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The effect of enzyme treatment on the *in vitro* fermentation of lucerne incubated with equine faecal inocula

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A series of experiments was conducted to determine the effects of a fibrolytic enzyme preparation (enzyme 1; E1) on the *in vitro* fermentation of lucerne incubated with equine faecal inocula. In experiment 1, high-temperature-dried (HT) lucerne was treated with five levels of E1 (0 to 2·4 ml/g DM) and incubated at 50°C for 20 h. Samples then received a simulated foregut digestion (SFD) treatment before DM and NSP analysis. In experiment 2, HT lucerne was treated with the same enzyme levels used in experiment 1. Samples were then split into two groups; plus or minus an SFD treatment before *in vitro* fermentation using an equine faecal inoculum. In experiment 3, fresh and wilted lucerne were treated with the same levels of E1 as experiments 1 and 2, incubated at 50°C for 20 h, then fermented *in vitro*. For experiment 4, fresh and wilted lucerne were treated with low levels (0 to 0·008 ml/g DM) of E1 before fermentation. E1 significantly ($P<0·05$) enhanced DM and NSP losses from HT lucerne following SFD treatment compared with the control. High levels of E1 significantly ($P<0·05$) enhanced the rate, but not extent, of fermentation of HT, wilted and fresh lucerne; however, low levels of E1 were ineffective. At higher application levels, E1 appears to have considerable potential to enhance the nutritive value of lucerne for horses. Information on the fermentation kinetics of the substrates was valuable; all end-point measurements showed no effect of enzyme treatment.

Enzyme treatment: *In vitro* fermentation: Equine inocula: Lucerne

There is increasing interest in feeding high-energy, fibre-based feeds as an alternative to feeding cereal grains as a means of meeting the energy demands of working horses. This is due to the association of the onset of metabolic disorders, such as laminitis and colic, with feeding high levels of cereal grains. Preparations of fibrolytic enzymes have the potential to initiate the hydrolysis of forage fibre (structural carbohydrates), rendering the fibrous fraction more amenable to degradation *in vivo*. Exogenous fibrolytic enzymes have been reported to enhance the nutritive value of forages fed to ruminants by enhancing the digestibility of plant structural carbohydrates, thus enhancing the overall energy balance in the animal (Beauchemin *et al.* 1995, 2000; Feng *et al.* 1996; McAllister *et al.* 1999; Rode *et al.* 1999; Yang *et al.* 1999; Kung *et al.* 2000; Colombatto *et al.* 2003). However, such effects have not been investigated in the horse. Furthermore, the potential benefits of exogenous enzymes in horse diets are likely to differ from those in the ruminant due to the fundamental differences in the anatomical structure of the digestive tracts of these herbivores. In the horse, the application of exogenous enzymes to forages may help release starches, sugars, proteins, vitamins and minerals more fully for digestion and absorption in the small intestine by making available nutrients that were previously protected against digestive activity by intact cell walls (Low & Longland, 1989; Lyons & Walsh, 1993). The advantages of this are potentially threefold: first, the efficiency of feed utilisation will be enhanced, since the digestion of carbohydrate in the foregut (in terms of ATP yield) is more efficient than its fermentation in the hindgut; second, protein supply to the horse will increase, as only the protein digested and absorbed in the small intestine can be efficiently utilised by the horse; third, increased degradation of plant material in the hindgut will enhance the overall energy balance in the horse.

Although representative of the ideal situation, the *in vivo* determination of the nutritive value of feedstuffs for horses is expensive, laborious and lengthy. Consequently, *in vitro* digestibility techniques allow a greater number of feedstuffs and feed additives to be rapidly and simultaneously evaluated at reduced cost. Furthermore, these techniques have progressed from end-point digestibility measurements to methods of assessing the degradation kinetics of feeds. The *in vitro* gas production (GP) technique of Theodorou *et al.* (1994) provides information on the extent and rate at which feedstuffs are degraded, the latter being of particular relevance to the horse, which, compared with ruminants, has a rapid total tract transit time, often in the region of 26 h (Moore-Colyer *et al.* 2003). Although

Abbreviations: A + B:P, acetate plus butyrate:propionate; DML, DM loss; E1, enzyme 1; ETOH, absolute ethanol; FRGP, fractional rate of gas production; GP, gas production; HT, high-temperature-dried; SFD, simulated foregut digestion; TVFA, total volatile fatty acid; VFA, volatile fatty acid.

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the technique of Theodorou *et al.* (1994) initially relied upon rumen fluid as the source of microbial inoculum, more recently faecal inocula have been successfully used in GP studies investigating the fermentation of feeds for both ruminants (Akhter *et al.* 1999) and equines (Lowman, 1998), precluding the need for surgically modified animals.

The effect of enzyme treatment on the degradation of forages incubated *in vitro* with an equine microbial inoculum has yet to be reported. The objectives of the experiments reported here were to examine the effects of various levels of exogenous enzyme treatments on the *in vitro* fermentation of high-temperature-dried (HT), wilted and fresh lucerne. Furthermore, in an attempt to simulate the digestion of feed in the equine gastrointestinal tract, a simulated foregut digestion (SFD) step was included to allow the pre-caecal degradation of feeds to be estimated.

Materials and methods

Enzyme preparations

The enzyme product used was a crude liquid commercial preparation (Kemin Europa, Herentals, Belgium), designated enzyme 1 (E1). Based on the information provided by the manufacturers, E1 was a concentrated cellulose of fungal origin with enzyme activities of 6222, 1039 and 2156 IU (quantity of enzyme that liberates 1 μg glucose equivalents per min) for cellulase, xylanase and β-glucanase, respectively. Previous work by the group showed E1 to have a pH optimum of 5 and a tolerable temperature range of 30–60°C.

Enzyme application to forages used

High-level application to high-temperature-dried lucerne. Pre-bloom lucerne (*Medicago sativa*; variety Daisy/Capri mix) was mown, left to wilt overnight, chopped to 75 mm lengths and dried at 800°C (rotary dryer; Van der Brock International, Utrecht, Holland) for 0·5 min. Five levels of enzyme preparation were each applied to twelve replicate samples of 0·5 g HT lucerne. Enzyme levels were 0 (L0; control), 0·3 (L1), 0·6 (L2), 1·2 (L3) and 2·4 (L4) ml enzyme/g DM herbage. Each treatment was prepared in water and applied (using a hand-held sprayer) at a level of 6 ml/g DM herbage. The control (L0) received a water-only treatment. Following treatment, samples were then maintained at 50°C for 20 h in an incubator. This herbage was used in experiments 1 and 2.

