

Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the Rumen Simulation Technique (Rusitec)

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The effects of an exogenous enzyme preparation, the application method and feed type on ruminal fermentation and microbial protein synthesis were investigated using the rumen simulation technique (Rusitec). Steam-rolled barley grain and chopped alfalfa hay were sprayed with water (control, C), an enzyme preparation with a predominant xylanase activity (EF), or autoclaved enzyme (AEF) 24 h prior to feeding, or the enzyme was supplied in the buffer infused into the Rusitec (EI). Microbial N incorporation was measured using $(^{15}\text{NH}_4)_2\text{SO}_4$ in the buffer. Spent feed bags were pummelled mechanically in buffer to segregate the feed particle-associated (FPA) and feed particle-bound (FPB) bacterial fractions. Enzymes applied to feed reduced neutral-detergent fibre content, and increased the concentration of reducing sugars in barley grain, but not alfalfa hay. Ruminal cellulolytic bacteria were more numerous with EF than with C. Disappearance of DM from barley grain was higher with EF than with C, but alfalfa was unaffected by EF. Treatment EF increased incorporation of ^{15}N into FPA and FPB fractions at 24 and 48 h. In contrast, AEF reduced the 24 h values, relative to C; AEF and C were similar at 48 h. Infused enzyme (EI) did not affect ^{15}N incorporation. Xylanase activity in effluent was increased by EF and EI, compared to C, but not by AEF. Xylanase activity in FPA was higher at 48 h than at 24 h with all treatments; it was higher with EF than C at 24 and 48 h, but was not altered by AEF or EI. Applying enzymes onto feeds before feeding was more effective than dosing directly into the artificial rumen for increasing ruminal fibrolytic activity.

Rusitec: Exogenous enzymes: Ruminal microbes: Digestion: ^{15}N incorporation

In ruminants, digestion of the structurally complex fibrous compounds in plant cell walls is accomplished through the enzymic action of the ruminal microflora, but it is far from complete. There is considerable room to enhance the utilization of fibrous feeds by ruminant livestock. Interest in using exogenous enzymes to complement or stimulate existing digestive activity in the rumen has increased recently, but production responses have been highly variable. Positive effects (Beauchemin *et al.* 1995; Lewis *et al.* 1995; Stokes & Zhang 1995; Feng *et al.* 1996; Treacher *et al.* 1996; McAllister *et al.* 1999), negative effects (Theurer *et al.* 1963; Svozil *et al.* 1989), and lack of

effects (Beauchemin *et al.* 1995; Chen *et al.* 1995; McAllister *et al.* 2000) of enzymes on ruminant production have been reported. These inconsistencies have been attributed to differences in crude enzyme preparations, type of diets and/or application methods (Beauchemin *et al.* 1998; McAllister *et al.* 2000). It is known that microbial attachment to and colonization of feeds is essential for their degradation in the rumen (McAllister *et al.* 1994; Flint & Forsberg, 1995), but the mechanism by which exogenous enzymes alter feed digestion has not been clearly defined. In theory, these enzymes could work synergistically with, complementarily with, antagonistically against, or

Abbreviations: AEF, autoclaved exogenous enzyme applied onto each feed type; C, control (no added enzyme); EF, exogenous enzyme applied onto each feed type; EI, exogenous enzyme included in infusion buffer; DMD, disappearance of DM; FPA, feed particle-associated; FPB, feed particle-bound; NAN, non-ammonia nitrogen; NDF, neutral-detergent fibre; RS, reducing sugars; VFA, volatile fatty acids.

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independently from endogenous microbial populations. In this study, the rumen simulation technique (Rusitec) was used to investigate specific effects of exogenous fibrolytic enzymes on rumen microbial populations and microbial protein synthesis in conjunction with fermentation and digestion of forage (alfalfa) and concentrate (barley grain).

