* cumulative gas production according to procedures outlined by Theodorou *et al*. (1994) to evaluate the kinetics of fermentation and to estimate digestibility. Samples were incubated in dark brown, 100-mL bottles with a Bromobutyl septa cap (20 mm diameter) and sealed with aluminium caps. Ruminal liquor (~solid : liquid 50 : 50 ratio) was collected before the morning feeding from two cannulated ewes fed to maintenance with a standard diet (alfalfa pellet : maize grain, 70 : 30; plus complete mineral salts). Incubation medium was prepared by mixing one part of ruminal liquor with 10 of carbonate-bicarbonate buffer (Theodorou *et al*. 1994). Samples were incubated in duplicate during 24 h at 39°C in a water bath, in three different periods (i.e. three different weeks) to generate three repetitions.

Pressure changes into the incubation bottles were measured using a pressure transducer (T443A model, Bailey and Mackey Ltd, Birmingham, England) connected with a three-ways valve at 2, 4, 8, 12 and 24 h of incubation (i.e. five times), pressure values were corrected by the amount of substrate DM incubated. Volume was then regressed on pressure records in order to fit a linear regression model (after checking for outliers – visibly out of pattern data), to calculate the actual volume record for every bottle and time. Raw gas production data were corrected by their respective blanks gas production (Table 1) in order to calculate net cumulative gas production (NCGP), and data were expressed per g of incubated DM basis (mL/g DM). The NCGP at 2 h was analysed independently in order to assess the immediate effect of enzymes addition. Similarly, NCGP were used at 8 and 24 h to evaluate the effects on intermediate and slow degrading fractions. In order to assess CH_4 concentration, gas produced at each sampling time was collected and preserved into gas-tight vials saturated with N_2 at atmospheric pressure. Samples were kept at 4°C until they were analysed through gas chromatography (Hewlett Packard 4890, equipped with a Porapak N 80/100 column 2 m long and using N_2 as carrier; injector temperature: 110°C; oven: 90°C; flame ionisation detector: 250°C). CH_4p at 24 h of incubation (after correction by the respective blanks, Table 1) was expressed by incubated DM (CH_4iDM, g/kg), digested DM (CH_4dDM, g/kg), and as a proportion of substrate gross energy (Ym, Mcal/100 Mcal of gross energy).

After 24 h of incubation, fermentation was terminated by adding 2–3 drops of a saturated thymol solution to each bottle, filtered through fibre filter bags (ANKOM #F57) previous to being sealed. The ivDMD was calculated from filter bags residues after being treated with neutral detergent solution according to Van Soest *et al*. (1966). Furthermore, NDFD was analysed according to Goering and Van Soest (1970).

True DMD at 24 h of incubation was therefore calculated as follows:

$$ivDMD\ (\%) = (100 - NDF\ residue) \times \frac{100}{DM\ incubated}$$

Statistical analyses

Data were analysed according to a randomised complete block design (blocking by incubation period), considering a factorial arrangement of Enzyme (R, PROTEX) and Dose (D30, D60 and D90), being the mathematical model:

$$y_{ijklm} = \alpha_i + \beta_j + \gamma_k + \delta_l(\gamma_k) + \alpha \times \delta(\gamma) + \rho_m(\gamma_k) + \delta \times \rho(\gamma) + \alpha \times \delta \times \rho(\gamma) + \varepsilon_{ijklm}$$

Where: y_{ijklm} is the response variable; α_i is the ith substrate type (Milium, Meadow); β_j is the jth block effect (1, 2, 3); γ_k is the kth effect of treatment (treated samples, Control); $\delta_l(\gamma_k)$ is the lth effect of the enzyme type (nested into γ_k; PROTEX and R); $\rho_m(\gamma_k)$ is the mth effect of the enzyme doses (nested into γ_k; 0, 30, 60 and 90 mg/100 mL); ε_{ijklm} is the residual random error.

