In vitro ruminal fermentation of low-quality forages as influenced by the treatment with exogenous fibrolytic enzymes

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SUMMARY – The effects of three fibrolytic enzymes on the *in vitro* ruminal fermentation of five low-quality forages (three cereal straws (barley, wheat and rice), corn stover and grass hay) were investigated using batch cultures of mixed ruminal microorganisms. Four different treatments were investigated: no additive (control; CON), cellulase from *Aspergillus niger* (CEL; Fluka Chemie GmbH), xylanase from ruminal microorganisms (XYL; Megazyme International Ireland Ltd), and a 1:1 mixture cellulase:xylanase (MIX). Enzymes (20 IU/g forage dry matter (DM)) were applied directly onto the forages 24 h before incubation with buffered ruminal fluid at 39ºC for 24 h. The treatment with CEL increased (P<0.05) gas production after 24 h of incubation for wheat straw, barley straw and grass hay, whereas the treatment with MIX and XYL did not affect (P>0.05) gas production for any forage. For all substrates, neutral-detergent fibre digestibility (NDFD) and NH_3-N concentration were not affected (P>0.05) by the addition of enzymes. The treatment of forages with CEL increased the production of propionate for grass hay, corn stover and barley straw, and increased (P<0.05) total VFA production for wheat straw and corn stover. No effects (P>0.05) of MIX and XYL treatments on VFA production were observed for any substrate. The results indicate that the pre-treatment of low-quality forages with fibrolytic enzymes under the conditions of the present experiment contributed little, if any, to ruminal fibrolytic activity.

Keywords: Cellulase, xylanase, cereal straws, batch cultures, *in vitro* degradability.

RESUME – *"Influence du traitement avec des enzymes fibrolytiques exogènes sur la fermentation* in vitro *de fourrages pauvres". Les effets de trois enzymes fibrolytiques exogènes sur la fermentation de cinq fourrages pauvres on été étudiés en utilisant une technique in vitro de type "batch". On a utilisé 4 traitements : témoin (control, CON), cellulase d'Aspergillus niger (CEL; Fluka Chemie GmbH), xylanase de microorganismes ruminaux (XYL; Megazyme International Ireland Ltd), et un mélange 1:1 cellulase:xylanase (MIX). Les enzymes (20 UI/g MS fourrage) on été appliquées directement sur les fourrages 24 h avant leur incubation avec du contenu ruminal mélangé avec une solution tampon à 39ºC pendant 24 h. Le traitement avec CEL a augmenté (P<0,05) la production de gaz, pour la paille de blé, la paille d'orge et le foin de graminées, tandis que les traitements MIX et XYL n'ont pas modifié (P>0,05) la production de gaz des fourrages. Pour tous les substrats, l'addition d'enzymes n'a pas affecté la DFND ni l'azote ammoniacal. Le traitement des fourrages avec CEL a augmenté la production de propionate pour le foin de graminées, les cannes de maïs et la paille d'orge, ainsi que la production totale d'AGV (P<0,05) pour la paille de blé et les cannes de maïs. Aucune différence significative (P>0,05) n'a été notée entre les traitements MIX et XYL et le témoin pour la production d'AGV. Ces résultats montrent que le pré-traitement des fourrages pauvres avec les trois enzymes fibrolytiques dans ces conditions expérimentales a très peu contribué à l'activité fibrolytique ruminale.*

Mots-clés : *Cellulase, xylanase, pailles de céréales, cultures en batch, dégradabilité* in vitro*.*

Introduction

In some small ruminant production systems, forages constitute the major portion of all available feed resources. In the case of low-quality forages (high fibre content and low digestibility), any improvement in their nutritive value would increase the productivity of the animals. The use of feed enzymes for ruminants has been viewed with considerable scepticism, but in recent years a considerable number of studies on this topic have been conducted. The majority of these experiments were designed with the expectation that a fibrolytic enzyme should increase the degradability of feed in the rumen, and this response has been observed in many of these studies (McAllister *et al.*, 2001; Phipps *et al.*, 2002). These effects, however, seem to be dependent on the enzyme source, dose and method of administration and the diet fed to the animals. Most of studies with enzyme-treated forages have been conducted with good-quality forages, but studies involving fibrous feeds are limited. The

objective of this study was to evaluate the effects of three different fibrolytic enzymes on the *in vitro* ruminal fermentation of five low-quality forages.

