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 (15NH4)2SO4 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 15N 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 15N 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: 15N 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...
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 (NH₄)₂SO₄ 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 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 Co., minimum 15N 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 15N enrichment in non-ammonia nitrogen (NAN). Effluent was preserved by placing 18 ml of 25 % (w/v) HgCl₂ 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 determination of 15N activity, and 15N in NAN. Washed feed residues (FPB fraction) were weighed for determination of DM and 15N enrichment. All samples were stored at −40°C until analysed.

For 15N 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 50 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 15N 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 15N 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 15N into microbial N in effluent, FPA and FPB fractions were calculated based on sample size (weight and volume), total N and 15N enrichments in the fractions produced daily. Total microbial 15N 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 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 each 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 15N 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 15N incorporation per d was observed. However, the amount of 15N incorporated in feed-related fractions (FPA+FPB) was higher (P < 0.05) with EF than with C, AEF or EI.

Incorporation of 15N 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 15N into FPB microbial N both at 24 and at 48 h, relative to C. In contrast, AEF decreased (P < 0.01) 15N incorporation into FPB microbial N, but only at 24 h. EI did not affect (P > 0.05) 15N incorporation into FPB microbial N at either time point.

Similar to the FPB, EF increased (P < 0.05), and AEF decreased (P < 0.01) 15N incorporation into FPA microbial N at 24 h, relative to C (Table 4). At 48 h, however,
incorporation was similar among treatments ($P > 0.05$). In effluent microbial N, the amount of $^{15}$N incorporated was numerically higher ($603.9 \mu g$ at 24 h, averaged across treatments) than that incorporated into microbial N in FPB or FPA fractions ($44.0$ and $181.1 \mu g$ respectively), but $^{15}$N incorporation in the effluent was unaffected ($P > 0.05$) by treatment.

Xylanase activity in the fermentation effluent was increased ($P, 0.05 \dagger$) 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 $50 \mu g$ 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 2. Effect of exogenous fibrolytic enzyme on DM disappearance (DMD), digestibility of neutral-detergent fibre (NDF), and gas production in the Rumen Simulation Technique†

<table>
<thead>
<tr>
<th>Item</th>
<th>C</th>
<th>EF</th>
<th>AEF</th>
<th>EI</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMD at 48 h (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolled barley grain</td>
<td>71.69</td>
<td>75.18</td>
<td>68.97</td>
<td>70.23</td>
<td>0.917</td>
</tr>
<tr>
<td>Chopped alfalfa hay</td>
<td>50.24</td>
<td>51.78</td>
<td>49.58</td>
<td>50.52</td>
<td>0.976</td>
</tr>
<tr>
<td>NDF digestibility at 48 h (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolled barley grain</td>
<td>50.21</td>
<td>47.59</td>
<td>46.91</td>
<td>48.15</td>
<td>1.502</td>
</tr>
<tr>
<td>Chopped alfalfa hay</td>
<td>24.41</td>
<td>23.09</td>
<td>23.55</td>
<td>25.71</td>
<td>1.258</td>
</tr>
<tr>
<td>Gas production (ml/24 h)‡</td>
<td>872</td>
<td>933</td>
<td>868</td>
<td>889</td>
<td>25-5</td>
</tr>
<tr>
<td>Proportion of methane in gas (%)</td>
<td>2.56</td>
<td>2.53</td>
<td>2.47</td>
<td>2.24</td>
<td>0.146</td>
</tr>
</tbody>
</table>