Initially, the above complete model was run, and terms were dropped whenever $P > 0.05$. Every model was tested by homoscedasticity and normality ($P = 0.05$). Multiple means comparisons were made by Tukey test (Steel and Torrie 1980). All procedures were carried out by using Proc GLM (SAS Institute 2013). Significance was declared at 5% probability level and trends were discussed when $P < 0.10$.

Results

All the explored interactions that were not significant ($P > 0.05$) were dropped from the final model. Meadow samples were higher to Milium ones ($P < 0.05$) for ivDMD and NDFD at 24 h (Table 3). The NCGP at 24 h showed a similar pattern (Table 4). However, at early stages of fermentation (NCGP at 2 h and 8 h), Milium samples were higher than Meadow samples. Likewise, digestible NDF (dNDF, %DM) was higher in Meadow than in Milium. In relation to CH_4 emissions, CH_4iDM and Ym of Meadow forage were ~13% higher than Milium ($P < 0.05$), but when expressed per unit of digested DM, Milium became ~11% superior to Meadow ($P < 0.05$). The addition of PROTEX enzyme reduced ($P < 0.05$) ivDMD and NDFD in both substrates at 6.8% and 14.7%, respectively. The same occurred with dNDF. The NCGP was significant at 8 h of incubation, when PROTEX reduced ($P < 0.05$) NCGP to 30.3 from 39.2 mL/g incubated DM (average of Control and R). However, after 24 h of incubation NCGP showed an Enzyme × Dose interaction ($P < 0.05$, Table 4), with differences ($P < 0.05$) within R enzyme treatment. The CH_4p was not affected by the type of enzymes or doses rates.

Table 3. *In vitro* dry matter digestibility (ivDMD, %), neutral detergent fibre digestibility (NDFD, % NDF) and digestible NDF (dNDF, % DM) after 24 h of incubation of *Milium coloratum* and Patagonian meadow forages after being treated with no enzyme (Control), and Protex or Rovabio at 30, 60 and 90 mg/100 mL

Significance factors: **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant ($P > 0.05$)

Variables	Substrates			Enzymes				Significant factors[B]				
	Milium $n = 21$	Meadow $n = 21$	s.e.m.[A]	Control $n = 6$	Protex $n = 18$	Rovabio $n = 18$	s.e.m.	Block	Subs	Enz	Doses	E × D
ivDMD	48.7b	62.8a	0.86	56.7a	53.1b	56.7a	1.61	***	***	***	n.s.	n.s.
NDFD	26.7b	40.9a	1.41	35.2a	30.7b	34.7ab	2.64	***	***	**	n.s.	n.s.
dNDF	37.5b	51.9a	1.78	46.5a	39.9b	46.0a	3.32	***	***	**	n.s.	n.s.

[A]s.e.m., standard error of the mean. Different letters within the same factor (Substrate or Enzyme) and line differ (Tukey test, $P < 0.05$).

[B]Proposed model = Subs + Enz + Block + Treat + Enz (Treat) + Doses (Treat) + E × D + ε; where: Subs, Substrate type; Enz, Enzyme type; Block, Block effect; Treat, Treatment; E × D, interaction between enzymes and doses, ε, residual random error.

Table 4. Net cumulative gas production (NCGP, mL/g DM) at 2, 8 and 24 h and methane emissions after 24 h of incubation of *Milium coloratum* and Patagonian meadow forages after being treated with no enzyme (Control), and Protex or Rovabio at 30, 60 and 90 mg/100 mL

Significance factors: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; [A], $P < 0.10$; n.s., not significant ($P > 0.05$)