Materials and methods

Rusitec, inoculum, diets and experimental design

Inoculum (ruminal fluid and solid digesta) for two eight-vessel Rusitec units (Czerkawski & Breckenridge, 1977) was obtained from three Holstein cows in early lactation, maintained on a 60:40 % concentrate:barley silage diet (as-fed basis). Inoculum was prepared, and fermentations were established in the Rusitec units as described by Wang *et al.* (1998), except that the slow-speed centrifugation of ruminal fluid was omitted and the buffer infused into the fermenter vessels (McDougall, 1948) was modified to contain $(\text{NH}_4)_2\text{SO}_4$ 0.3 g/l. Diet (DM basis) for each of the 16 vessels comprised 5 g chopped (0.5 cm) alfalfa hay and 5 g steam-rolled barley grain, contained in separate feed bags. The four experimental treatments (n 4) were: no exogenous enzyme (control, C), exogenous enzyme delivered in the infused buffer (EI), exogenous enzyme applied onto each feed type (EF) and autoclaved enzyme applied onto each feed type (AEF). Before commencing data collection, a 12 d adaptation period was allowed during which gas and effluent volumes and fermenter liquid pH were monitored in order to assess the stability of the fermentation.

The enzyme used in this study was a powdered preparation from *Trichoderma longibrachiatum* (Biovance Technologies Inc., Omaha, NB, USA) that contained a mixture of hydrolases. The main enzyme activity present was xylanase (817 units/g, with one unit defined as release of 1 mmol xylose per min from oat spelt xylan (X-0627, Sigma Chemical Company, St Louis, MO, USA) under incubation conditions of 39°C and pH 6.5). Feeds for C and EI vessels were sprayed with deionized water (10 ml/100 g DM) using a hand-held, single-nozzle spray bottle. In the same manner, feeds for EF and AEF were sprayed with 1 % aqueous solutions of enzyme (10 ml/100 g DM), except that, for AEF, the enzyme solution was autoclaved (20 min, 121°C), cooled to room temperature, and homogenized in a blender before application. After spraying, the DM content of the barley grain and alfalfa hay were 79.1 (SD 0.49) % and 83.7 (SD 0.81) %, respectively. The treated feeds were weighed into feed bags (5 g DM each), then stored at room temperature for 24 h prior to transfer to a 4°C cooler. Feeds were treated every 3 d throughout the experiment. For EI vessels, the infusate was prepared daily by dissolving the enzyme (15 mg/l) in the McDougall (1948) buffer. The EI infusate was stored on ice throughout the experiment. The enzyme concentration was calculated from the infusion rate and DM content of the feeds, so that approximately equal amounts of enzyme were delivered to each vessel daily by the two methods (EI and EF/AEF).

Sample collection and analyses

Fermentation characteristics and DM disappearance. Effluent and gas produced by each fermentation vessel were measured daily over the 25 d of the study (Wang *et al.* 1998). Methane in the gas was determined on days 17 and 18 on subsamples of gas from the collection bags, using a Varian 3400 gas chromatograph equipped with a CTR I column (Alltech Associates, Inc., Deerfield, IL, USA). The injection volume was 100 μ l. Fermenter liquids were subsampled daily from day 16 to day 21, and analysed for ammonia and volatile fatty acids (VFA) as described by Wang *et al.* (1998). Disappearances of DM (DMD) from alfalfa hay and barley grain were determined using the 48 h bags from day 16 to day 21 (6 d) as described by Wang *et al.* (1998).

Microbial protein synthesis. To estimate microbial protein synthesis, effluent and feed residue solids were sampled on day 14 for determination of background ^{15}N , and the $(\text{NH}_4)_2\text{SO}_4$ in the McDougall's buffer was replaced with ^{15}N -enriched $(\text{NH}_4)_2\text{SO}_4$ (Sigma Chemical Co., minimum ^{15}N enrichment 10.01 atom %) for the remainder of the experiment. On days 22 and 25, 24 h accumulations of preserved effluent from each vessel were sampled for determination of ^{15}N enrichment in non-ammonia nitrogen (NAN). Effluent was preserved by placing 18 ml of 25 % (w/v) HgCl_2 in the collection flask for each vessel immediately after emptying on the day before sampling.