### 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†

<table>
<thead>
<tr>
<th>Item</th>
<th>C</th>
<th>EF</th>
<th>AEF</th>
<th>EI</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulolytic bacteria ($\times 10^6$/ml)</td>
<td>0.49</td>
<td>6.70</td>
<td>1.32</td>
<td>2.52</td>
<td>0.903</td>
</tr>
<tr>
<td>Protozoa ($\times 10^7$/ml)</td>
<td>2.78</td>
<td>2.56</td>
<td>2.78</td>
<td>2.44</td>
<td>0.223</td>
</tr>
<tr>
<td>Ammonia (mg N/l)</td>
<td>126.0</td>
<td>116.3</td>
<td>120.9</td>
<td>132.2</td>
<td>2.64</td>
</tr>
<tr>
<td>Volatile fatty acids (mm)</td>
<td>34.97</td>
<td>36.20</td>
<td>35.32</td>
<td>38.57</td>
<td>1.876</td>
</tr>
<tr>
<td>Molar proportions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>0.53</td>
<td>0.56</td>
<td>0.53</td>
<td>0.57</td>
<td>0.0274</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.289</td>
<td>0.339</td>
<td>0.276</td>
<td>0.334</td>
<td>0.0166</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.141</td>
<td>0.157</td>
<td>0.152</td>
<td>0.154</td>
<td>0.0216</td>
</tr>
<tr>
<td>Atom % excess of $^{15}$N in microbial N</td>
<td>1.793</td>
<td>1.794</td>
<td>1.846</td>
<td>1.815</td>
<td>–‡</td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feed particle-associated fraction</td>
<td>0.769</td>
<td>0.923</td>
<td>0.889</td>
<td>0.861</td>
<td>–‡</td>
</tr>
<tr>
<td>48 h</td>
<td>0.936</td>
<td>1.053</td>
<td>0.968</td>
<td>0.919</td>
<td>–‡</td>
</tr>
<tr>
<td>Microbial incorporation of $^{15}$N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ($\mu g/24 h$)</td>
<td>638.2</td>
<td>667.0</td>
<td>676.0</td>
<td>621.3</td>
<td>25-86</td>
</tr>
<tr>
<td>Feed related ($\mu g$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>212.5</td>
<td>290.4</td>
<td>158.7</td>
<td>238.9</td>
<td>11-61</td>
</tr>
<tr>
<td>48 h</td>
<td>280.7</td>
<td>336.9</td>
<td>343.1</td>
<td>302.4</td>
<td>15-56</td>
</tr>
</tbody>
</table>

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.

\[k\] 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.


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 (Dehoriy & 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

### Table 4. Incorporation of $^{15}$N into microbial N ($\mu$g) 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†

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Treatment*</th>
<th>C</th>
<th>EF</th>
<th>AEF</th>
<th>EI</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent (24 h)</td>
<td></td>
<td>605.7</td>
<td>619.6</td>
<td>640.2</td>
<td>550.2</td>
<td>27.71</td>
</tr>
<tr>
<td>Feed particle-associated fraction</td>
<td></td>
<td>169.6b</td>
<td>236.7a</td>
<td>128.5c</td>
<td>189.7b</td>
<td>14.83</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td>232.7</td>
<td>280.4</td>
<td>289.7</td>
<td>267.5</td>
<td>22.24</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td>42.9</td>
<td>53.7</td>
<td>50.3c</td>
<td>54.9b</td>
<td>3.58</td>
</tr>
<tr>
<td>Feed particle-bound fraction</td>
<td></td>
<td>48.2</td>
<td>56.5</td>
<td>53.4b</td>
<td>54.9b</td>
<td>1.58</td>
</tr>
</tbody>
</table>

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

†Within a row, means bearing different superscripts differ ($P < 0.05$).

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

### 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†

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment*</th>
<th>C</th>
<th>EF</th>
<th>AEF</th>
<th>EI</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylanase activity ‡</td>
<td></td>
<td>0.95b</td>
<td>1.91a</td>
<td>0.79b</td>
<td>1.91a</td>
<td>0.159</td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>79.7</td>
<td>133.3a</td>
<td>73.9b</td>
<td>73.1b</td>
<td>7.25</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td>134.2b</td>
<td>237.5a</td>
<td>144.1b</td>
<td>123.1b</td>
<td>7.60</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td>69.2</td>
<td>120.5a</td>
<td>59.3b</td>
<td>73.1b</td>
<td>7.25</td>
</tr>
</tbody>
</table>

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

†Within a row, means bearing different superscripts differ ($P < 0.05$).

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

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 enzyme 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

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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 cellulose, hemicellulose 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; Frumholz 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; Odeny 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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Exogenous enzymes in the Rusitec