Variables	Substrates			Enzymes				Significant factors[B]				
	Milium $n = 21$	Meadow $n = 21$	s.e.m.[A]	Control $n = 6$	Protex $n = 18$	Rovabio $n = 18$	s.e.m.	Block	Subs	Enz	Doses	E × D
					Net cumulative gas production							
2 h	9.6a	6.0b	1.35	7.9	6.3	9.1	2.52	***	*	n.s.[A]	n.s.	n.s.
8 h	40.4a	34.7b	2.37	41.4a	30.3b	37.0a	4.44	***	*	*	n.s.	n.s.[A]
24 h[C]	97.6b	110.5a	4.66	110.5a	89.9b	105.4a	8.72	***	**	**	n.s.	*
					Methane production							
Methane (g/kg incubated DM)	4.3b	4.9a	0.26	4.6	4.7	4.4	0.47	***	*	n.s.	n.s.	n.s.
Methane (g/kg digested DM)	8.0a	7.2b	032	7.9	7.2	7.5	0.58	***	***	n.s.	n.s.	n.s.
Ym (Mcal/100 Mcal)	13.0b	14.7a	0.77	14.0	14.1	13.3	2.02	***	*	n.s.	n.s.	n.s.

[A]s.e.m., standard error of the mean. Different letters within the same factor (Substrate or Enzyme) and line differ (Tukey test, $P < 0.05$).

[B]Proposed model = Subs + Enz + Block + Treat + Enz (Treat) + Doses (Treat) + E × D + ε; where: Subs, Substrate type; Enz, Enzyme type; Block, Block effect; Treat, Treatment; E × D, interaction between enzymes and doses, ε, residual random error.

[C]E × D, means for doses within Rovabio: R30 = 104.0, R60 = 95.1 and R90 = 117.0. Tukey test, $P < 0.05$; s.e.m. = 12.33. No differences within Protex ($P > 0.05$).

Discussion

Substrate analyses

The chemical characteristics reported here agreed with previous reports for Milium (Rhodes and Udén 1998; Tomás *et al*. 2009; Asa *et al*. 2010). Although the Patagonian meadow showed comparable NDF concentration to previous reports (Jaurena *et al*. 2009, 2010), it showed considerably higher ADF_{OM} values, which were reflected in the lower ivDMD reported here. These discrepancies are the natural consequence of the different botanical composition and environment. Both forages (Milium and Meadow) presented high cell wall contents, but the Patagonian meadow showed a DM and NDF digestibility that were 29% and 53% higher than Milium, respectively (in agreement with the higher NCGP at 24 h). In relation to this, Milium showed 18% higher ADF_{OM} concentration than Meadow suggesting different cell wall characteristics, as the type of lignin and characteristics of cell wall carbohydrates explained almost all the variation in NDF rate and extent of digestion (Raffrenato *et al*. 2009). These so far intriguing patterns have already been seen in previous works with these types of grasses (Jaurena *et al*. 2009, 2010). In spite of the relatively lower cell content (1-NDF) and dNDF of Milium, the higher NCGP at early fermentation times would suggest a greater amount of rapidly soluble components than those of Meadow grasses.

We are unaware of previous studies reporting associated CH_4 emissions for similar forages, hence no comparisons were possible. The Meadow had a CH_4p higher than Milium when expressed per kg of DM incubated and concurrently higher Ym values, as a consequence of the higher ivDMD. However, when corrected per kg of DM digested, the relationship was reversed (Milium exceeded Meadow), suggesting that some compositional characteristic of Milium (e.g. fibre content) favoured CH_4p per unit of degraded substrate. Taking into account that CH_4 comes exclusively from the ruminally fermentable carbohydrates fraction (and therefore actually degraded) this expression (CH_4p per unit of digested substrate) suggests that it should be preferred when comparing different substrates.