High-level application to fresh and wilted lucerne. Pre-bloom lucerne (*Medicago sativa*; variety Daisy/Capri mix) was mown, chopped to 75 mm lengths and transported on ice to the laboratory. The herbage was then thoroughly mixed and split into two equal amounts, one of which was stored on ice, and the other wilted (at 18°C) to a DM content of 370 g/kg. The DM content of the fresh material was determined as 170 g/kg. Enzyme treatment and levels of enzyme applied to the fresh and wilted lucerne herbage were identical to that described for the treatment of the HT lucerne. Each level of enzyme preparation was applied to four replicate samples (0·5 g DM) of fresh and wilted lucerne in GP culture bottles, and samples were then maintained at 50°C for 20 h in an incubator. This herbage was used in experiment 3.

Low-level application to fresh and wilted lucerne. Herbage preparation was identical to that described for the high-level application to fresh and wilted lucerne. Five levels of enzyme preparation were added to the fresh and wilted lucerne herbage; 0 (CL0), 0·5 (CL1), 1·0 (CL2), 2·0 (CL3) and 4·0 (CL4) ml/500 g DM lucerne (equivalent to 0·000, 0·001, 0·002, 0·004 and 0·008 ml/g DM). Each treatment was prepared in water and applied (using a hand-held sprayer) at a level of 10 ml/500 g DM herbage. Four 0·5 g DM representative samples, taken from each forage and enzyme treatment combination, were then placed in GP culture bottles and maintained at 50°C for 20 h in an incubator. This herbage was used in experiment 4.

Experiment 1

Following incubation with high application levels of enzyme, four replicates of HT lucerne herbage from each of the five enzyme application levels were subjected to a SFD treatment. The SFD treatment was adapted from the method of Furuya *et al.* (1979), whereby 10 ml pepsin HCl (0·075 mol HCl/l; 2 mg pepsin/ml (Fisher Scientific UK, Loughborough, UK)) was added to the samples, which were then thoroughly mixed and incubated for 1 h at 37°C. Following incubation, samples were neutralised by the addition of 0·2 M-NaOH. An aqueous solution of porcine pancreatin (1·5 ml) was added to each tube, samples were mixed and incubated at 37°C for 2 h. The pancreatin (α-amylase, lipase and protease) solution was prepared by mixing the contents of two Pancrex V capsules (approximately 9000 British Pharmacopaeial units α-amylase per capsule; Paines & Byrne Ltd, Greenford, UK) with 9 ml distilled water. The mixture was then thoroughly mixed and centrifuged at 1500 ***g*** for 10 min and the supernatant fraction used as the pancreatic enzyme solution. Following incubation, 50 ml absolute ethanol (ETOH) was added to two of the four replicates from each enzyme application level, with the remaining two receiving 50 ml distilled water. Tubes were then left at room temperature (about 20°C) for 1 h before centrifugation at 1500 ***g*** for 10 min. The resultant supernatant fraction was removed by aspiration and discarded. The ETOH precipitated any soluble NSP released as a result of enzyme treatment. Samples that received the ETOH treatment were then washed twice more by centrifugation at 1500 ***g*** for 10 min in 85 % ethanol (50 ml). Samples were then lyophilised for DM determination, ground in liquid N_2 using a pestle and mortar, and analysed for NSP content and composition using a modified version of the method of Englyst & Cummings (1984). The uronic acid content of the samples was determined by the colorimetric method of Scott (1979).

Experiment 2

Following incubation with high application levels of enzyme, a further four replicates of HT lucerne herbage from each of the five enzyme application levels were subjected to an SFD treatment as described in experiment 1, using ETOH during the washing phase (+SFD), whilst the remaining samples were not (−SFD). Previous work by the group showed ETOH washing procedures to have little effect on the *in vitro* fermentation of HT lucerne. Both sample groups were then fermented *in vitro* with an equine faecal inoculum using the GP technique of Theodorou *et al.* (1994). Methods employed for the GP technique were as described by Theodorou *et al.* (1994), with the exception of the preparation of the faecal inoculum. Freshly voided faeces were collected from a horse maintained on a basal diet of *ad libitum* grass hay supplemented with 1 kg molassed sugar-beet pulp and 1 kg HT molassed chopped lucerne (Alfa-A; Dengie Crops Ltd, Maldon, Essex, UK) and combined with an equal weight of

culture medium, then homogenised in a stomacher (Laboratory blender stomacher 400; Seward, London, UK) for 90 s. The resultant suspension was strained through a triple layer of muslin and collected in a CO_2-filled flask. The faecal inoculum was dispensed immediately after extraction. Treatment arrangements during *in vitro* GP included five levels of enzyme application and two pre-treatment groups; with or without the SFD treatment. A total of forty-four bottles were included for GP; forty containing substrate (twenty per pre-treatment group) and four inoculum blanks (no substrate). Head-space gas pressure readings were taken at 2, 5, 7, 9, 12, 15, 19, 23, 28, 36, 47, 55, 72, 96 and 120 h post-inoculation with the accumulated gas volume measured and then released to zero following each reading. After the final reading, bottles were refrigerated at 4°C to arrest fermentation. Vessel contents were subsequently analysed for DM, volatile fatty acid (VFA) production and pH measurements. Samples (1·2 ml) of culture fluid were acidified with orthophosphoric acid (5 μl) and stored at −20°C before VFA analysis according to the method of Merry *et al.* (1995). The remaining culture fluid was separated from residual plant particles and adherent microbial biomass by vacuum filtration through sintered glass crucibles (porosity 1) and the residue rinsed with two volumes of distilled water. The washed residues were then lyophilised to constant weight for the determination of residual DM.

Experiment 3

Following incubation with high application levels of enzyme, the fresh and wilted lucerne samples were fermented *in vitro* as described in experiment 2, with the exception of the head-space gas pressure readings, which were taken at 2, 4·5, 7, 10, 13, 16, 19, 26·5, 33·5, 45·5, 52·5, 67·5, 91·5 and 120 h post-inoculation. No SFD treatment was used for these samples. Treatment arrangements during *in vitro* GP included five levels of enzyme application and two degrees of wilting; fresh or wilted. A total of forty-four bottles were included for GP; forty containing substrate (twenty fresh and twenty wilted) and four inocula blanks. Following incubation, vessel contents were analysed for DM, VFA concentration and pH as described in experiment 2.

