Proposed mechanisms for enzymes as modifiers of ruminal fermentation

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Introduction

Beauchemin and Rode (1996) recently reviewed the use of enzymes in ruminant diets, however, enzymes in the form of crude whole fungal cell preparations have been used to enhance ruminant production for many years. This paper aims to investigate the similarities between the emerging feed enzymes and fungal feed additives, and to use this information to speculate on potential modes of action of dietary added enzymes in the rumen.

Feed enzymes

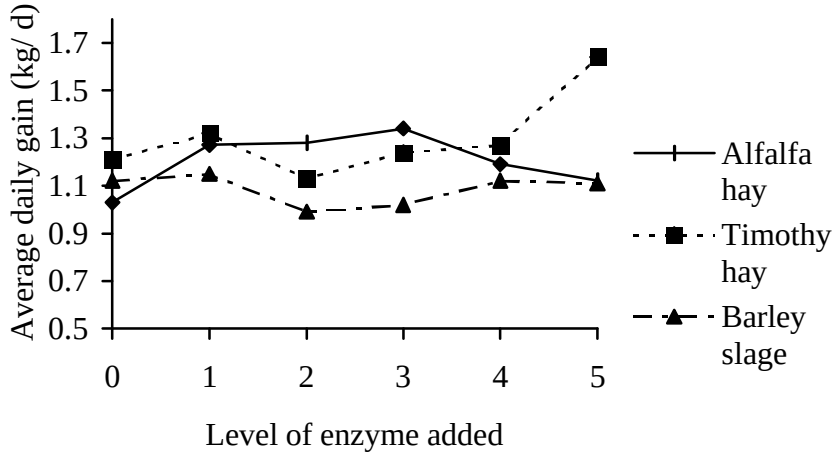
The use of dietary enzymes in poultry, and to a lesser extent pig diets, is now well established. Increases in productivity are attributed to increased substrate availability due to a decrease in digesta viscosity, in addition to the direct action of the enzymes on the dietary substrate (Cowan *et al*., 1996, Graham and Balnave, 1995).

The use of enzymes in ruminants is less well accepted. There have been sporadic reports of the use of proteolytic and amylolytic preparations in ruminant diets (Ralston *et al.,* 1962, Rust *et al.,* 1965, Krause *et al.,* 1989, Boyles *et al.,* 1992), while Graham and Balnave (1995) have suggested lipases capable of disrupting the waxy cuticle on leaf surfaces may be beneficial. However, the majority of studies have focused on preparations containing cellulolytic and xylanatic activities. Perhaps surprisingly, one area in which such preparations have proven useful is in the feeding of high grain diets (Beauchemin *et al.,* 1996, Iwaasa *et al*., 1996). An explanation for this apparent contradiction came from the work of Beauchemin *et al* (1996) who demonstrated that enzyme supplementation was more beneficial on barley as opposed to corn diets. It was suggested that the added enzymes had a role in the degradation of the fibrous husk of the barley grain making the starch more available for degradation. In agreement with this hypothesis, Hristov *et al*. (1996a) found that cellulase and xylanase preparations enhanced sugar release from barley but not corn when incubated *in vitro.*

However, it is the role of added enzymes in supplementing the cell wall degrading activity in the rumen of animals fed forage based rations that has received the most attention. The addition of fibrolytic enzymes to the diet has been reported to stimulate average daily gain by between 5 and 30% (Beauchemin *et al*., 1995, Beauchemin and Rode, 1996, Michal *et al*., 1996, Pritchard *et al*., 1996, Treacher *et al.,* 1996) and milk yield by between 2 and 15 % (Lewis *et al*., 1995, Stokes and Zheng, 1995, Higginbotham *et al.,* 1996, Sanchez *et al*., 1996) in cattle fed mixed forage/concentrate rations. In some (Lewis *et al*., 1995 Stokes and Zheng, 1995, Michal *et al*., 1996) but not all studies (Treacher *et al.,* 1996, Sanchez *et al*., 1996) this has been associated with an increase in dry matter intake.