Feed particle-associated (FPA) and feed particle-bound (FPB) fractions were prepared from the 48 h feed residues on days 22 and 25, and from the 24 h residues on day 25. Upon removal from the fermenter vessel and gentle squeezing to expel excess liquid, the alfalfa hay and barley grain feed bags were sealed together in a plastic bag with 20 ml of McDougall (1948) buffer and processed for 60 s in a Stomacher 400 laboratory blender (Seward Medical Limited, London, UK). The processed liquid was squeezed out and retained, and the feed residues were washed twice with 10 ml buffer. The two 10 ml washings were combined with the initially expressed liquid, and the total volume was recorded. Subsamples of this fraction (FPA) were taken for determinations of xylanase activity and ^{15}N in NAN. Washed feed residues (FPB fraction) were weighed for determination of DM and ^{15}N enrichment. All samples were stored at -40°C until analysed.

For ^{15}N determinations, liquid samples were centrifuged (20 000 g; 30 min; 4°C), and the pellets were washed three times with 7 M-phosphate buffer (pH 7.2), centrifuging (20 000 g; 30 min; 4°C) after each wash. The resulting pellet was resuspended in 5.0 ml water, combined with 1.0 ml 5 % (w/v) NaOH and dried at 75°C. Feed residue samples were resuspended in 10 ml buffer, and washed, dried and treated with base as for liquid samples. Dried materials were weighed and ground for measurement of total N and ^{15}N enrichment by mass spectrometry using an NA 1500 nitrogen analyser (Carlo Erba Instruments, Rodano, MI, Italy).

Xylanase activity. On days 22 and 25, fermenter liquids from each vessel were sampled for determination of xylanase activity, as were the FPA fractions prepared for ^{15}N determinations. Xylanase activity was measured as release

of reducing sugars (RS) from oat spelt xylan during standardized incubation conditions, and was expressed as μg glucose equivalents released/min per ml, for liquid fractions, or per g DM, for FPA fractions. Liquid and FPA samples were centrifuged (20 000 g; 30 min; 4°C) and xylanase activity was determined in the supernatant. Three millilitres of sample were combined with 3 ml substrate solution (2 % suspension (w/v) oat spelt xylan in 0.2 M-phosphate buffer, pH 6.0), and incubated at 39°C, with shaking, for 2 h. Incubations were terminated by placing the tubes into boiling water for 10 min. Incubation solutions were centrifuged (20 000 g; 15 min; 4°C) and the supernatants were assayed for RS (Nelson 1944) against a glucose standard.

Microbial enumeration. On day 13, homogenate was prepared from two fermenters in each treatment group by blending together fermenter liquid (20 ml) and samples (0.5 g) from each of the 48 h feed bags (alfalfa hay and rolled barley grain) as described by Wang *et al.* (1998). Cellulolytic bacteria in the homogenates ($n = 2$) were enumerated by the most probable number method, and protozoa by light microscopy, also as described by Wang *et al.* (1998). The averages of values from the two fermenters are reported.

Feed and feed residues. Feed and feed residues were freeze-dried and ground (through a 1-mm screen), then analysed for DM (105°C for 3 h) and organic matter (after ashing). Neutral-detergent fibre (NDF) was determined using the method of Van Soest *et al.* (1991). To solubilize starch and facilitate filtering during NDF analysis, heat-stable α -amylase (A-3306, Sigma Chemical Co.) was included at 0.1 % (v/v) in the NDF solution added to feed samples (50 ml/0.5 g sample). Total N was measured by MS (NA 1500, Carlo Erba Instruments). Reducing sugars in feeds were measured by mixing 5 g sample with 100 ml boiling water, simmering for 5 min, cooling in ice water, centrifuging (10 000 g; 10 min) and analysing the supernatant for RS as described by Nelson (1944).