Application of enzymes

As stated in the underlying hypothesis of this work, if any of the assayed enzymes were effective in degrading some of the refractory components of the cell walls, an increase in NCGP

at early incubation times would have been expected, but R did not bring about any significant ($P < 0.05$) changes at any of the assayed doses (i.e. 30, 60 and 90 mg/100 mL). This outcome agreed with a previous report of Jalilvand *et al*. (2008) who did not find any difference in *in vitro* cumulative gas production at 24 h due to the incorporation of an enzyme mixture (cellulase, xylanase, alfa-glucanase, protease and amylase activity) in alfalfa hay, corn silage and wheat straw, though the lag phase was lower in wheat straw and corn silage treated with enzymes. On the contrary, some authors have found an improvement during the first hours of *in vitro* fermentation using fibrolytic enzymes in leaves and stems of alfalfa (Colombatto *et al*. 2003*d*), in fibrous forage (Elghandour *et al*. 2013), corn silage (Colombatto *et al*. 2004*b*), and legume and grass mixture silage (Stokes 1992). Colombatto *et al*. (2003*c*) demonstrated that the addition of exogenous enzymes improved cellulose and xylan fermentation *in vitro* mainly due to an increase in hydrolytic activity. The beneficial potential of using enzymes in dairy cows diets has also been reported by improvements in DM, NDF and CP degradability (Rode *et al*. 1999; Elghandour *et al*. 2013). Discrepancies among authors who tested similar additives could be associated with factors which are not related with the enzymatic activity *per se*, such as the method of application, application level (Beauchemin *et al*. 2003) or concentration (Jalilvand *et al*. 2008).

The addition of proteolytic enzymes to ruminant feeds used to be considered not important, mainly because it was commonly argued that proteolytic enzymes could increase dietary and endogenous proteins degradation with the consequent raise in N waste (Eun and Beauchemin 2008). In this experiment, the decrease of ivDMD generated by the addition of PROTEX would support this hypothesis, even taking into account the decrease in NDFD. Similar reductions in ivDMD were found in silages (Colombatto *et al*. 2004*a*), although the authors attributed the decrease of ivDMD to the starch degradation during the ensiling caused by the activity of the α-amylase present in the additive. Collectively, these findings highlight the need to adequately characterise the enzyme additives before use in ruminant feeds.

In contrast, positive responses to the same proteolytic enzyme have been found in *in vitro* and *in vivo* studies. For example, Colombatto *et al*. (2003*b*) observed in a screening study that this commercial product improved the degradability of forage, and in another *in vitro* study with a dual-flow continuous culture system, they found improvements in the degradability of NDF and hemicellulose of a dairy total mixed ration (Colombatto *et al*. 2003*a*). Similarly, using PROTEX, improvements in ivDMD at 22 h were observed (11.6%, 7.7% and 6.7% of improvement for alfalfa hay, corn silage and dry-rolled corn, compared with the Control; Colombatto and Beauchemin 2009). *In vivo* studies have reported improvements in OM, N and hemicellulose digestion with high and low forage inclusion rates (Eun and Beauchemin 2005), as well as in a study with sheep and goats fed with wheat straw and a commercial concentrate (increments in digestibility of OM, NDF and CP of up to 11%; Salem *et al*. 2011). In a study with dairy cows fed with a total mixed ration with the addition of an enzyme mix including protease activity, an improvement of intake and digestibility was also found (Gado *et al*. 2009). In a meta-analysis on protease enzymes carried out by Eun and Beauchemin (2008), improvements on *in vitro* degradability linked to protease activity have been found, but they also observed that high levels of protease inclusion could have detrimental effects on ruminal forage degradation. From this review, it becomes evident the need for further studies on proteases, and the need to develop a more accurate measurement methodology of the enzyme activity.