Experiment 4

Following incubation with low application levels of enzyme, the fresh and wilted lucerne samples were fermented *in vitro* as described in experiment 3. Following incubation, vessel contents were also analysed for DM, VFA concentration and pH as described in experiment 3. No SFD treatment was used for these samples. Treatment arrangements during *in vitro* GP included five levels of enzyme application and two degrees of wilting; fresh or wilted. A total of forty-four bottles were included for GP; forty containing substrate (twenty fresh and twenty wilted) and four inoculum blanks.

Statistical analyses

The spreadsheet software Excel 2000 (Microsoft Corp., Redwood, WA, USA) was used for data handling. The maximum likelihood programme (Ross, 1987) was used for non-linear regression, to fit curves to experimentally derived gas accumulation profiles using the model of France *et al.* (1993):

$$y = A - BQ^{t}Z^{\sqrt{t}},$$

where Q is e^{-b}, Z is e^{-c}, and B is $Ae^{bLT+c\sqrt{Lt}}$.

In this equation, y denotes cumulative GP (ml), t is incubation time (h), A is the predicted asymptotic value for gas pool size (ml), LT is the lag time (h), and b (per h) and c (per $h^{0.5}$) are rate constants. The mean control profiles for gas produced in the inoculated culture bottles in the absence of substrate was subtracted before curve-fitting analysis. The time-dependent fractional rate of GP (FRGP) per h was also calculated:

$$\mathrm{FRGP} = b + c/2\sqrt{T_{50}},$$

where T_{50} is the time taken to reach 50 % of the total GP.

Table 1. Dry matter loss (DML) and total non-starch polysaccharide loss from high-temperature-dried lucerne incubated for 20 h with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) and a simulated foregut digestion treatment using either water or absolute ethanol (ETOH) during sample washing (mg/g) (*n* 2)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
DML							
Water	311[d]	360[e]	361[e]	391[f]	380[f]	360	
ETOH	255[c]	265[c]	260[c]	242[b]	221[a]	248	
Level mean	283[j]	312[k]	310[k]	316[k]	300[j]		
Level SED	5·5						0·001
Treatment SED	2·5						0·001
Level × treatment SED	4·0						0·001*
Total NSP							
Water	193[b,c]	184[b]	235[c]	442[f]	359[e]	283	
ETOH	191[b,c]	134[a]	174[a,b]	288[d]	319[d,e]	221	
Level mean	192[j,k]	159[j]	205[k]	365[l]	339[l]		
Level SED	19·5						0·001*
Treatment SED	8·7						0·001
Level × treatment SED	13·8						0·001†

[a,b,c,d,e,f] Mean values within a column or row within the same parameter (DML or TNSP) with unlike superscript letters were significantly different ($P<0·05$).

[j,k,l] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

Data from experiment 1 (values for DM and NSP loss), and the GP modelled parameters (as described earlier with the addition of T_{95}; the time taken to produce 95 % of the total GP), DM and NSP loss, and VFA concentration and pH for experiments 2, 3 and 4, were analysed for significant differences by two-way ANOVA with polynomial contrasts. Comparisons between treatment groups were made by least significant difference equations. All statistical analyses were carried out using Genstat release 5.1 (Lawes Agricultural Trust, Harpenden, UK).

Results

Experiment 1

Overall, DM loss (DML) was significantly ($P<0·001$) greater in the water-washed lucerne than the ETOH-washed lucerne (Table 1). There was a significant interaction ($P<0·001$) between enzyme level and wash treatment; DML from the water-washed lucerne increased quadratically with increasing enzyme level, whilst that from the ETOH-washed lucerne decreased quadratically. Overall, total NSP loss was significantly ($P<0·001$) greater in the water-washed lucerne in comparison with the ETOH-washed herbage. Total NSP loss also showed a significant interaction ($P<0·01$) between enzyme level and wash treatment; total NSP loss increased quadratically in response to increasing enzyme level in the water-washed lucerne, whereas there was a linear increase in the ETOH-washed samples.

Experiment 2

Analysis of curve-fitted parameters showed a significant ($P<0·001$) interaction between enzyme level and SFD treatment for total GP (*A*) values; whilst total GP increased linearly with increasing enzyme level in the −SFD lucerne, no increases were noted in the +SFD lucerne (Table 2). In fact, a significant ($P<0·05$) decrease in total GP was noted in the +SFD treatment group at level 1 enzyme addition in comparison with the control (Table 2). Enzyme treatment significantly ($P<0·001$) increased the degradation rate of the lucerne (Fig. 1). The FRGP increased

Table 2. Gas production curve-fitted parameters; total gas volume (*A*), lag time (*LT*), fractional rate of gas production (FRGP) and time taken to produce 50 % (T_{50}) and 95 % (T_{95}) of total gas production for high-temperature-dried lucerne treated with different levels of a fibrolytic enzyme treatment (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) with (+SFD) or without (−SFD) a simulated foregut digestion treatment before *in vitro* fermentation (n 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (P)
A (ml)							
+SFD	190^{bc}	167^{a}	188^{b}	181^{a,b}	194^{b,c}	184	
−SFD	191^{b,c}	205^{c}	236^{d}	283^{e}	353^{f}	254	
Level mean	191^{j}	186^{j}	212^{k}	232^{l}	273^{m}		
Level SED	5·7						0·001†
Treatment SED	3·6						0·001
Level × treatment SED	8·1						0·001†
LT (h)							
+SFD	2·91	3·22	3·01	2·91	2·74	2·96	
−SFD	1·47	2·23	2·12	2·09	1·53	1·89	
Level mean	2·19^{j,k}	2·72^{l}	2·57^{l}	2·50^{k,l}	2·14^{j}		
Level SED	0·158						0·01*
Treatment SED	0·010						0·001
L × T SED	0·223						NS
FRGP (per h)							
+SFD	0·061^{a}	0·072^{b}	0·078^{d}	0·076^{c}	0·084^{g}	0·074	
−SFD	0·061^{a}	0·080^{f}	0·075^{c}	0·080^{f}	0·079^{e}	0·075	
Level mean	0·061^{a}	0·076^{b}	0·077^{b}	0·078^{b,c}	0·081^{c}		
Level SED	0·0022						0·001†
Treatment SED	0·0014						NS
L × T SED	0·0031						0·05
T_{50} (h)							
+SFD	15·53^{d}	13·45^{c}	12·27^{b}	12·17^{b}	11·55^{a,b}	12·99	
−SFD	14·10^{c}	11·08^{a}	11·16^{a}	10·84^{a}	11·70^{a,b}	11·78	
Level mean	14·81^{l}	12·27^{k}	11·71^{jk}	11·51^{j}	11·62^{j,k}		
Level SED	0·344						0·001†
Treatment SED	0·218						0·001
L × T SED	0·487						0·05†
T_{95} (h)							
+SFD	51·32	44·52	41·22	42·46	38·02	43·51	
−SFD	50·17	39·96	42·13	39·47	38·70	42·09	
Level mean	50·75	42·24	41·68	40·97	38·36		
Level SED	2·189						0·001†
Treatment SED	0·678						0·05
Level × treatment SED	1·156						NS

^{a,b,c,d,e,f,} Mean values within a column or row within the same parameter (A, *LT*, FRGP, T_{50} or T_{95}) with unlike superscript letters were significantly different ($P<0·05$).