Calculations and statistics. Disappearances of DM from feed bags were determined gravimetrically and separately for rolled barley grain and alfalfa hay. Incorporation of ^{15}N into microbial N in effluent, FPA and FPB fractions were calculated based on sample size (weight and volume), total NAN and ^{15}N enrichments in the fractions produced daily. Total microbial ^{15}N incorporation was the sum of incorporations in effluent and in 48 h feed bags. For measurements repeated on different days, data were combined across days for each fermenter vessel.

Data were analysed by one-way ANOVA, with treatment included as a main effect. Differences among treatments were tested using the least square means procedure of the SAS programming language, with the PDIF (difference between P values) option invoked (1991, Statistical Analysis Systems Institute Inc., Cary, NC, USA).

Results

Applying exogenous enzyme onto feed (EF) reduced ($P < 0.05$) NDF content and increased ($P < 0.01$) RS content in barley grain but not in alfalfa hay (Table 1). Autoclaved

Table 1. Chemical composition (DM basis) of rolled barley grain and chopped alfalfa hay 24 h after treatment with water (control) or 1 % solutions of a crude xylanase preparation (EF) or autoclaved enzyme (AEF)

	Treatment			SEM
	Control	EF	AEF	
Rolled barley				
Organic matter (%)	97.28	97.26	97.10	0.939
Neutral-detergent fibre (%)	29.13 ^a	26.18 ^b	29.06 ^a	0.572
Total N (%)	1.90	1.95	1.96	0.037
Reducing sugars (mg/g DM)	1.68 ^d	5.58 ^c	1.99 ^d	0.352
Chopped alfalfa hay				
Organic matter (%)	92.15	91.39	91.42	0.817
Neutral-detergent fibre (%)	54.53	52.67	54.07	0.785
Total N (%)	2.32	2.37	2.46	0.072
Reducing sugars (mg/g DM)	33.96	37.91	36.19	2.683

^{a,b} Within a row, values followed by different superscripts differ ($P < 0.05$).

^{c,d} Within a row, values followed by different superscripts differ ($P < 0.01$).

enzyme did not affect the NDF or RS content of grain or hay ($P > 0.05$). No differences in organic matter or total N contents were observed ($P > 0.05$) among C, EF or AEF treatments for either feed type.

Disappearance of DM from barley grain was higher ($P < 0.05$) with EF than with C, AEF or EI, but treatment did not affect ($P > 0.05$) DMD from alfalfa hay or digestibility of NDF in either feed type (Table 2). Also unaffected ($P > 0.05$) by treatment were volume of gas and proportion of methane produced from either substrate.

Cellulolytic bacteria were ten times more numerous in EF vessels than in C, but protozoal numbers were similar among treatments (Table 3). Ammonia concentration in effluent from EF vessels was lower ($P < 0.05$) than from C or EI, but was similar ($P > 0.05$) to that in effluent from AEF vessels (Table 3). Total concentrations of VFA in vessel liquids were similar ($P > 0.05$) among treatments, but the molar proportion of propionate was higher ($P < 0.05$) with EF than with C or AEF. Treatment did not affect ($P > 0.05$) molar proportions of acetate and butyrate (Table 3) or other minor VFA (data not shown). Application of enzyme to the feed numerically increased the ^{15}N enrichment in the microbial N (by 20 % at 24 h and by 13 % at 48 h) of the FPA fraction, but not of the effluent, thus no significant effect of treatment on total ^{15}N incorporation per d was observed. However, the amount of ^{15}N incorporated in feed-related fractions (FPA+FPB) was higher ($P < 0.05$) with EF than with C, AEF or EI.