The effect of enzymes on structural carbohydrates and proteins could help to decrease CH_4 emissions of substrates (Beauchemin *et al*. 2008). To date, there have been few studies analysing the relationship between the addition of exogenous enzymes and ruminal CH_4, with contradictory results. For example, increases in CH_4p (i.e. 43% in fermented forages) associated to the addition of enzymes with cellulolytic activity, were found using the Rumen Simulation Technique (Dong *et al*. 1999). Giraldo *et al*. (2008) found differences between cellulolytic enzymes derived from different microorganisms in diets with high and low forage inclusion. When the enzyme was derived from *Trichoderma longibrachiatum*, there was an increase in *in vitro* CH_4p ($P < 0.05$) whereas no differences ($P > 0.10$) were detected when the enzyme mixture was derived from *Aspergillus niger*. In other experiments, although fibrolytic activity (i.e. xylanase) induced improvements in the disappearance of DM, NDF and ADF, there was no response in CH_4p (i.e. g/kg DM incubated, $P < 0.05$; Giraldo *et al*. 2007*a*, 2007*b*). Using a continuous-flow dual culture system to study the addition of an enzyme with proteolytic activity in a total mixed diet for dairy cows [i.e. corn silage, rolled corn and alfalfa hay (30%, 40% and 30% of inclusion)], no differences were found in *in vitro* CH_4p though the treatment increased the NDF degradability (Colombatto *et al*. 2003*a*). Additionally, in an *in vivo* study with steers fed with barley silage, the application of a proteolytic enzyme did not change CH_4 emissions, or NDF and ADF digestibility (McGinn *et al*. 2004). In this study, none of the enzymes assessed altered the *in vitro* CH_4 emissions. Although we are unaware of similar studies analysing the methanogenic capacity of substrates studied here, in a previous study Milium yielded slightly more CH_4 than here [8.7 g CH_4/kg digested DM and ivDMD (24 h) = 653 g/kg DM; J. M. Cantet, pers. comm.].

Conclusions

The aim of this study was to analyse the impact of adding two types of enzymes on digestion parameters to two forages scarcely described. Although there were differences between the two forage species (Meadow presented higher ivDMD, NDFD and NCGP at 24 h of incubation, but emitted less CH_4 per unit of digested DM than Milium), no effects associated with the addition of R were found for any of the rated doses. In contrast, the addition of PROTEX induced a decrease in ivDMD, NDFD and dNDF, though no effects were detected in relation to CH4p.

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References

Adesogan AT, Ma ZX, Romero JJ, Arriola KG (2014) Improving cell wall digestion and animal performance with fibrolytic enzymes. *Journal of Animal Science* **92**, 1317–1330. doi:10.2527/jas.2013-7273

AOAC International (formerly the Association of Official Analytical Chemists) (1990) 'Official methods of analysis.' (AOAC: Arlington, VA)

Asa R, Tanaka A, Uehara A, Shinzato I, Toride Y, Usui N, Hirakawa K, Takahashi J (2010) Effects of protease-resistant antimicrobial substances produced by lactic acid bacteria on rumen methanogenesis. *Asian-Australasian Journal of Animal Sciences* **23**, 700–707. doi:10.5713/ajas.2010.90444

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

Beauchemin KA, Kreuzer M, O' Mara F, McAllister TA (2008) Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture* **48**, 21–27. doi:10.1071/EA07199

Bedford MR (2000) Exogenous enzymes in monogastric nutrition – their current value and future benefits. *Animal Feed Science and Technology* **86**, 1–13. doi:10.1016/S0377-8401(00)00155-3

Colombatto D, Beauchemin KA (2009) A protease additive increases fermentation of alfalfa diets by mixed ruminal microorganisms *in vitro*. *Journal of Animal Science* **87**, 1097–1105. doi:10.2527/jas.2008-1262

Colombatto D, Hervás G, Yang WZ, Beauchemin KA (2003*a*) Effects of enzyme supplementation of a total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. *Journal of Animal Science* **81**, 2617–2627.

Colombatto D, Morgavi DP, Furtado AF, Beauchemin KA (2003*b*) Screening of exogenous enzymes for ruminant diets: relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal of Animal Science* **81**, 2628–2638.