^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

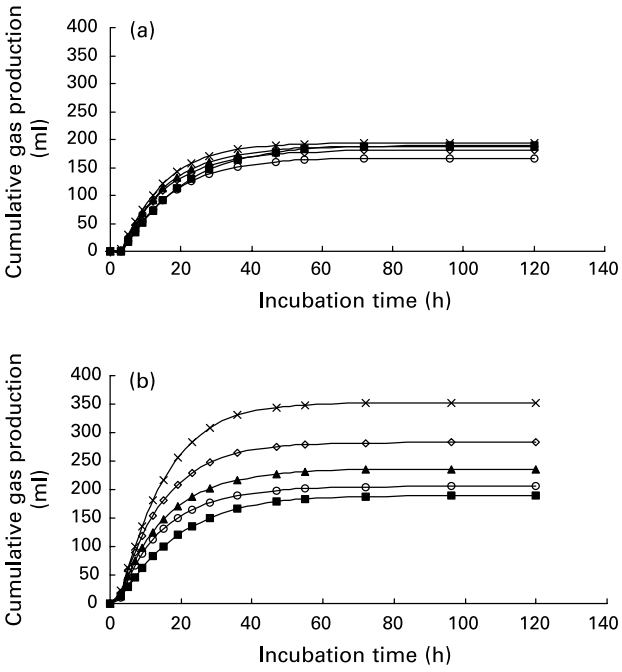

Fig. 1. Fitted cumulative gas production curves for high-temperature-dried lucerne treated with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0 (−■−), L1 (−○−), L2 (−▲−), L3 (−◇−) and L4 (−X−), respectively) with (a) and without (b) a simulated foregut digestion treatment before *in vitro* fermentation (*n* 4).

linearly in both the +SFD and −SFD lucerne with increasing level of enzyme application, with no apparent effect of SFD treatment on the rate at which the lucerne was degraded (Table 2). T_{50} and T_{95} values were significantly ($P<0·001$) greater in the +SFD lucerne in comparison with the −SFD group (Table 2). Enzyme treatment did not affect DML in either the +SFD or −SFD lucerne (Table 3).

Values for pH were significantly ($P<0·001$) lower overall in the −SFD lucerne compared with the +SFD group; total VFA (TVFA) concentration was almost twofold greater in the −SFD lucerne in comparison with the +SFD group (Table 3). However, whilst TVFA values increased linearly with increasing enzyme level in the −SFD lucerne, TVFA concentration only increased significantly ($P<0·05$; Table 3) at L4 in the + SFD samples. Molar proportions of acetate decreased linearly with increasing enzyme level, whilst the proportion of propionate increased significantly ($P<0·001$; Table 4) with increasing enzyme level. Molar proportions of butyrate were similar across all treatments; therefore, increasing enzyme application level resulted in a significant ($P<0·001$) decrease in the acetate plus butyrate:propionate (A + B:P) proportion (Table 4).

Experiment 3

Analysis of curve-fitted parameters revealed a significant ($P<0·05$) linear increase in both the fresh and wilted lucerne with increasing enzyme level for total GP, with the fresh lucerne producing significantly ($P<0·001$) more gas overall in

Table 3. Dry matter loss (DML), pH and total volatile fatty acid (TVFA) concentration of the culture fluid following the *in vitro* fermentation of high-temperature-dried lucerne treated with different levels of a fibrolytic enzyme treatment (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) with (+SFD) or without (−SFD) a simulated foregut digestion treatment before *in vitro* fermentation (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
DML (mg/g)							
+SFD	657	646	674	655	653	657	
−SFD	678	669	670	687	662	673	
Level mean	667	658	672	671	657		
Level SED	14·8						NS
Treatment SED	9·3						NS
Level × treatment SED	20·1						NS
pH							
+SFD	6·85^{e}	6·76^{b}	6·78^{c}	6·81^{d}	6·87^{e}	6·81	
−SFD	6·84^{e}	6·80^{d}	6·77^{bc}	6·76^{b}	6·71^{a}	6·78	
Level mean	6·85^{m}	6·78^{k}	6·77^{j}	6·79^{l}	6·79^{l}		
Level SED	0·007						0·001*
Treatment SED	0·004						0·001
Level × treatment SED	0·009						0·001†
TVFA (mmol/l)							
+SFD	26·1^{a}	25·6^{a}	26·1^{a}	26·2^{a}	29·9^{b}	26·8	
−SFD	34·2^{c}	42·1^{d}	47·3^{e}	56·1^{f}	71·2^{g}	50·2	
Level mean	30·2^{j}	33·8^{k}	36·7^{l}	41·2^{m}	50·6^{n}		
Level SED	0·87						0·001†
Treatment SED	0·55						0·001
Level × treatment SED	1·23						0·001†

^{a,b,c,d,e,f,} Mean values within a column or row within the same parameter (DML, pH or TVFA) with unlike superscript letters were significantly different ($P<0·05$).
^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* Significant quadratic response ($P<0·05$).
† Significant linear response ($P<0·05$).