Incorporation of ^{15}N into the microbial protein associated with effluent, FPA and FPB fractions was affected by both exogenous fibrolytic enzyme and by application method (Table 4). Applying exogenous enzyme to feed before feeding (EF) increased ($P < 0.05$) incorporation of ^{15}N into FPB microbial N both at 24 and at 48 h, relative to C. In contrast, AEF decreased ($P < 0.01$) ^{15}N incorporation into FPB microbial N, but only at 24 h. EI did not affect ($P > 0.05$) ^{15}N incorporation into FPB microbial N at either time point.

Similar to the FPB, EF increased ($P < 0.05$), and AEF decreased ($P < 0.01$) ^{15}N incorporation into FPA microbial N at 24 h, relative to C (Table 4). At 48 h, however,

Table 2. Effect of exogenous fibrolytic enzyme on DM disappearance (DMD), digestibility of neutral-detergent fibre (NDF), and gas production in the Rumen Simulation Technique†

Item	Treatment*				SEM
	C	EF	AEF	EI	
DMD at 48 h (%)					
Rolled barley grain	71.69 ^b	75.18 ^a	68.97 ^b	70.23 ^b	0.917
Chopped alfalfa hay	50.24	51.78	49.58	50.52	0.976
NDF digestibility at 48 h (%)					
Rolled barley grain	50.21	47.59	46.91	48.15	1.502
Chopped alfalfa hay	24.41	23.09	23.55	25.71	1.258
Gas production (ml/24 h)‡	872	933	868	889	25.5
Proportion of methane in gas (%)	2.56	2.53	2.47	2.24	0.146

C, control (no enzyme); EF, enzyme applied to feed; AEF, autoclaved enzyme applied to feed; EI, enzyme infused with buffer.

^{a,b} Within a row, means bearing different superscripts differ ($P < 0.05$).

* Enzyme comprised xylanase concentrate applied at 1 mg/g DM.

† For details of procedures, see p. 326.

‡ Average of volumes produced on days 16 to 21 inclusive.

incorporation was similar among treatments ($P > 0.05$). In effluent microbial N, the amount of ^{15}N incorporated was numerically higher (603.9 μg at 24 h, averaged across treatments) than that incorporated into microbial N in FPB or FPA fractions (44.0 and 181.1 μg respectively), but ^{15}N incorporation in the effluent was unaffected ($P > 0.05$) by treatment.

Xylanase activity in the fermentation effluent was increased ($P < 0.05$) by enzymes, whether supplied on feed or in buffer (Table 5). Autoclaved enzyme did not affect xylanase activity. In all fermenter vessels, xylanase activity in the FPA fraction was higher at 48 h than at 24 h ($P < 0.05$), and at both time points it was higher ($P < 0.05$) with EF than with AEF, EI or C. In the FPA fraction, the increase in xylanase activity between 24 and 48 h was

numerically greater (104) in EF vessels than in C, AEF or EI (55, 69 and 50 μg RS/g DM per min respectively).

Discussion

Applying a crude xylanase preparation to feed 24 h before fermentation increased microbial protein production in FPA and FPB fractions, increased ruminal cellulolytic bacterial numbers, increased xylanase activity in liquid and FPA fractions and increased DMD from rolled barley grain. Similar responses were not observed when enzyme was infused, suggesting that interaction between the enzyme and the feed before contact with ruminal fluid is required for enhancement of feed digestion.

Table 3. Effect of exogenous fibrolytic enzyme on protozoal and cellulolytic bacterial numbers, fermentative characteristics and incorporation of ^{15}N into microbial N in the Rumen Simulation Technique†