Responses to enzyme addition appear to be influenced by the basal diet fed. Beauchemin *et al* (1995) reported that while a mixed cellulase and xylanase preparation stimulated liveweight gain in cattle fed hay based rations, no benefit was observed when the enzymes were included in a barley silage based ration. It may also be important to match enzymes to diet. Beauchemin and Rode (1996) demonstrated that the relative effectiveness of various commercial enzyme preparations in an *in vitro* system varied dependent on the dietary substrate used. Such diet dependency in the action of enzyme preparations may help explain situations in which no benefits are seen when enzymes are added (Zheng *et al*., 1996, Beauchemin and Rode, 1996). Sanchez *et al* (1996) suggested that the response to fibrolytic enzymes added to an alfalfa based total mixed ration fed to cattle, in early lactation, was quadratic. Maximal benefit was observed when enzyme was added at 2.5 ml / kg diet, with no observed benefit, compared to the control (no addition), when the enzyme was added at twice that level. Beauchemin *et al* (1995) also found evidence of a quadratic response to enzyme addition, but found that the optimal level of enzyme addition varied with the diet fed (Fig 1). Obviously it will be important to match both type of enzyme and inclusion rate to diet composition. Higginbotham *et al* (1996) found that a fibrolytic enzyme preparation caused a stimulation in milk yield in early but not late lactation, however it is likely that this difference reflects a change in the physiological status of the animal rather than a change in the biological effectiveness of the enzyme.

Fig 1 Effect of enzyme addition on live weight gain in cattle fed alfalfa hay, timothy hay or barley silage diets (data taken from Beauchemin *et al*., 1995)



There appears to be a general assumption that the production benefits seen when fibrolytic enzymes are added to the diet result from the action of these added enzymes in the rumen. Increases in rumen degradability of fiber have been observed in some studies (Feng *et al*., 1992, Hristov *et al*., 1996b, Lewis *et al*., 1996), but others have found no effect of enzyme addition on ruminal fermentation (Suzuki *et al*., 1994, Treacher *et al.*, 1996, Hristov *et al., 1996c)* and it has been suggested that the site of action of added enzymes is on the feed prior to consumption (Feng *et al*., 1992). Lewis (1995) found the application of enzyme to grass hay prior to feeding to be more effective than direct infusion of the same enzyme into the rumen of steers 2h after feeding. Treacher (1996) suggested that this was due to binding of the enzyme to the forage prior to it entry into the rumen and that extra-ruminal contact of the enzyme

with the forage enhanced the action of the enzyme in the rumen. More data is needed to confirm that the rumen is the site of action of the added enzymes.

Crude fungal preparations and earlier studies with added enzymes

In 1960 Burroughs *et al* reported that an enzyme supplement increased weight gain in feedlot steers by 9%. Theuer *et al* (1963) found no effect of the same supplement in sheep. And while Leatherwood *et al* (1960) found that an enzyme preparation from *Aspergillus niger* enhanced the cellulolytic activity of rumen fluid *in vitro*, the preparation did not affect live weight gain in calves. In view of the variable responses to enzyme addition interest in the use of enzymes in ruminant diets faded until the mid 1980's when the idea of adding crude fungal preparations to ruminant diets was suggested.

By far the most commonly investigated fungal additives have been those based on *Aspergillus oryzae* (AO), however as will be seen other fungi may be equally as suitable. Products are typically a mixture of fungal cells and spent fermentation fluid, and in at least one case would appear to contain few if any live fungi (Fondevila *et al*., 1990). It has been suggested that the conditions used in the growth and harvesting of the fungi may have an important influence on the stimulatory activity of the additive (Kistner, 1966, Gomez-Alarcon, 1990); however this does not appear to have been scientifically evaluated. Rates of inclusion of AO in the diet are typically 3 g/d in dairy cattle (Kellems *et al.*, 1990, Gomez-Alarcon *et al.*, 1991, Higginbotham *et al.*, 1993) and studies by Wiedmeier (1989) and Denigan *et al* (1992) suggest little benefit is likely to accrue from inclusion rates greater than 10 g/d.

Responses to AO have been recorded in lactating and growing cattle (Wiedmeier, 1989, Gomez-Alarcon *et al*., 1991) and sheep (Judkins and Stobart, 1988). Williams and Newbold (1990) summarized a number of studies in which AO had been added to the diet of lactating dairy cows and found that milk yield increased on average by 5% in response to the addition of the fungi. However, Newbold (1995) noted that responses were highly variable ranging from 12% increase in output to a reduction in yield of 9%. As with recent trials with diet added enzymes, there were indications that the effectiveness of AO was influenced by the diet and nutritional demands of the host. Responses to AO, appeared to be greater in early as opposed to mid or late lactation (Wallentine *et al.*, 1986, Kellems *et al.*, 1987, Denigan *et al.*, 1992), when presumably the cow can make more use of any increase in nutrient supply resulting from fungal inclusion. The ratio of forage to concentrate also appeared to influence responses, with Huber *et al* (1985) recording larger responses in milk yield in response to AO addition as the level of concentrates in the ration increased. Response to AO may also be modified by more subtle variations in the diet. Harris *et al* (1983) recorded a 5 % increase in fat corrected milk yield in response to AO when the diet contained sugar cane and corn silage but a 9 % depression in yield when the silage was replaced by cotton seed hulls. In common to the data on the effect of diet added enzymes several studies have reported an increase in dry matter intake in animals supplemented with AO (Van Horn *et al.*, 1984, Gomez-Alarcon *et al.*, 1991, Caton *et al*., 1993). Williams and Newbold (1990) suggested that in many studies the increase in production, observed when fungal feed additives were added to the diet, could be largely explained by an increase in dry matter intake.

