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Effects of treating high forage and high concentrate diets with exogenous fibrolytic enzymes on their *in vitro* ruminal fermentation*

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ABSTRACT

Batch cultures of mixed rumen microorganisms were used to study the effects of a 24 h pre-treatment with a xylanase from *Trichoderma viride* (XYL) and a cellulase from *Trichoderma longibrachiatum* (CEL) on the *in vitro* fermentation of two diets composed by grass hay and concentrate in the proportions of 70:30 (HF) and 30:70 (HC). In 8 h incubations with HF, CEL increased ($P<0.05$) the production of acetate, propionate and total volatile fatty acids (VFA) and gas, but no effects ($P>0.05$) were observed for XYL. In contrast, with HC both enzymes increased ($P<0.05$) the production of propionate and total VFA and the true DM degradability of substrate, and decreased the CH_4/VFA ratio ($P<0.05$). After 24 h incubation, some of the observed effects disappeared, but CEL still increased ($P<0.05$) the production of acetate, propionate and total VFA for HF. For HC, both enzymes increased ($P<0.05$) the production of total VFA and XYL also increased ($P<0.05$) acetate production. The results indicate that effects of enzymes on *in vitro* fermentation were influenced by the nature of the diet, and that this influence varied with the incubation time, being more pronounced at short incubation times.

KEY WORDS: exogenous fibrolytic enzymes, ruminal fermentation, batch cultures

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INTRODUCTION

The use of fibrolytic enzymes as feed additives in ruminant diets has received considerable attention in the last years, despite observed responses have been highly variable. The effects of enzymes are influenced by factors such as type and dose of enzyme, type of diets fed to the animals, and enzyme application method (Beauchemin et al., 2003). To our knowledge, however, no study has been conducted to investigate how the forage:concentrate ratio in the diet can affect the response to enzyme treatment. The objective of this study was therefore to evaluate the effects of two fibrolytic enzymes on the *in vitro* ruminal fermentation of two diets differing in their forage:concentrate ratio. The study examined the effects of the enzymes at two incubation times, since it has been shown that the response to enzymes usually becomes less marked as incubation time increases (Beauchemin et al., 2003).

MATERIAL AND METHODS

The diets were composed of grass hay and a commercial concentrate based on barley, maize, soyabean meal and a vitamin-mineral mixture in the proportions (DM basis) of 70:30 (high-forage diet; HF) and 30:70 (high-concentrate diet; HC). Crude protein (CP), neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) contents of diets were 143, 496 and 253 g/kg DM for HF and 176, 376 and 161 g/kg DM for HC. Two enzyme preparations commercialized by Fluka Chemie GmbH (Germany) were tested: xylanase from *Trichoderma viride* (XYL) and cellulase from *Trichoderma longibrachiatum* (CEL). Enzyme preparations were assayed for endoglucanase, exoglucanase, xylanase and amylase activities following the procedures described by Colombatto and Beauchemin (2003). All activities were measured at pH 6.5 and 39ºC in order to resemble optimal ruminal conditions. At pH 6.5 and 39ºC, 1 mg of XYL liberated per min 15.0 μmol of xylose from oat spelt xylan and 0.237 μmol of glucose from carboxymethylcellulose, but no amylase and exoglucanase activities were detected. One mg of CEL liberated 1.72 μmol of xylose from oat spelt xylan, and 2.40, 0.385 and 0.040 μmol of glucose from carboxy-methylcellulose, soluble starch and Avicel PH-101, respectively. 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 39ºC and pH 6.5, and both enzyme preparations were added at a rate of 40 enzymatic units/g substrate DM.

Samples (500 mg) of ground diet (1-mm screen) were accurately weighed into 120-ml serum bottles. Solutions of each enzyme preparation were prepared in 0.1 M sodium phosphate buffer (pH 6.5), 0.5 ml of the corresponding solution were

added directly to each bottle 24 h before starting the incubation, and bottles were kept at room temperature (21-23ºC) until incubation. The inoculum was obtained from four rumen-cannulated Merino sheep fed 500 g of grass hay and 500 g of concentrate per day administered in two equal portions at 09.00 and 18.00 h. Ruminal contents of each sheep were obtained immediately before the morning feeding, mixed and strained through four layers of cheesecloth. 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 39ºC under continuous flushing with CO_2. Fifty ml of buffered ruminal fluid were added into each bottle under CO_2 flushing, bottles were sealed with rubber stoppers and aluminium caps, and incubated at 39ºC for 8 and 24 h. Four incubation runs were performed on different days, and in each of them one bottle per treatment was included.