Material and methods

Samples of barley straw, wheat straw, rice straw, corn stover and grass hay were ground through a 1-mm screen and fermented *in vitro* with buffered ruminal fluid. The chemical composition of forages is given in Table 1. Three different enzymes preparations were tested: cellulase from *Aspergillus niger* (CEL; Cellulase 22178; Fluka Chemie GmbH), xylanase from ruminal microorganisms (XYL; XylanaseM6; Megazyme International Ireland Ltd), and a 1:1 mixture cellulase:xylanase (MIX). Solutions of each enzyme containing 5 units per ml were prepared in 0.1 M sodium phosphate buffer (pH 6.5). Samples of 500 mg of forage dry matter (DM) were accurately weighed into 120-ml glass bottles and 2 ml of the corresponding solution (20 IU/g forage DM) were added into each bottle 24 h before starting the incubation. Bottles were kept at room temperature (21-23ºC) until incubation. This pre-treatment of forages with enzymes was selected as previous *in vitro* studies have shown that this enzyme-feed interaction enhanced the efficacy of enzymatic treatments (Giraldo *et al.*, 2004). Two ml of buffer were added to bottles corresponding to control treatment.

Table 1. Chemical composition (g/kg DM) of forages incubated *in vitro* with buffered ruminal fluid

	Organic matter	Crude protein	Neutral-detergent fibre	Acid-detergent fibre	Lignin
Barley straw	929	54	707	387	63.2
Wheat straw	931	35	757	544	77.1
Rice straw	920	37	708	407	70.1
Grass hay	950	57	710	419	78.3
Corn stover	940	53	653	450	65.3

Ruminal fluid was obtained from four rumen-cannulated Merino sheep fed forage (medium-quality alfalfa hay) *ad libitum*. Ruminal contents of each sheep were obtained before the morning feeding, mixed and strained through four layers of cheese-cloth 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) in a proportion 1:4 (v:v) at 39ºC under continuous flushing with CO_2. Bottles were prewarmed (39ºC) prior to the addition of 50 ml of buffered ruminal fluid into each bottle under CO_2 flushing. Bottles were sealed with rubber stoppers and aluminium caps and incubated at 39ºC for 24 h. Four incubation runs were performed on different days, so that each treatment was conducted in quadruplicate. In each incubation run, two blanks (bottles without substrate but with the corresponding enzymatic treatments) were included. Bottles were withdrawn from the incubator 24 h after inoculation and gas production was measured with a calibrated syringe and a pressure transducer. Bottles were uncapped, the pH was measured immediately and the fermentation was stopped by swirling the bottles in ice. One ml of the bottle content was added to 1 ml of deproteinizing solution (10% of metaphosphoric acid and 0.06% crotonic acid; w/v) for volatile fatty acids (VFA) analysis and another 1 ml was added to 1 ml of HCl for NH_3-N analysis. The contents of the bottles were then transferred to previously weighed filter crucibles. Each solid residue of incubation was washed with 50 ml of hot (50ºC) distilled water and crucibles were dried at 50ºC for 48 h. Residues were analysed for neutral-detergent fibre (NDF) to estimate fibre degradability (NDFD).

Dry matter, ash and N were determined according to the AOAC (1999). Neutral-detergent fibre, acid-detergent fibre and acid-detergent lignin analyses were carried out according to Van Soest *et al.* (1991) and Goering and Van Soest (1970). NH_3-N concentration was determined by a colorimetric method and VFA concentrations by gas chromatography as described by Carro *et al.* (1999). The amounts of VFA produced were obtained by subtracting the amounts present initially in the incubation medium from those determined at the end of the incubation period. Because interactions forage x enzyme ($P<0.05$) were detected for some variables, data for each forage were analysed independently as an ANOVA with four enzymatic treatments (control (CON), CEL, MIX and XYL) and four ruminal inocula as main effects. When a significant effect of the treatment ($P<0.05$) were detected, differences between means were assessed by the LSD test.