Can fungal feed additives be considered as enzymes ?

There is general agreement that increases in productivity when fungal feed additives are added to the diet result from their action in rumen, and specifically an increase in the number and activity of bacteria, including cellulolytic bacteria, in the rumen (Martin and Nisbet, 1992, Wallace and Newbold, 1992, Dawson, 1993, Newbold, 1995). What is less clear is how small amounts of fungi included in the diet stimulate bacterial activity in the rumen.

Newbold *et al* (1996) suggested that feed additives based on *Saccharomyces cerevisiae* might help stimulate the growth of anaerobic bacteria in the rumen by reducing potentially inhibitory levels of oxygen, however AO had no effect on oxygen uptake by rumen fluid. Nisbet and Martin (1990) found that malic acid stimulated the growth and activity of the prominent gram negative rumen bacterium *Selenomonas ruminantium* and it was suggested that a stimulation in the numbers of *S. ruminantium* by malic acid within the fungi might occur *in vivo*. Malic acid at relatively high levels of addition has been found to stimulate rumen fermentation and milk yield (Kung *et al.*, 1982, Martin and Streeter, 1995). However the concentration of malic acid in fungi is low. Nisbet and Martin (1990) reported that the malic acid concentration in 10% filtrate of AO was 1.45 mM suggesting that at an inclusion rate of 3 g/d, cattle might receive less than 10 mg/ d malic acid in the diet. Newbold *et al* (1996) found that 100 mg/d malic acid fed to sheep had no effect on ruminal fermentation. Also autoclaving AO destroyed its ability to stimulate bacterial numbers in a rumen simulating fermentor (Newbold *et al.*, 1991) and it is unlikely that malic acid would be destroyed by autoclaving.

Table 1. The effect of preparations based on *Aspergillus oryzae* and *Aspergillus foetidus* on bacterial numbers and fiber degradation in the rumen of sheep.

	Control	*A. oryzae*	*A. foetidus*
Total bacteria ($x10^8$/ ml)	2.43	3.14	2.61
Cellulolytic bacteria ($x10^7$/ ml)	2.73	5.30	5.53
Potential degradability of hay in the rumen (%)	62.8	63.0	62.9
Rate of degradation of hay (%/ h)	2.3	3.4	3.2

In order to identify the ingredients within fungal feed additives capable of stimulating microbial activity within the rumen, we investigated the effects of a commercial product based on *A. oryzae* and a preparation of *Aspergillus foetidus*, grown in our own laboratory, on rumen fermentation in sheep (Newbold, McIntosh and Wallace, unpublished observation). Both fungal preparations stimulated bacterial numbers, and in particular numbers of cellulolytic bacteria in rumen (Table 1). Both fungi also stimulated the rate, but not the extent, of fiber degradation in the rumen (Table 1). To identify the active component within the fungi, an aqueous extract was prepared from *A. foetidus* and fractionated by dialysing against deionized water to prepare higher molecular weight material remaining after dialysis and a low molecular weight fraction washed out by dialysis. The molecular cut-off of the dialysis tubing used was around 10,000 Da. Thus it is to be expected that low molecular weight material such as malic acid (Mr = 134) would be recovered in the washings from outside the bag. Material remaining within the bag would be high molecular weight material. Fractions were tested in the rumen simulating fermentor Rusitec. Total and cellulolytic bacterial numbers were slightly, but not significantly, higher in vessels supplemented with the low molecular weight fraction (Table 2). A significant increase in both fiber digestion and bacterial numbers was observed in vessels supplement with the high molecular weight fraction (Table 2). Thus while low molecular weight products, such as malic acid, may have a minor role in stimulating bacterial activity when *A. foetidus* is fed, it appears that as yet unidentified factors, retained in the higher molecular weight fraction, are of greater importance in mediating the effects of the fungi.