Item	Treatment*				SEM
	C	EF	AEF	EI	
Cellulolytic bacteria ($\times 10^6/\text{ml}$)	0.49 ^b	6.70 ^a	1.32 ^b	2.52 ^b	0.903
Protozoa ($\times 10^3/\text{ml}$)	2.78	2.56	2.78	2.44	0.223
Ammonia (mg N/l)	126.0 ^{a,b}	116.3 ^c	120.9 ^{b,c}	132.2 ^a	2.64
Volatile fatty acids (mm)	34.97	36.20	35.32	38.57	1.876
Molar proportions					
Acetate	0.53	0.56	0.53	0.57	0.0274
Propionate	0.289 ^{b,c}	0.339 ^a	0.276 ^c	0.334 ^{a,b}	0.0166
Butyrate	0.141	0.157	0.152	0.154	0.0216
Atom % excess of ^{15}N in microbial N					
Effluent	1.793	1.794	1.846	1.815	–‡
Feed particle-associated fraction					
24 h	0.769	0.923	0.889	0.861	–‡
48 h	0.936	1.053	0.968	0.919	–‡
Microbial incorporation of ^{15}N					
Total ($\mu\text{g}/24\text{ h}$)	638.2	667.0	676.0	621.3	25.86
Feed related (μg)					
24 h	212.5 ^b	290.4 ^a	158.7 ^b	238.9 ^b	11.61
48 h	280.7 ^b	336.9 ^a	343.1 ^a	302.4 ^{a,b}	15.56

C, control (no enzyme); EF, enzyme applied to feed; AEF, autoclaved enzyme applied to feed; EI, enzyme infused with buffer.

^{a,b} Within a row, means followed by unlike letters differ ($P < 0.05$).

* Enzyme comprised xylanase concentrate applied at 1 mg/g DM.

† For details of procedures, see p. 326.

‡ Standard error of the mean not available because values were obtained from pooled samples.

|| Total incorporation is the sum of incorporations in effluent, feed particle-associated (FPA) and feed particle-bound (FPB) fractions prepared from feed bags. Feed-related incorporation is the sum of incorporation in FPA and FPB fractions.

Table 4. Incorporation of ¹⁵N into microbial N (μg) in effluent and in the feed particle-associated and feed particle-bound fractions during 24 and 48 h incubations of alfalfa hay or rolled barley grain in the Rumen Simulation Technique†

Sample type	Treatment*				SEM
	C	EF	AEF	EI	
Effluent (24 h)	605.7	619.6	640.2	550.2	27.71
Feed particle-associated fraction					
24 h	169.6 ^b	236.7 ^a	128.5 ^c	189.7 ^b	14.83
48 h	232.7	280.4	289.7	267.5	22.34
Feed particle-bound fraction					
24 h	42.9 ^b	53.7 ^a	30.2 ^c	49.2 ^{a,b}	2.55
48 h	48.2 ^b	56.5 ^a	53.4 ^{a,b}	54.9 ^{a,b}	1.58

C, control (no enzyme); EF, enzyme applied to feed; AEF, autoclaved enzyme applied to feed; EI, enzyme infused with buffer.

^{a,b,c} Within a row, means bearing different superscripts differ ($P < 0.05$).

* Enzyme comprised xylanase concentrate applied at 1 mg/g DM.

† For details of procedures, see p. 326.

Responses to enzyme applied to the feed were related to both pre-incubation and post-incubation effects (Table 1). Other studies have also indicated that spraying aqueous enzyme onto feed before consumption (to moisture levels equalling 10 % of the total DM) enables release of RS from substrate by the fibrolytic enzyme(s) before contact with rumen microbial populations (Beauchemin & Rode, 1996; Hristov *et al.* 1996). Supplying enzyme in the buffer afforded no pre-feeding opportunity for enzymic hydrolysis of substrate. Metabolic products of primary colonizers of feedstuffs are thought to attract secondary colonizers to feed surfaces and stimulate attachment (Cheng & McAllister, 1997). Thus, the released products of hydrolysis that accumulated on the surface of the feed particles may have elicited a similar chemotactic response that enhanced attachment of ruminal microbes to feed particles. Ruminal micro-organisms typically must circumvent physical barriers to colonization (e.g. cuticle, pericarp) and access internal, readily digestible tissues via stomata, lenticels or damaged areas (Cheng *et al.* 1983/84). By cleaving specific bonds in structural polymers, applied enzymes may weaken the surface of feed particles, thereby removing some of these physical barriers that impede microbial attachment. This possibility is supported by the observations that