Table 4. Volatile fatty acid composition of the culture fluid following the *in vitro* fermentation of high-temperature-dried lucerne treated with different levels of a fibrolytic enzyme treatment (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) with (+SFD) or without (−SFD) a simulated foregut digestion treatment before *in vitro* fermentation (molar proportions) (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
Acetate							
+SFD	0·65^{f}	0·65^{f}	0·64^{e}	0·64^{e}	0·62^{c}	0·64	
−SFD	0·65^{f}	0·65^{f}	0·63^{d}	0·59^{b}	0·55^{a}	0·61	
Level mean	0·65^{j}	0·65^{j}	0·63^{k}	0·62^{l}	0·58^{m}		
Level SED	0·004						0·001†
Treatment SED	0·002						0·001
Level × treatment SED	0·005						0·001†
Butyrate							
+SFD	0·09^{k}	0·10^{l}	0·10^{l}	0·10^{l}	0·10^{l}	0·10	
−SFD	0·08^{j}	0·08^{j}	0·08^{j}	0·08^{j}	0·08^{j}	0·08	
Level mean	0·09	0·09	0·09	0·09	0·09		
Level SED	0·001						NS
Treatment SED	0·001						0·01
Level × treatment SED	0·003						0·05*
Propionate							
+SFD	0·21^{b}	0·20^{a}	0·21^{b}	0·21^{b}	0·22^{c}	0·21	
−SFD	0·23^{d}	0·23^{d}	0·25^{e}	0·28^{f}	0·31^{g}	0·26	
Level mean	0·22^{l}	0·21^{j}	0·23^{l}	0·24^{m}	0·27^{n}		
Level SED	0·003						0·001†
Treatment SED	0·001						0·001
Level × treatment SED	0·004						0·001†
Valerate							
+SFD	0·05^{b}	0·06^{c}	0·06^{c}	0·06^{c}	0·07^{d}	0·06	
−SFD	0·04^{a}	0·05^{b}	0·05^{b}	0·06^{c}	0·06^{c}	0·05	
Level mean	0·05^{j}	0·05^{j}	0·06^{k}	0·06^{k}	0·06^{k}		
Level SED	0·002						0·001†
Treatment SED	0·001						0·001
Level × treatment SED	0·003						NS*
A + B:P							
+SFD	3·61^{e}	3·80^{f}	3·62^{e}	3·58^{e}	3·26^{d}	3·57	
−SFD	3·17^{d}	3·21^{d}	2·77^{c}	2·42^{b}	2·01^{a}	2·71	
Level mean	3·39^{m}	3·50^{n}	3·19^{l}	3·00^{k}	2·63^{j}		
Level SED	0·048						0·001†
Treatment SED	0·030						0·001
Level × treatment SED	0·068						0·001†

A + B:P, acetate plus butyrate:propionate.

[a,b,c,d,e,f] Mean values within a column or row within the same parameter (acetate, butyrate, propionate, valerate or A + B:P) with unlike superscript letters were significantly different ($P<0·05$).

[j,k,l] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

comparison with the wilted material (Table 5). The degradation rate of the lucerne was also enhanced by enzyme addition (Fig. 2). A significant ($P<0·05$) interaction between enzyme level and degree of wilting was noted for the FRGP (Table 5), whereby values for the wilted lucerne increased linearly with increasing level of enzyme addition, whilst the fresh material produced a more quadratic response to increasing levels of enzyme treatment. A similar pattern was observed for T_{50} and T_{95} measurements, whereby these values decreased linearly in the wilted lucerne but quadratically in the fresh material. There was a significant ($P<0·001$) interaction between enzyme level and degree of wilting for the pH of the culture fluid (Table 6). Whilst the pH of the fresh lucerne decreased in response to enzyme application and remained the same between enzyme levels, the pH of the wilted material only decreased with the application of enzyme at level 4. Results for DML revealed a significant ($P<0·05$) interaction between enzyme level and degree of wilting, whereby there was a general trend towards increased DML with increased enzyme addition in the fresh material, whilst the inverse occurred in the wilted lucerne. However, although not significant, DML decreased at the highest level of enzyme addition in both the fresh and wilted herbage (Table 6).

Enzyme treatment resulted in a highly significant ($P<0·001$) linear increase in the TVFA concentration of the culture fluid in both the fresh and wilted lucerne (Table 6). VFA molar proportions were also affected by enzyme addition (Table 7). The proportion of acetate present decreased linearly with increasing enzyme level, whilst propionate concentration increased. Butyrate values also decreased linearly ($P<0·001$), whilst the amount of valerate present in the culture fluid increased ($P<0·001$). As a consequence, the A + B:P ratio decreased significantly ($P<0·001$) in response to increases in enzyme level (Table 7).

Table 5. Gas production curve-fitted parameters; total gas volume (*A*), lag time (*LT*), fractional rate of gas production (FRGP) and time taken to produce 50 % (T_{50}) and 95 % (T_{95}) of total gas production for fresh and wilted lucerne treated with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
A (ml)							
Fresh	169	195	221	289	375	250	
Wilted	140	155	195	224	340	211	
Level mean	155^{j}	175^{k}	208^{l}	256^{m}	358^{n}		
Level SED	6·8						0·001†
Treatment SED	4·3						0·001
Level × treatment SED	9·7						NS
LT (h)							
Fresh	2·16	2·05	2·02	2·03	2·08	2·06	
Wilted	1·65	1·78	1·84	1·80	1·86	1·79	
Level mean	1·88	1·91	1·93	1·91	1·97		
Level SED	0·056						NS
Treatment SED	0·035						0·001
Level × treatment SED	0·079						NS
FRGP (per h)							
Fresh	0·052^{a}	0·075^{c,d}	0·072^{c,d}	0·074^{c,d}	0·073^{c,d}	0·069	
Wilted	0·049^{a}	0·055^{a,b}	0·065^{b,c}	0·071^{c,d}	0·077^{d}	0·063	
Level mean	0·051^{j}	0·065^{k}	0·068^{k,l}	0·073^{l,m}	0·075^{m}		
Level SED	0·0035						0·001†
Treatment SED	0·0022						0·001
Level × treatment SED	0·0050						0·05*
T_{50} (h)							
Fresh	15·45^{e}	11·60^{a,b}	11·47^{a}	11·55^{a,b}	11·85^{a,b}	12·39	
Wilted	15·51^{e}	14·42^{d,e}	13·36^{c,d}	12·77^{b,d}	11·89^{a,b}	13·59	
Level mean	15·48^{l}	13·01^{m}	12·42^{m,n}	12·16^{m,n}	11·87^{n}		
Level SED	0·444						0·001†
Treatment SED	0·280						0·001
Level × treatment SED	0·627						0·05*
T_{95} (h)							
Fresh	59·76^{d,e}	44·15^{b,c}	44·72^{b,c}	42·19^{a,b}	42·62^{a,b}	46·69	
Wilted	63·14^{e}	56·39^{d}	47·48^{c}	43·38^{a,b,c}	39·91^{a}	50·06	
Level mean	61·45^{j}	50·27^{k}	46·10^{l}	42·79^{l,m}	41·26^{m}		
Level SED	1·82						0·001†
Treatment SED	1·15						0·01
Level × treatment SED	2·57						0·01†

^{a,b,c,d,e,f} Mean values within a column or row within the same parameter (*A*, *LT*, FRGP, T_{50} or T_{95}) with unlike superscript letters were significantly different ($P<0·05$).