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

Colombatto D, Mould FL, Bhat MK, Owen E (2003*d*) 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. doi:10.1016/S0377-8401(03)00126-3

Colombatto D, Mould FL, Bhat MK, Phipps RH, Owen E (2004*a*) *In vitro* evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage. I. Effects of ensiling temperature, enzyme source and addition level. *Animal Feed Science and Technology* **111**, 111–128. doi:10.1016/j.anifeedsci.2003.08.010

Colombatto D, Mould FL, Bhat MK, Phipps RH, Owen E (2004*b*) *In vitro* evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage. III. Comparison of enzymes derived from psychrophilic, mesophilic or thermophilic sources. *Animal Feed Science and Technology* **111**, 145–159. doi:10.1016/j.anifeedsci.2003.08.012

Dong Y, Bae HD, McAllister TA, Mathison GW, Cheng KJ (1999) Effects of exogenous fibrolytic enzymes, alfa-bromoethanesulfonate and monensin on fermentation in a rumen simulation (RUSITEC) system. *Canadian Journal of Animal Science* **79**, 491–498. doi:10.4141/A99-024

Elghandour MMY, Salem AZM, Gonzalez-Ronquillo M, Bórquez JL, Gado HM, Odongo NE, Peñuelas CG (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. doi:10.1016/j.anifeedsci.2012.11.010

Eun JS, Beauchemin KA (2005) Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation and milk production. *Journal of Dairy Science* **88**, 2140–2153. doi:10.3168/jds.S0022-0302(05)72890-3

Eun JS, Beauchemin KA (2007) Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. *Journal of Dairy Science* **90**, 2839–2851. doi:10.3168/jds.2006-820

Eun JS, Beauchemin KA (2008) Relationship between enzymic activities and *in vitro* degradation of alfalfa hay and corn silage. *Animal Feed Science and Technology* **145**, 53–67. doi:10.1016/j.anifeedsci.2007.05.039

Feng P, Hunt CW, Pritchard GT, Julien WE (1996) Effect of enzyme preparations on *in situ* and *in vitro* degradation and *in vivo* digestive characteristics of mature cool-season grass forage in beef steers. *Journal of Animal Science* **74**, 1349–1357.

Gado HM, Salem AZM, Robinson PH, Hassan M (2009) Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Animal Feed Science and Technology* **154**, 36–46. doi:10.1016/j.anifeedsci.2009.07.006

Giraldo LA, Carro MD, Ranilla MJ, Tejido ML (2007*a*) Influence of fibrolytic enzymes on *in vitro* methane production and rumen fermentation of a substrate containing 60% of grass hay. *Livestock Research for Rural Development* **19**, 185–187.

Giraldo LA, Ranilla MJ, Tejido ML, Carro MD (2007*b*) Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. *British Journal of Nutrition* **98**, 753–761. doi:10.1017/S0007114507744446

Giraldo LA, Tejido ML, Ranilla MJ, Carro MD (2008) 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. doi:10.1016/j.anifeedsci.2007.06.013

Goering HK, Van Soest PJ (1970) 'Forage fiber analysis (apparatus, reagents, procedures and some applications) laboratory handbook. No. 379.' (USDA, Agricultural Research Services: Washington, DC)

Grainger C, Beauchemin KA (2011) Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal Feed Science and Technology* **166–167**, 308–320. doi:10.1016/j.anifeedsci.2011.04.021

He ZX, He ML, Walker ND, McAllister TA, Yang WZ (2014) Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains with solubles: ruminal fermentation, digestibility, and growth performance of feedlot steers. *Journal of Animal Science* **92**, 3978–3987. doi:10.2527/jas.2014-7707

Jalilvand G, Odongo NE, López S, Naserian A, Valizadeh R, Shahrodi FE, Kebreab E, France J (2008) Effects of different levels of an enzyme mixture on *in vitro* gas production parameters of contrasting forages. *Animal Feed Science and Technology* **146**, 289–301. doi:10.1016/j.anifeedsci.2008.01.007