Results and discussion

The effects of exogenous fibrolytic enzymes on *in vitro* rumen fermentation of forages are shown in Table 2. The treatment with CEL increased (P<0.05) gas production for barley straw, wheat straw and grass hay, but no effects (P>0.05) of MIX and XYL treatments were observed for any forage. The increase in gas production for CEL treatment could not stem from enzyme fermentation itself, as there were no differences (P>0.05) between CON and CEL treatments in the amount of gas produced in the blanks (mean values of 5.7 and 5.5 ml, respectively); these results might therefore indicate that CEL treatment enhanced the *in vitro* fermentation of these forages. Indeed, the treatment of barley straw, grass hay and corn stover with CEL increased (P<0.05) propionate production by 4.6, 5.9 and 6.3%, respectively, but did not affect (P>0.05) the production of acetate. Wang *et al.* (2004) showed that the treatment of wheat straw with an enzyme preparation containing xylanase and ß-gluconase activities increased VFA at 4 h of incubation, but no differences due to the enzyme treatment were observed after 30 h of incubation. In agreement with these results, previous research (Giraldo *et al.*, 2005) showed that the effects of treating substrates with fibrolytic enzymes on *in vitro* VFA production by ruminal microorganisms were more marked at 6 than at 24 h of incubation. In the present experiment, VFA production was measured at 24 h and only CEL treatment increased significantly (P<0.05) this parameter for wheat straw and corn stover. There were, however, no effects (P>0.05) of any enzymatic treatment on molar proportions of acetate, propionate and butyrate or acetate:propionate ratio.

Final pH values ranged from 6.55 to 6.69 (values not shown) and were not affected (P>0.05) by any of the enzymatic treatments. For all forages, there was no change (P>0.05) in NH_3-N concentration with added enzymes, indicating no differences in protein degradability and/or ammonia-N incorporation by ruminal microorganisms. Although all forages presented a low protein content (35-57 g crude protein/kg DM), NH_3-N concentrations after 24 h of incubation were in the range of those considered as optimal for ruminal microbial growth (Mehrez *et al.*, 1977) due to the use of a N-enriched buffer (Goering and Van Soest, 1970).

The sterified bonds between cellulose, hemicellulose and lignin restrict the digestion of cell walls by ruminal microorganisms; however, it has been shown (Nsereko *et al.*, 2000; Giraldo *et al.*, 2004) that exogenous fibrolytic enzymes could potentially improve fibre degradation through a hydrolytic action prior to feeding or *in vitro* incubation. In the present experiment, the 24 h pre-treatment with enzymes of the five forages did not affect (P>0.05) their NDF content (see Table 3). On the other hand, the 24 h pre-treatment of a good-quality substrate (mixture grass hay:concentrate; 60:40) with the cellulase used in this study (cellulase from *Aspergillus niger;* Fluka Chemie GmbH) at rates of 15 and 30 IU/g substrate DM decreased (P<0.05) by 7.6 and 10.2% its NDF content (Giraldo *et al.*,2005). These results would indicate that effectiveness of fibrolytic enzymes varies with the substrate (McAllister *et al.*, 2001). The ability of cellulases and xylanases to increase the extent of fibre digestion may be limited by the lack of enzymes that degrade the core structure of lignin-cellulose complexes in low-quality forages. Krueger *et al.* (2003) showed that an enzymatic complex containing high esterase, cellulose and endogalacturonase activities enhanced the digestion of low-quality tropical hays and suggested that the use of enzymes such as ferulic acid esterases could made the digestible xylans in the cell wall more susceptible to enzymatic degradation. In the present study, the lack of effects of enzymes on forages NDF content was in agreement with the observed inefficacy of enzymatic treatments to increase forages NDFD (P>0.05).

Conclusions

Under the conditions of the present experiment, the pre-treatment of low-quality forages with cellulase and xylanase enzymes produced subtle effects on *in vitro* ruminal fermentation, suggesting that the used enzymes contributed little, if any, to ruminal fibrolytic activity. Moreover, the use of a mixture of both enzymes did not produce any further beneficial effects on ruminal fermentation. Future work is required to investigate the possible contribution of these enzymes to forage degradation and to find the ideal combination of highly active enzymes for optimizing the degradation of low-quality forages.