^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

Experiment 4

The rate and extent of GP was not affected by any of the enzyme levels in either the fresh or wilted lucerne (Fig. 3). DML, TVFA concentration and pH were also unaffected by enzyme application (Table 8).

Discussion

The aim of the present study was to assess the effects of various levels of exogenous enzyme treatments on the *in vitro* fermentation of HT, wilted and fresh lucerne. Enzyme treatment of HT lucerne significantly increased DM and total NSP loss following the SDF treatment using water during the washing phase compared with the control, indicating a loss of structural carbohydrate as a consequence of enzyme treatment. Conversely, the substitution of water with ETOH decreased DML with increasing enzyme level. However, in addition to precipitating oligosaccharides, ETOH also precipitates proteins; therefore, the decrease in DML in the ETOH-washed samples may be partly attributable to the precipitation of enzyme protein. Since NSP determination is unaffected by protein content, NSP values were unaffected by enzyme content, and the differences in total NSP losses noted between the enzyme-treated water and ETOH-washed samples were presumably due to the presence of oligosaccharides precipitated by the ETOH. The ETOH losses are more representative of those encountered *in vivo,* since short-chain NSP would not be digested in the small intestine of the horse, but predominantly fermented in the large intestine (Argenzio, 1990).

Enzyme treatment appeared to increase the proportion of short-chain NSP polymers present in the HT lucerne following SFD treatment, evident by the significant differences in total NSP loss noted between the water and ETOH wash treatments as enzyme level increased (averaging 62 mg/g DM across all enzyme levels). Total NSP losses from the ETOH-washed samples ranged from 191 mg/g DM in the control lucerne to 319 mg/g DM at the highest level of enzyme addition, which is a crude representation of the amount of NSP that may be digested in the distal small intestine of the horse.

Due to the NSP losses encountered during the SFD treatment, the +SFD samples showed significantly lower total GP and DML values compared with the −SFD group. Within the +SFD samples, although some partially degraded NSP was present in the material before fermentation, total GP and DML values were unaffected by enzyme treatment. Nevertheless, the ability to measure the fermentation patterns of these samples is a useful tool. Although all end-point digestibility measurements

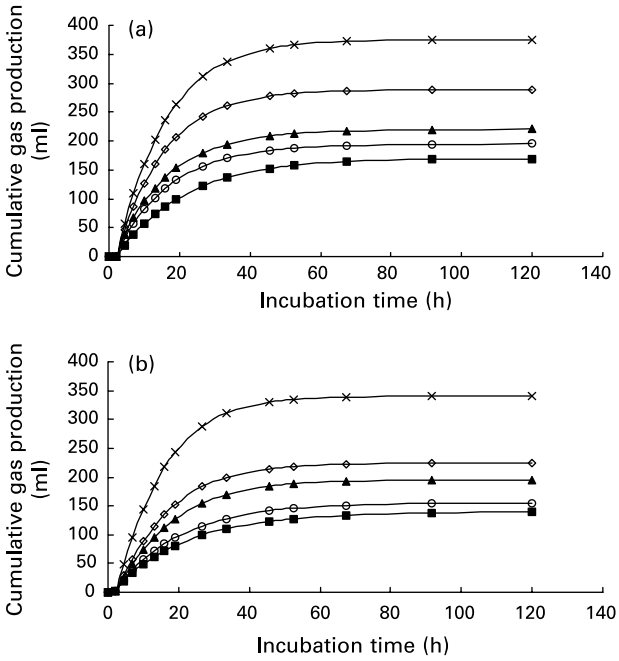

Fig. 2. Fitted cumulative gas production curves for (a) fresh and (b) wilted lucerne with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0 (−■−), L1 (−○−), L2 (−▲−), L3 (−◇−) and L4 (−X−), respectively) (*n* 4).

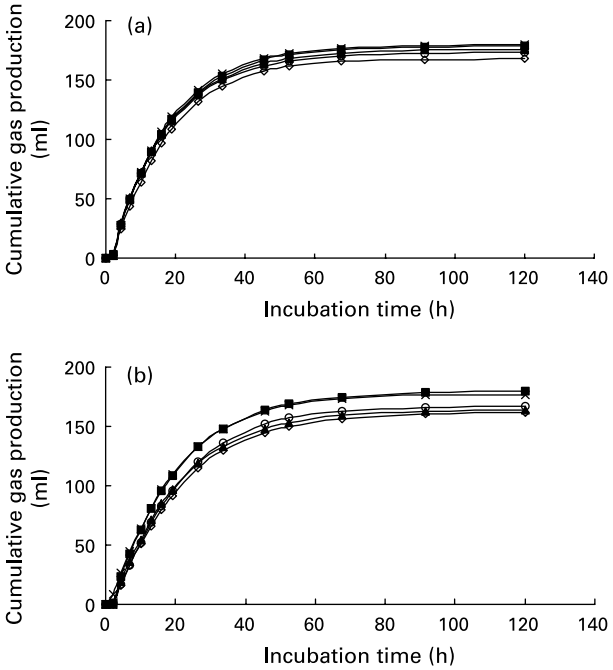

Fig. 3. Fitted cumulative gas production curves for (a) fresh and (b) wilted lucerne with different commercially viable levels of a fibrolytic enzyme preparation (0·000, 0·001, 0·002, 0·004 and 0·008 ml/g dry matter for CL0 (−X−), CL1 (−◇−), CL2 (−▲−), CL3 (−○−) and CL4 (−■−), respectively) (*n* 4).