Arambel *et al* (1987) speculated that endogenous polysaccharidase enzymes might play an important role in the stimulation of ruminal digestion in animals fed fungi from the genus *Aspergillus*. In the experiment reported in Table 2, the high molecular weight fraction stimulated bacterial numbers and fiber digestion. The same fraction had the highest carboxymethylcellulase and amylase activity. When the high molecular weight fraction was incubated with a wide spectrum protease prior to feeding, its ability to stimulate fiber digestion and bacterial numbers was destroyed (Table 2). Thus it appears that proteins are important factors in the ability of *A. foetidus* to stimulate rumen digestion. In view of the enzyme activity in the high molecular weight fraction, the involvement polysaccharidase enzymes the action of *A. foetidus* seems likely. Thus the effects of *A. foetidus* and Cellulase AC (a commercial enzyme preparation prepared from *Aspergillus niger*) on fermentation in Rusitec were compared.

Table 2. Effect of low and high molecular weight fraction prepared from an aqueous extract of *Aspergillus foetidus* the high molecular weight fraction after incubation with pronase on bacterial numbers and dry matter digestion in Rusitec

	Control	Low molecular weight fraction	High molecular weight fraction	High molecular weight fraction treated with pronase
Total bacteria ($x10^8$/ml)	3.5	4.5	6.4	3.9
Cellulolytic bacteria ($x10^6$/ml)	2.5	5.1	9.9	2.2
Digestion of DM (g) after 24 h incubation	5.95	7.31	8.90	6.6

Cellulase AC was added at a concentration calculated to supply a similar xylanase activity to the vessels as *A. foetidus* (500 mg of *A. foetidus* produced 1 mg of glucose /min from 1 mg/ml xylan at pH 6.8 and 39°C, representing 50 mg of Cellulase AC). As in previous experiments, *A. foetidus* stimulated total and cellulolytic bacterial numbers (Table 3). Cellulase AC also stimulated microbial numbers.

Table 3. Effect of *Aspergillus foetidus* and Cellulase AC on bacterial numbers in Rusitec

	Control	*A.foetidus*	Cellulase AC
Total viable bacteria $x10^8$ /ml	4.03	4.63	5.87
Cellulolytic bacteria $x10^6$ /ml	1.92	4.33	4.20

In summary, our results show that the fraction in crude fungal preparations capable of stimulating bacterial activity in the rumen is heat labile (Newbold *et al*., 1991), of high molecular weight, destroyed by proteases and similar effects can be obtained with a

purified enzyme prepared from *A. niger*. Thus it seems reasonable to conclude that the activity of fungal feed additives based on *Aspergillus* is due to the presence of polysaccharidase enzymes in the crude fungal preparations.

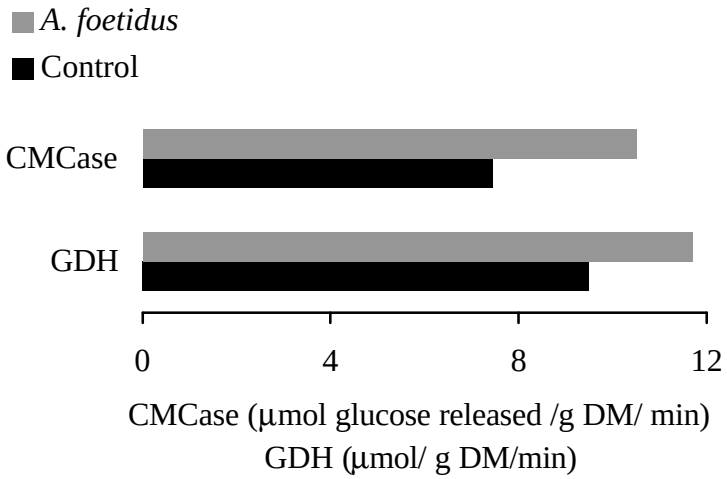
One apparent difference between products based on crude fungal preparations and the trials that have been reported with dietary enzymes is the amount of enzyme activity added to the diet. As reported above, *A. foetidus* contained 10 units of xylanase/ g of fungi as fed (1 unit equals 1 μm of reducing sugar produced/ min from 1 mg/ml xylan at pH 6.8, 39°C). Assuming cattle received a supplement of 10 g *A. foetidus* /d this implies the fungi would add 100 units of xylanase to the diet each day. The enzyme activity added in commercial AO based products may be as much as 10x higher than this (Newbold, 1995). Even with the higher activity of commercial products, this is an order of magnitude lower than the activities apparently added in the studies summarized by Beauchemin and Rode (1996). However, in the studies quoted by Beauchemin and Rode (1996) the enzyme are measured at lower pH and at higher temperatures than those encountered in the rumen. Thus the activities quoted are considerably higher than those which would be measured at a pH and temperature similar to that in the rumen. Indeed our own measurements suggest that when measured under rumen like conditions (pH 6.5, 39°C) dietary enzymes are likely to supply enzymic activities of a similar magnitude to those suggested above for *A. foetidus* (Newbold, Hristov, Beauchemin and Rode, unpublished observation).