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Table 2. Influence of different enzymatic treatments on production of gas (ml) and volatile fatty acids (VFA; µmol), NH_3-N concentration (mg/l) and neutral-detergent fibre degradability (NDFD; %) after *in vitro* fermentation of forage samples (500 mg) in batch cultures of mixed ruminal microorganisms for 24 h (n=4)

Substrate and item	Enzymatic treatment†				sed††
	CON	CEL	XYL	MIX	
Barley straw					
Gas	70.4 [a]	72.8 [b]	69.3 [a]	71.9 [ab]	1.25
Total VFA	1656	1692	1675	1703	18.1
Acetate	1129	1139	1144	1165	15.5
Propionate	394 [a]	412 [b]	399 [a]	404 [ab]	5.1
Butyrate	111	118	114	113	3.0
NH_3-N	202	182	180	171	7.2
NDFD	46.7	46.4	45.6	45.4	1.53
Wheat straw					
Gas	50.9 [a]	54.5 [b]	52.6 [ab]	53.4 [ab]	1.31
Total VFA	1298 [a]	1395 [b]	1323 [a]	1347 [ab]	31.1
Acetate	895 [a]	958 [b]	890 [a]	923 [ab]	19.5
Propionate	302	324	317	313	10.6
Butyrate	82.3	95.0	96.5	94.0	5.16
NH_3-N	207	194	183	184	8.9
NDFD	40.9	41.7	42.3	41.3	1.30
Rice straw					
Gas	48.5	50.2	48.6	48.8	0.96
Total VFA	1253	1270	1222	1242	32.3
Acetate	843	862	807	834	21.4
Propionate	313	312	309	308	8.8
Butyrate	77.8	78.8	84.5	80.0	3.46
NH_3-N	185	197	187	208	9.1
NDFD	42.1	43.1	42.8	43.5	0.92
Grass hay					
Gas	58.1 [a]	60.6 [b]	59.5 [ab]	59.7 [ab]	0.89
Total VFA	1419	1493	1454	1476	35.2
Acetate	967	1013	1003	1010	26.1
Propionate	324 [a]	343 [b]	325 [a]	335 [ab]	7.7
Butyrate	102	110	102	106	4.4
NH_3-N	212	213	193	193	8.8
NDFD	34.8	34.5	36.4	36.0	1.22
Corn stover					
Gas	71.6	73.7	72.0	72.7	2.76
Total VFA	1833 [a]	1907 [b]	1826 [a]	1820 [a]	29.5
Acetate	1205	1235	1208	1196	26.8
Propionate	463 [a]	492 [b]	448 [a]	461 [a]	10.9
Butyrate	139 [a]	151 [b]	140 [a]	138 [a]	3.2
NH_3-N	224	224	217	215	8.3
NDFD	42.6	42.3	40.2	43.2	1.73

†Treatments: CON: control; CEL: cellulase from *Aspergillus niger;* XYL: xylanase from ruminal microorganisms; MIX: cellulase:xylanase mixture (1:1); all enzymatic treatments were applied at a rate of 20 IU/g forage DM.
††Standard error of the difference.
[a, b]: mean values within a row with unlike superscript letters differ (P<0.05).

Table 3. Influence of 24 h pre-treatment of forages with different enzymatic treatments on its neutral-acid-detergent fibre (NDF; g/kg DM) content (n=4)

Forage	Enzymatic treatment[†]				sed[††]
	CON	CEL	XYL	MIX	
Barley straw	707	693	700	695	0.96
Wheat straw	757	744	739	740	0.85
Rice straw	708	704	699	708	0.71
Grass hay	710	700	700	697	0.79
Corn stover	653	646	650	661	0.68

[†]Treatments: CON: control; CEL: cellulase from *Aspergillus niger;* XYL: xylanase from ruminal microorganisms; MIX: cellulase:xylanase mixture (1:1); all enzymes were applied at a rate of 20 IU/g forage DM.
[††]Standard error of the difference.

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