Table 6. Dry matter loss (DML), pH and total volatile fatty acid (TVFA) concentration of the culture fluid following the *in vitro* fermentation of fresh and wilted lucerne treated with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
DML (mg/g)							
Fresh	747^{c,d}	785^{d}	808^{d}	800^{d}	703^{a,b,c}	768	
Wilted	705^{a,b,c}	717^{b,c}	650^{a}	664^{a,b}	661^{a,b}	679	
Level mean	726^{j,k}	751^{j}	729^{j}	732^{j}	682^{k}		
Level SED	22·1						0·05
Treatment SED	14·0						0·001
Level × treatment SED	31·2						0·05*
pH							
Fresh	6·90^{c}	6·81^{a}	6·82^{a,b}	6·81^{a}	6·79^{a}	6·83	
Wilted	6·93^{d}	6·95^{d}	6·93^{d}	6·91^{c,d}	6·83^{b}	6·91	
Level mean	6·91^{m}	6·88^{l}	6·87^{kl}	6·86^{k}	6·81^{j}		
Level SED	0·008						0·001†
Treatment SED	0·005						0·001
Level × treatment SED	0·012						0·001*
TVFA (mmol/l)							
Fresh	37·5	43·2	46·0	52·8	68·8	49·6	
Wilted	31·7	32·6	40·1	47·2	64·2	43·2	
Level mean	34·6^{j}	37·9^{k}	43·0^{l}	50·0^{m}	66·5^{n}		
Level SED	1·03						0·001†
Treatment SED	0·65						0·001
Level × treatment SED	1·46						NS

^{a,b,c,d,e,f,} Mean values within a column or row within the same parameter (DML, pH or TVFA) with unlike superscript letters were significantly different ($P<0·05$).

^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

Table 7. Volatile fatty acid composition of the culture fluid following the *in vitro* fermentation of fresh and wilted lucerne treated with different levels of a fibrolytic enzyme preparation (0, 0·3, 0·6, 1·2 and 2·4 ml/g dry matter for L0, L1, L2, L3 and L4, respectively) (molar proportions) (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	L0	L1	L2	L3	L4	Treatment mean	Significance (*P*)
Acetate							
Fresh	0·64^{f}	0·61^{f}	0·60^{e}	0·55^{c}	0·51^{a}	0·58	
Wilted	0·63^{f}	0·64^{f}	0·61^{e}	0·57^{d}	0·53^{b}	0·59	
Level mean	0·63^{n}	0·62^{m}	0·60^{l}	0·56^{k}	0·52^{j}		
Level SED	0·006						0·001†
Treatment SED	0·003						0·01
Level × treatment SED	0·009						0·05†
Butyrate							
Fresh	0·10^{b}	0·11^{c}	0·10^{b}	0·10^{b}	0·09^{a}	0·10	
Wilted	0·11^{c}	0·11^{c}	0·11^{c}	0·10^{b}	0·09^{a}	0·10	
Level mean	0·11^{j}	0·11^{j}	0·10^{k}	0·10^{k}	0·09^{l}		
Level SED	0·002						0·001†
Treatment SED	0·001						NS
Level × treatment SED	0·003						0·05
Propionate							
Fresh	0·19	0·21	0·24	0·29	0·33	0·25	
Wilted	0·21	0·19	0·23	0·27	0·32	0·24	
Level mean	0·20^{j}	0·20^{j}	0·23^{k}	0·28^{l}	0·32^{m}		
Level SED	0·006						0·001†
Treatment SED	0·004						0·05
Level × treatment SED	0·009						NS
Valerate							
Fresh	0·07	0·07	0·07	0·08	0·08	0·07	
Wilted	0·06	0·06	0·06	0·06	0·07	0·06	
Level Mean	0·06^{j}	0·06^{j}	0·07^{k}	0·07^{k}	0·08^{l}		
Level SED	0·002						0·001†
Treatment SED	0·001						0·001
Level × treatment SED	0·003						NS
A + B:P							
Fresh	3·92^{h}	3·39^{e,f}	2·99^{d}	2·24^{b,c}	1·85^{a}	2·88	
Wilted	3·57^{f,g}	3·85^{g,h}	3·16de	2·46^{c}	1·93^{a,b}	2·99	
Level mean	3·74^{m}	3·62^{m}	3·07^{l}	2·35^{k}	1·89^{j}		
Level SED	0·113						0·001†
Treatment SED	0·071						NS
Level × treatment SED	0·159						0·05*

A + B:P, acetate plus butyrate:propionate.

^{a,b,c,d,e,f,} Mean values within a column or row within the same parameter (acetate, butyrate, propionate, valerate or A + B:P) with unlike superscript letters were significantly different ($P<0·05$).

^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

† Significant linear response ($P<0·05$).

showed no response to enzyme treatment, mathematical analysis of the cumulative GP curves showed distinct differences in the fermentation profiles of the +SFD samples. Rate parameter values (FRGP, T_{50} and T_{95}) showed a significant increase in the rate of GP with increasing enzyme level, indicating that the partially degraded cell-wall material entering the GP system was more accessible to microbial degradation. Therefore, enzyme treatment appears to increase the *in vitro* pre-caecal degradability of the NSP fraction of lucerne, concomitant with an enhancement of the *in vitro* degradation rate of the residue remaining following the SFD treatment. This has important implications for the overall energy balance of the horse since the digestion of carbohydrate in the small intestine, in terms of ATP yield, is more efficient than its fermentation in the large intestine (Moore-Colyer *et al.* 1997). An increased degradation rate is also of particular relevance to the horse, which has low mean digesta retention times compared with those encountered in ruminants (Moore-Colyer *et al.* 2003). Consequently, enhancement of the early fermentation of feedstuffs in the equine hindgut will further benefit the overall energy balance of the animal. Therefore, when evaluating the effects of enzyme treatment on the *in vitro* degradation of lucerne for horses, it appears necessary to use an SFD treatment before GP to mimic the changes that would occur in the forage *in vivo* after foregut digestion before fermentation in the large intestine.

The linear increase in the rate and extent of GP with increasing enzyme level in the −SFD samples indicated an increase in the fermentability of this group of substrates. However, although a similar response was observed for fresh and wilted material treated with the same levels of E1, the addition of commercially viable enzyme levels showed no effect. Although the higher enzyme application levels appeared effective in the degradation of lucerne, they are not commercially viable due to the enzyme purchase costs. Consequently, the practical applications of fibrolyitc enzymes to lucerne are currently limited; however, developments in genetic technology, and the use of molecular techniques

Table 8. Dry matter loss (DML), total volatile fatty acid (TVFA) concentration and pH of the culture fluid following the *in vitro* fermentation of fresh and wilted lucerne treated with different commercially viable levels of a fibrolytic enzyme preparation (0·000, 0·001, 0·002, 0·004 and 0·008 ml/g dry matter for CL0, CL1, CL2, CL3 and CL4, respectively) (*n* 4)

(Mean values and standard errors of difference)