Table 5. Effect of exogenous fibrolytic enzyme on xylanase activity in fermenter effluent and in feed particle-associated (FPA) fractions in the Rumen Simulation Technique†

Item	Treatment*				SEM
	C	EF	AEF	EI	
Xylanase activity ‡					
Effluent	0.95 ^b	1.91 ^a	0.79 ^b	1.91 ^a	0.159
FPA fraction					
24 h	79.7 ^b	133.3 ^a	73.9 ^b	73.1 ^b	7.25
48 h	134.2 ^b	237.5 ^a	144.1 ^b	123.1 ^b	7.60

C, control (no enzyme); EF, enzyme applied to feed; AEF, autoclaved enzyme applied to feed; EI, enzyme infused with buffer.

^{a,b} Within a row, means bearing different superscripts differ ($P < 0.05$).

* Enzyme comprised xylanase concentrate applied at 1 mg/g DM.

† For details of procedures, see p. 326.

‡ Expressed as release of reducing sugars (RS). In effluent, activity is expressed as μg RS/ml per min. On feed particles, activity is expressed as μg RS/g DM per min.

exogenous enzymes tend to lower the NDF and acid-detergent fibre contents of feeds (Gwayumba & Christensen, 1996; Hristov *et al.* 1996), and also by the decrease in NDF content of barley grain observed in the present study, but not by the observation that NDF content of alfalfa hay remained unchanged.

The observation that, in effluent, xylanase activity was higher with either EF or EI than with C, but that, in FPA fractions, it was higher only with EF relative to C (Table 5) may have resulted both from the attachment of exogenous enzyme to the feed particles, and from endogenous production of xylanases by bacteria attached to the feed. It is possible that binding to feed particles increases the resistance of exogenous enzyme to ruminal proteolysis, as compared to enzyme introduced into effluent. These factors suggest that applying exogenous enzymes to feed prior to consumption may be advantageous compared to feeding them directly.

This present study indicated that the efficacy of exogenous enzymes is affected not only by the method of application, but also by the type of feed to which they are applied. Applied to feeds prior to feeding, enzymes significantly increased 48 h DMD from barley grain, but not from alfalfa hay (Table 2). This distinction between feed types probably arises from both pre- and post-incubation responses to the exogenous enzyme. Enzymes applied to feed increased RS and decreased NDF content in barley grain but not alfalfa hay (Table 1). Diet-specific effects have been observed with other enzyme preparations (Hristov *et al.* 1996; Beauchemin *et al.* 1997), and we have also observed a similar phenomenon with pure cultures of bacteria, conceivably due to the profile of bacterial enzymes produced.

The extent to which complex feed substrates are degraded by pure cultures of bacteria is dependent upon the bacterial species (Dehority & Scott, 1967; Kudo *et al.* 1987). In our laboratory, *Fibrobacter succinogenes* more readily digested barley straw than alfalfa hay, whereas *Ruminococcus flavefaciens* was more effective at degrading alfalfa hay than barley straw (Y Wang, D Morgavi and T McAllister, unpublished results), and in a related experiment, the enzyme preparation used in the present study was stimulatory to *F. succinogenes* but not to *R. flavefaciens*. In agreement with that pure culture work, scanning electron microscopy of 12 h feed residues collected during the present Rusitec study confirmed that the predominant colonizing bacteria comprised different morphotypes on barley grain than on alfalfa hay, and that the morphological diversity among bacteria colonizing alfalfa hay was enhanced on EF, compared to C, AEF and EI (data not shown). It is possible, therefore, that the pre-feeding effects of exogenous enzyme may influence the digestive rumen microbiota, and thereby contribute to the differing effects of enzyme treatment between diets.