Jaurena G, Livraghi E, Alvarez Ugarte D, Ceccaldi E, Molina R (2009) Conservación de forraje de vegas como heno enrollado en la isla de Tierra del Fuego, Argentina. *Revista Argentina de Producción Animal* **29**, 99–110. [in Spanish, English abstract]

Jaurena G, Livraghi E, Alvarez Ugarte D, Ceccaldi E, Molina R (2010) Ensiling natural meadow forage in Tierra del Fuego, Argentina. *Spanish Journal of Agricultural Research* **8**, 722–728. doi:10.5424/sjar/2010083-1270

Johnson KA, Johnson DE (1995) Methane emissions from cattle. *Journal of Animal Science* **73**, 2483–2492.

McGinn SM, Beauchemin KA, Coates T, Colombatto D (2004) Methane emissions from beef cattle: effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *Journal of Animal Science* **82**, 3346–3356.

Moe PW, Tyrrell HF (1979) Methane production in dairy cows. *Journal of Dairy Science* **62**, 1583–1586. doi:10.3168/jds.S0022-0302(79)83465-7

Pinos-Rodríguez JM, González SS, Mendoza GD, Bárcena R, Cobos MA, Hernández A, Ortega ME (2002) Effect of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science* **80**, 3016–3020.

Raffrenato E, Fievisohn R, Cotanch KW, Grant RJ, Van Soest PJ, Chase L, Van Amburgh ME (2009) aNDF, NDFd, iNDF, ADL and kd: what have we learned? In 'Proceeding of the Cornell nutrition conference for feed manufacturers', 71st Meeting, 20–22 October 2009. pp. 77–93.

(Department of Animal Science, NY State College of Agriculture and Life Sciences, Cornell University: Ithaca, NY)

Rhodes NM, Udén P (1998) Promising tropical grasses and legumes as feed resources in Central Tanzania. III. Effect of feeding level on digestibility and voluntary intake of four grasses by sheep. *Spanish Journal of Agricultural Research* **70**, 79–95.

Rode LM, Yang WZ, Beauchemin KA (1999) Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science* **82**, 2121–2126. doi:10.3168/jds.S0022-0302(99)75455-X

Salem AZM, El-Adawy M, Gado H, Camacho LM, González-Ronquillo M, Alsersy H, Borhami B (2011) Effects of exogenous enzymes on nutrients digestibility and growth performance in sheep and goats. *Tropical and Subtropical Agroecosystems* **14**, 867–874.

SAS Institute (2013) 'SAS user's guide: statistics, 12.3.' (SAS Institute, Inc.: Cary, NC)

Steel RGD, Torrie JH (1980) Multiple comparisons. In 'Principles and procedures of statistics, a biometrial approach.' 2nd edn. (Eds C Napiel, JW Maisel) pp. 185–186. (McGraw-Hill Publisher: New York, NY)

Stokes MR (1992) Effects of an enzyme mixture, an inoculant, and their interaction on silage fermentation and dairy production. *Journal of Dairy Science* **75**, 764–773. doi:10.3168/jds.S0022-0302(92)77814-X

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J (1994) A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology* **48**, 185–197. doi:10.1016/0377-8401(94)90171-6

Tomás A, Gastaldi L, Gaggiotti M, Romero L (2009) Rendimiento y valor nutritivo de materiales de *Panicum coloratum* var. *Makarikariensis Revista Argentina de Producción Animal* **29**(Suppl. 1), 564–565 [in Spanish, English abstract].

Van Soest PJ (1994) 'Nutritional ecology of the ruminant.' 2nd edn. (Cornell University Press: Ithaca, NY)

Van Soest PJ, Wine RH, Moore LA (1966) Estimation of the true digestibility of forages by the *in vitro* digestion of cell walls. In 'Proceedings of the 10th international grasslands congress'. (Ed. HA Steppler) pp. 438–441. (Finish Grassland Association: Helsinki, Finland)