	Enzyme level						
	CL0	CL1	CL2	CL3	CL4	Treatment mean	Significance (*P*)
DML (mg/g)							
Fresh	720	654	731	717	636	692	
Wilted	705	662	654	658	660	668	
Level mean	713	658	693	688	648		
Level SED	23·1						NS
Treatment SED	14·6						NS
Level × treatment SED	32·7						NS
pH							
Fresh	6·87^{d}	6·94^{h}	6·92^{f,g}	6·93^{g,h}	6·89^{e}	6·91	
Wilted	6·87^{d}	6·86^{c,d}	6·83^{a}	6·84^{a,b}	6·85^{b,c}	6·85	
Level mean	6·87^{j}	6·90^{l}	6·88^{j,k}	6·89^{k,l}	6·87^{j}		
Level SED	0·009						0·05*
Treatment SED	0·005						0·001
Level × treatment SED	0·013						0·001*
TVFA (mmol/l)							
Fresh	39·5	37·2	37·8	35·8	40·0	38·1	
Wilted	35·5	36·4	36·3	36·6	38·5	36·7	
Level mean	37·6	36·8	37·1	36·2	39·3		
Level SED	1·05						NS
Treatment SED	0·66						0·05
Level × treatment SED	1·48						NS

A + B:P, acetate plus butyrate:propionate.

^{a,b,c,d,e,f,} Mean values within a column or row within the same parameter (acetate, butyrate, propionate, valerate or A + B:P) with unlike superscript letters were significantly different ($P<0·05$).

^{j,k,l} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Significant quadratic response ($P<0·05$).

in enzyme production, may lead to lower-cost enzyme production (Bickerstaff, 1995) and thus greater commercially viable enzyme application levels in the future.

The linear increase in GP noted at the higher levels of enzyme application are consistent with the findings of Wallace *et al.* (2001), who reported significant linear increases in GP from grass silage treated with increasing enzyme levels ranging from 0·002 to 5 ml/g DM. The observed increases in GP could also be attributed, at least in part, to the additional protein provided by the enzyme solutions. However, Wallace *et al.* (2001) showed that whilst enzyme treatment significantly enhanced the rate and extent of GP in grass and maize silage, the addition of autoclaved enzymes produced similar GP profiles to the control material. Moreover, Colombatto (2000) only detected very small amounts of GP from controls containing enzyme and inoculum in the absence of substrate. Increases in GP could also be a consequence of the fermentation of sugars present in the enzyme solution; however, previous results from a study by our group showed very low amounts of reducing sugars in this enzyme solution. Moreover, the results of the SFD treatment showed significant losses of NSP from enzyme-treated lucerne compared with the control, supporting the premise that the observed increases in GP were due to enhanced degradation of the fibrous fraction of the lucerne.

Mathematical analysis of the GP curves showed significant differences between the fermentation profiles of the HT, fresh and wilted enzyme-treated lucerne; linear in the wilted and HT-lucerne, which concurs with the work of Wallace *et al.* (2001), but quadratic in the fresh lucerne. The latter is not unknown; others have reported positive quadratic responses to enzyme treatment *in vitro* (Colombatto *et al.* 2003) and *in vivo* (Beauchemin *et al.* 1995; Lewis *et al.* 1999). Morgavi *et al.* (2000) proposed the hypothesis of competitive inhibition, after finding that elevated enzyme levels decreased the attachment of the rumen bacterium *Fibrobacter succinogenes* to pure cellulose.

The greater fermentation rate in the enzyme-treated fresh lucerne compared with the wilted material may reflect a greater partial degradation of cell-wall components in the enzyme-treated fresh lucerne. Therefore, the greater moisture content of the fresh lucerne would appear more conducive to enzyme hydrolysis during the 20 h incubation period before GP, which is consistent with the fundamental requirement for water in the hydrolysis of soluble sugars from complex polymers (Chesworth *et al.* 1998). However, this is in contrast to the findings of Feng *et al.* (1992, 1996) who observed greater *in vitro* degradation parameters in enzyme-treated wilted grass compared with fresh grass incubated with a rumen microbial inoculum. These authors postulated that this may be explained by enhanced binding of the enzymes to the wilted grass, thereby increasing the resistance of the enzymes to proteolysis in the rumen. However, these studies were conducted in ruminants with no forage and enzyme incubation period before fermentation; thus exogenous enzymes were in direct competition with microbial enzymes. Conversely, this effect was eliminated in the present study due to the incubation period of 20 h employed before *in vitro* fermentation, allowing the enzymes the opportunity to elicit their effect before fermentation. Furthermore, these studies were in grass, not lucerne, which may also explain the different results.

The greater DML from the −SFD compared with the +SFD samples in experiment 1 was almost certainly due to the loss of soluble components and NSP from the +SFD samples during the SFD treatment. Consequently, longer lag times were encountered in the +SFD samples compared with the −SFD lucerne, signifying a lack of readily degradable material for microbial growth in the early stages of fermentation. However, despite the significant increases in GP with increasing enzyme level in the −SFD, fresh and wilted lucerne, there was no corresponding increase in DML. This lack of effect of enzyme treatment on DML following *in vitro* fermentation has been recognised by others (Colombatto *et al.* 2003) and may be a result of increased microbial biomass. Total bacterial numbers in ruminal fluid have been seen to increase with increasing enzyme levels (Nsereko *et al.* 2002), and since DML in this experiment was in fact apparent DML, due to microbial biomass adhering to the residue, an increase in microbial mass due to enzyme treatment is conceivable.

The pH of the culture medium in the experiments reported here ranged between 6·71 and 6·95, remaining within the physiological levels (about 6·7) encountered in the large intestine of the horse (Argenzio, 1990). However, the culture medium used in the *in vitro* GP method is heavily buffered and may not entirely reflect the situation *in vivo*. The significant linear increase in TVFA with enzyme treatment resulted in amounts twofold greater than the control, with concomitant decreases in the proportion of A + B:P. Such changes in VFA proportions are consistent with a saccharolytic fermentation (Beever, 1993), and similar decreases in the A + B:P ratio in response to enzyme treatment have been observed by others (Colombatto *et al.* 2003), along with increases in TVFA production.

Conclusion

The addition of fibrolytic enzymes to HT, fresh and wilted lucerne appears to have considerable potential to enhance the nutritive value of lucerne for horses. However, it must be noted that the levels of enzyme application used in these studies were significantly greater than those generally applied *in vivo* and when commercially viable enzyme application levels were investigated no significant effects were noted. It is possible, however, that the *in vitro* GP technique was not sufficiently sensitive to measure the effects of these low levels of enzyme application. Therefore, the effect of enzyme treatment on the degradation of lucerne by equids requires *in vivo* investigation.

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