After 8 and 24 h of incubation, total gas production was measured in all bottles using a pressure transducer and a calibrated syringe, and a gas sample (about 15 ml) from each bottle was stored in a vacuum tube before analysis for CH_4 concentration. Bottles were then uncapped, the pH was immediately measured and the fermentation was stopped by swirling the bottles in ice. Two ml of the bottle content were taken for VFA and NH_3-N analyses. Finally, the content of the bottles was transferred to previously weighed filter crucibles, the solid residue was washed with 50 ml of hot distilled water (50ºC) and the crucibles were dried at 50ºC for 48 h. Residues were analysed for NDF to estimate true DM degradability (TDMD) and NDF degradability (NDFD). Procedures for analysis of CP, NDF, ADF, VFA, CH_4 and NH_3-N have been described by Carro et al. (1999).

Data from each incubation time were analysed independently. When data from both enzymatic treatments and diets were analysed together, significant enzyme x diet interactions ($P<0.05$) were observed for some variables. Therefore, data were analysed separately for each diet as an ANOVA with three enzyme treatments (control (CON), XYL and CEL) and four incubation runs (blocking factor) as main effects. When a significant effect of the enzyme treatment ($P<0.05$) were detected, differences between means were assessed by the LSD test.

RESULTS AND DISCUSSION

The effects of the treatment with fibrolytic enzymes on *in vitro* fermentation of diets after 8 and 24 h of incubation with rumen mixed microorganisms are shown in Tables 1 and 2, respectively. For HF, there were no effects ($P>0.05$) of enzymes on final pH, CH_4 production, NH_3-N concentration and TDMD at any incubation time. After 8 h of incubation and with HF, CEL treatment increased ($P<0.05$) the production of gas, acetate, propionate and total VFA by 11, 10, 23 and 13%, respectively, but no effects of XYL ($P>0.05$) were detected. In

contrast, for HC both enzymes decreased (P<0.05) final pH and CH_4:VFA ratio, and increased (P<0.05) TDMD and the production of propionate and total VFA. These results indicate that CEL was more effective than XYL with HF, but only subtle differences were observed between the effects of both enzymes on *in vitro* fermentation of HC. The treatment with XYL implied mainly xylanase activity, as endoglucanase and amylase activities were negligible. Compared to XYL, CEL presented lower xylanase activity, but greater endoglucanase, exoglucanase and amylase activities. Eun and Beauchemin (2007) suggested that limiting enzymatic activity is likely substrate dependent. The lack of effect of XYL on fermentation of HF diet might be partly explained if endoglucanase activity would have been the limiting activity for this diet. However, for HC diet XYL and CEL produced similar effects on some ruminal variables at 8 h. These results indicate again that the enzymatic activities required to enhance ruminal degradation are influenced by the nature of the incubated substrates. As pointed out by Wallace et al. (2001), a precisely identification of the enzymatic activity causing a positive response in ruminal fermentation might make possible to develop more effective fibrolytic enzyme products.

Table 1. Influence of two enzymatic treatments on *in vitro* fermentation of two diets (500 mg DM) in batch cultures of mixed rumen microorganisms for 8 h (n=4)

Treatment[1]	High-forage diet (HF)				High-concentrate diet (HC)			
	CON	XYL	CEL	s.e.d.[2]	CON	XYL	CEL	s.e.d.[2]
PH	6.73	6.73	6.73	0.016	6.59^{b}	6.54^{a}	6.54^{a}	0.013
Gas, µmol	2127^{a}	2235^{ab}	2350^{b}	47.8	2465^{a}	2480^{a}	2563^{b}	16.3
CH_4, µmol	379	395	393	10.0	482	498	497	12.0
NH_3-N, mg/l	160	159	168	8.0	186	178	185	2.43
TDMD, %	497	502	510	0.6	591^{a}	626^{b}	619^{b}	0.573
Total VFA, µmol	1572^{a}	1538^{a}	1772^{b}	29.7	1829^{a}	2045^{b}	2158^{b}	46.5
Acetate, µmol	973^{a}	960^{a}	1070^{b}	16.3	1056^{a}	1108^{ab}	1239^{b}	49.7
Propionate, µmol	400^{a}	391^{a}	492^{b}	11.3	507^{a}	617^{b}	640^{b}	22.7
Butyrate, µmol	148	139	156	5.9	214	248	207	18.4
Ac/Pr, mol/mol	2.43^{b}	2.45^{b}	2.18^{a}	0.047	2.09	1.82	1.95	0.096
CH_4/VFA, mol/mol	0.242^{b}	0.257^{a}	0.222^{c}	0.0053	0.263^{b}	0.244^{a}	0.232^{a}	0.0069