How do polysaccharidase activities within fungal feed additives stimulate bacterial fermentation in the rumen ?

If polysaccharidase activities in products such as *A. foetidus* are responsible for the stimulation in bacterial activity, then presumably the enzymes must function within a few hours of feeding before being degraded by the proteolytic activity of the rumen microbes (Kopecny *et al*., 1987). Also, the quantities of added enzyme activity is small compared to endogenous microbial activities in the rumen (Newbold, 1992). The enzymes present in fungi must therefore act synergistically with rumen microbial activities rather than additively.

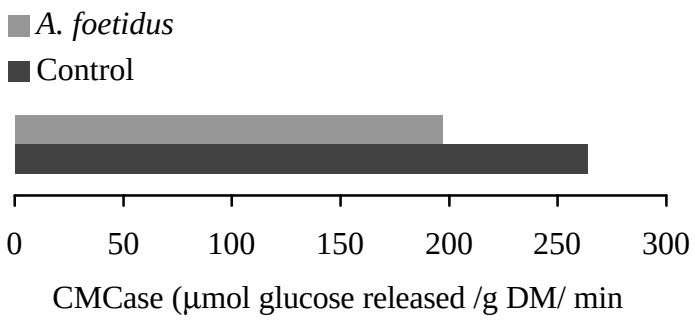
Close association between micro-organisms and fiber is essential for the digestion of forage in the rumen (Cheng *et al.*, 1991), and the rate of degradation of fiber *in vivo* depends on the rate at which the adherent cellulolytic microbial population develops (Silva *et al.*, 1987). If *Aspergillus* were to stimulate the attachment of rumen microbes to plant fiber, this might explain how small quantities of fungi can have a significant effect on fiber degradation *in vivo.*

Fig 2. Effect of *Aspergillus foetidus* on the association of microbial glutamate dehydrogenase (GDH) and carboxymethylcellulase (CMCase) with straw *in vitro.*



To test this hypothesis straw was pre-incubated in anaerobic phosphate buffer plus or minus an aqueous extract of *A. foetidus*. Rumen fluid was added and incubations continued for 30 min. The attachment of glutamate dehydrogenase (GDH) and carboxymethylcellulase (CMCase) to the fibres was used as an indicator of the attachment of the total and cellulolytic microbial populations respectively. GDH and CMCase activities extracted from straw which had been pre-incubated with *A. foetidus* then mixed with strained rumen fluid *in vitro* were significantly higher than when untreated straw were added to rumen fluid (Fig. 2). Straw pre-incubated with *A. foetidus* alone had activities less than 15% of the values observed when rumen fluid was present. Thus the enzymes associated with the feedstuffs were mainly of rumen microbial origin, and initial attachment of all microorganisms, of which GDH is an index, and the attachment of cellulolytic organisms (CMCase), were both enhanced by *A. foetidus*. These results were confirmed *in vivo* where including *A. foetidus* in the diet of sheep stimulated the attachment of cellulolytic bacteria, as indicated by the amount of CMCase that could be extracted from the fibre, to straw incubated in the rumen for 24 h (Fig 3).

Fig 3. Effect of *Aspergillus foetidus* on the association of microbial carboxymethylcellulase (CMCase) with straw *in vivo.*



The mechanism by which *A. foetidus* stimulates the attachment of rumen microbes to plant fibers remains unknown. However chemotactic responses to soluble sugars released from plant fibers have been shown to initiate the attachment of fungi and protozoa to plant cell walls in the rumen (Orpin ,1979 a, b; Orpin and Bountiff, 1978). It is possible that polysaccharidase enzymes from the fungi released soluble sugars from the fiber thus increasing the chemotactic attraction and eventual attachment of cellulolytic rumen microbes to the plant surface. Enzymic attack may also have altered the surface structure of the plants making them more suitable for microbial attachment.

Conclusion

It can be argued that fungal feed additives based on *Aspergillus* can be considered as crude enzyme preparations. Evidence has been presented suggesting that one possible mode of action of exogenous enzymes in the rumen would be to stimulate the attachment of rumen microbes to plant fiber. In order that we might more accurately match enzymes to dietary substrates, more work is need to identify the enzyme induced changes in the plant cell wall that help stimulate such attachment.

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