The hydrolytic release of RS from feed at 90 % DM occurred immediately following the application of enzyme solutions that increased moisture content by a further 10 % DM. Evaporation of this moisture, or its penetration into the feed particle, may limit the duration of this pre-ruminal hydrolysis. Some studies have shown that most of the RS release from high DM feeds affected by exogenous

enzymes occurs within 2 h of application (Nsereko *et al.* 2000). This pre-ruminal hydrolysis, albeit short-term, may play a critical role in the subsequent microbial colonization in mixed cultures. Although EF treatment decreased dietary NDF content in this present study, the extent of NDF digestion by ruminal microbes was not affected. This increase in initial solubilization of NDF independent of overall true digestibility was also reported by Chademana & Offer (1990), Erasmus *et al.* (1992) and Kumar *et al.* (1994), when *Saccharomyces cerevisiae* was used as a feed additive. A number of studies with various enzyme products showed that most of the preparations did not affect the extent of the digestion (Chen *et al.* 1995; Feng *et al.* 1996; Hristov *et al.* 1996, 1998; McAllister *et al.* 2000), although some showed that the rate of digestion was improved to a certain degree (Yang *et al.* 1999). Increasing the extent of digestion of feeds may be key to improving the efficacy of an enzyme for ruminant diets.

Increased incorporation of ^{15}N into both FPA and FPB microbial N with EF indicates that this treatment increased microbial attachment and increased colony growth following attachment, as was indicated by electron microscopy. Microbial attachment to substrate is thought to be the main factor in determining digestibility of fibre; it has been shown that over 85 % of cellulase, hemicellulase and glycosidase activities are associated with feed particles in the rumen (Williams & Strachan, 1984). Similar proportions of micro-organisms have been shown to be associated with solid digesta *in vivo* (Craig *et al.* 1987; Legay-Carmier & Bauchart, 1989) and in the Rusitec (Cheng & McAllister, 1997). Higher cellulolytic bacterial numbers and xylanase activity in the FPA fraction in the present study indicate that the increased attachment probably comprises mainly cellulolytic species. The increase in xylanase activity between 24 and 48 h, which was due to microbial activity, was notably larger with EF than it was with other treatments (104 v. 58 μg RS released/g DM per min, on average). Enhanced cellulolytic activity with enzyme treatment was also observed in *in vitro* studies with *Aspergillus niger* (Leatherwood *et al.* 1960) and with a whole cell product based on *A. oryzae* (Newbold *et al.* 1991, 1996), as well as with a number of exogenous fibrolytic preparations *in vivo* (Yang *et al.* 1999). These findings may also reflect a shift in the species profile of colonizing bacteria in response to pre-feeding treatment of the feeds with enzyme.

Increased cellulolytic bacterial numbers typically occur concurrently with generally increased bacterial numbers (Dawson 1987; Wiedmeier *et al.* 1987; Harrison *et al.* 1988; Frumholtz *et al.* 1989; Newbold *et al.* 1996). However, in the present study, similar 24 h levels of ^{15}N incorporation into microbial N across treatments indicated that total microbial mass was not changed. This observation, together with increased cellulolytic activity and reduced ammonia concentration in fermenter vessel liquid (Table 2), suggests that certain microbial populations, rather than total biomass, were affected by EF treatment.

Reduced ^{15}N incorporation into FPA fractions at 24 h with AEF (Table 4) is difficult to explain, but may be related to non-enzymic components in the crude fungal

extract. Some fungal extracts contain metabolic intermediates that stimulate ruminal bacteria (Nisbet & Martin, 1989), but some also contain preservatives that inhibit microbial activity (Alexander, 1971; Stewart *et al.* 1992; Odenyo *et al.* 1994). It is possible that some of these inhibitory substances impeded initial colonization of the substrate. This may have been reflected in the observation of decreased ^{15}N incorporation into microbial N at 24 h in the feed-related (FPB and FPA) fractions treated with AEF. Similar levels of incorporation between AEF and C at 48 h suggest that the inhibition was temporary, but the presence of these inhibitory substances may have prevented realization of the full benefits of supplemental enzymes.

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