[1] treatments, CON - control, XYL - xylanase from *Trichoderma viride*, CEL - cellulase from *Trichoderma longibrachiatum*. Both enzymes were applied at a level of 40 enzymatic units/g of substrate DM

[2] standard error of the difference

[a,b,c] for each diet, mean values within a row with unlike superscript letters differ (P<0.05)

Several studies (Wang et al., 2001; Giraldo et al., 2007) have reported that treating different feeds with fibrolytic enzymes produced a shift in the molar proportions of VFA, but shifts in pattern of VFA seem to be influenced by the type

of diet and enzyme preparations. In the present study, CEL treatment decreased significantly the acetate:propionate ratio at 8 h of incubation for HF, indicating a change in fermentation pattern. Wang et al. (2001) suggested that changes in fermentation pattern may reflect a shift in the species profile of colonizing bacteria in response to pre-treatment of feed with exogenous enzymes.

Table 2. Influence of two enzymatic treatments on *in vitro* fermentation of two diets (500 mg DM) in batch cultures of mixed rumen microorganisms for 24 h (n=4)

Treatment[1]	High-forage diet (HF)				High-concentrate diet (HC)			
	CON	XYL	CEL	s.e.d.[2]	CON	XYL	CEL	s.e.d.[2]
pH	6.65	6.62	6.61	0.054	6.45	6.44	6.43	0.014
Gas, µmol	5143	5017	5322	47.5	4868	4922	4963	23.3
CH_4, µmol	820	857	889	10.9	879	923	923	29.3
NH_3-N, mg/l	296	287	284	22.8	351	337	328	8.7
TDMD, %	63.5	64.0	66.9	1.29	75.5	75.0	75.5	0.57
NDFD, %	26.5[a]	30.5[ab]	33.4[b]	1.54	33.8	33.5	35.2	1.23
Total VFA, µmol	2812[a]	2888[a]	3080[b]	57.3	3444[a]	3551[b]	3560[b]	38.5
Acetate, µmol	1697[a]	1788[ab]	1869[b]	33.4	1957[a]	2100[b]	2028[ab]	44.1
Propionate, µmol	642[a]	630[a]	715[b]	15.7	834[a]	843[a]	885[b]	12.5
Butyrate, µmol	322	324	332	8.0	469	466	448	9.0
Ac/Pr, mol/mol	2.65[b]	2.84[b]	2.62[a]	0.046	2.35	2.50	2.31	0.077
CH_4/VFA, mol/mol	0.291	0.297	0.288	0.0072	0.254	0.261		0.0087

[1] treatments, CON - control, XYL - xylanase from *Trichoderma viride*, CEL - cellulase from *Trichoderma longibrachiatum*. Both enzymes were applied at a level of 40 enzymatic units/g of substrate DM

[2] standard error of the difference

[a,b,c] for each diet, mean values within a row with unlike superscript letters differ ($P<0.05$)

Some of the observed effects of enzymes disappeared after 24 h of incubation, but CEL still increased ($P<0.05$) the production of acetate, propionate and total VFA by 10, 11 and 9.5%, respectively, for HF; in contrast, no effects ($P>0.05$) of XYL were detected for these diet. Regarding HC, both CEL and XYL increased total VFA by 2.8 and 3.1%, respectively. For both diets, the effects of enzymes were more marked at 8 than at 24 h of fermentation, thus indicating that enzymes produced their effects at early stages of fermentation. Previous studies (Beauchemin et al., 2003; Giraldo et al., 2007) have also shown that effects of fibrolytic enzyme on *in vitro* fermentation were generally larger during the initial stages of degradation.

In general, effects of CEL on *in vitro* fermentation of diets were more marked than those of XYL. Although CEL was effective with both substrates, lower effects were observed for HC compared to HF. The results of this study indicate that effects of enzymes on *in vitro* fermentation were influenced by the nature

of the diet, and that this influence varied with the incubation time, being more pronounced at short incubation times.

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