EFFECT OF FIBROLYTIC ENZYMES ON THE NUTRITIVE VALUE OF TROPICAL GRASSES AND DAIRY CATTLE PERFORMANCE

By

DERVIN BARTOLO DEAN

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To my lovely wife Domenicchella, and my dear kids, Sheryll, Homer, and Stephanie

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LIST OF ABREVIATIONS

A-20	Biocellulase A-20
ADF	acid detergent fiber
ADFD	acid detergent fiber digestibility
BA	bahiagrass
BCS	body condition score
BE	bermudagrass
BHBA	beta hydroxybutyrate
BUN	blood urea nitrogen
BW	body weight
BWG	body-weight gain
CA	Cattle-Ase
CP	crude protein
CPP	crude protein production
CPD	crude protein digestibility
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
FCM	fat-corrected milk
FP	fat production
Glc	glucose
IVADFD	*in vitro* acid detergent fiber digestibility
IVDMD	*in vitro* dry matter digestibility
IVNDFD	*in vitro* neutral detergent fiber digestibility
MCF	milk crude fat
MCP	milk crude protein
NDF	neutral detergent fiber
NDFD	neutral detergent fiber digestibility
NFC	non-fiber carbohydrates
NH_3	ammonia
NH_3-N	ammonia nitrogen
Pr	Promote
RIN	relative intake
SCC	somatic cell counts
TDN	total digestible nutrients
TMR	total mixed ration
VFA	volatile fatty acids
WSC	water soluble carbohydrates
WSN	water soluble nitrogen
X-20	Biocellulase X-20

Abstract of Dissertation Presented to the Graduate School
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Five experiments were conducted to determine whether the nutritive value of hay or silage made from tropical grasses and animal performance can be improved by addition of fibrolytic enzymes. Experiments 1 and 2 determined the effect on the digestibility of Coastal bermudagrass and Pensacola bahiagrass hays of applying NH_3 or four fibrolytic enzymes: Promote (Pr), Biocellulase X-20 (X-20), Biocellulase A-20 (A-20), and Cattle-Ase (CA) at 0, 0.5, 1, and 2x the rates recommended by the respective manufacturers. Biocellulase X-20 and A-20 improved the 6-h and 48-h digestion of the forages, but ammoniation was more effective. In Experiment 3, Tifton 85 bermudagrass was ensiled without treatment (Control), or after treatment with the enzymes used in Experiments 1 and 2. Compared to Control silages, Promote-treated silages had lower pH and dry matter (DM) losses, and lower concentrations of ammonia-N, neutral (NDF) and acid detergent fiber (ADF), and greater concentrations of residual water soluble carbohydrates (WSC), *in vitro* DM, NDF, and ADF digestibility. The other enzymes also increased

fiber hydrolysis, but did not improve indices of fermentation quality. Therefore, Promote was the most promising enzyme for improving the fermentation and nutritive value of silages. Experiments 4 and 5 tested the effect of Promote on the performance of thirty Holstein lactating dairy cows fed a ration consisting of bermudagrass silage, corn silage, and concentrate *ad libitum* for two 28-d periods. Treatments were the following: Control, enzyme applied at ensiling to the bermudagrass (TS), or at feeding to the concentrate (EC), the total mixed ration (ETMR) or the bermudagrass silage (EF). Voluntary intake, apparent digestibility, milk production, and blood glucose concentration were unaffected by treatment. Cows fed ETMR tended to have lower beta hydroxybutyrate and blood urea-N concentrations and greater milk fat and protein concentrations than cows fed the control diet. In Experiment 5, five ruminally fistulated lactating cows were fed the same diets as in Experiment 4 for three, 15-d periods. Ruminal pH was decreased by feeding EC, whereas acetate:propionate ratios were reduced by feeding ETMR. In situ DM disappearance was unaffected by enzyme treatment. These experiments suggest that ETMR was the most promising treatment.

CHAPTER 1
INTRODUCTION

Forages represent the most important, cost effective feed resource in ruminant nutrition (Jung and Allen, 1995). However, the relatively low quality of tropical forages militates against their use as the sole feed for actively growing or high-performing ruminants. Several attempts have been made to improve forage quality genetically or by chemical or biological treatments. One of the most important goals in this regard is to improve the fiber digestibility of the forages. Some chemical and biological treatments have been effective at achieving this objective.

Ammoniation is one of the most studied chemical treatments for enhancing fiber digestibility and several reports have described its effectiveness for improving both forage quality and animal performance. Ammoniation increases forage crude protein (CP) concentration and substantially reduces the concentration of neutral detergent fiber (NDF) in forages. Most of the loss of NDF is due to hydrolysis of hemicellulose, though the disruption of chemical linkages between lignin and hemicellulose also occurs (Weiss and Underwood, 1995; Barrios-Urdaneta and Ventura, 2002). Additional benefits of ammoniation include reduced yeast and mold growth, and less aerobic deterioration of high moisture hay and silage (Woolford and Tetlow, 1984; Bates et al., 1989b). Consequently, feeding ammoniated forage often results in increased daily gain and dry matter (DM) intake in beef cattle (Vagnoni et al., 1995; Brown and Pate, 1997). However, the use of ammonia for improving forage quality has been limited because of the corrosive nature of the alkali which can be hazardous to operators and their

equipment (Lalman et al., 2005). Ammoniation also contributes to N importation to farms and therefore represents a small but important threat to air quality because of its contribution to surface water eutrophication and nitrate contamination of ground water (Ishler, 2005). In developing countries, the labor required for ammoniation and problems of delivering anhydrous ammonia have limited adoption of the technique.

Fibrolytic enzyme application is one of the most studied biological treatments for improving forage quality and animal performance. Such enzymes have been effective at improving the utilization of a wide range of diets containing roughages (Rode and Beauchemin, 1998) due to improved fiber hydrolysis (Colombatto et al., 2003b) which often results in increased digestibility (Christensen, 1997; Rode et al., 1999) and voluntary intake (Pinos-Rodriguez et al., 2002). Nevertheless, another study has shown that exogenous enzymes did not consistently improve forage utilization by ruminants (Lewis et al., 1999). This inconsistency is attributable to several factors such as differences in enzyme type and activity, treatment duration, application method, diet composition and level of animal performance.

Additional factors that may be implicated include suboptimal prevailing temperature and pH for enzyme action, presence of inhibitors or absence of cofactors and inadequate enzyme to substrate ratios. Nevertheless, feed enzymes have been used to improve the utilization of a wide range of diets containing legumes, grasses, haylage, straw and other feedstuffs (Beauchemin et al., 2003). The mode of action of these enzymes in ruminants is not fully understood. In addition to increasing fiber hydrolysis, they can enhance feed colonization by increasing the numbers of ruminal fibrolytic microbes (Morgavi et al., 2000; Nsereko et al., 2000a) and thereby increase the rate of

degradation of feeds in the rumen (Yang et al., 1999). Additionally, modes of action include improved palatability (Adesogan, 2005), changes in gut viscosity (Officer, 2000), and changes in the site of digestion (Rode and Beauchemin, 1998; Hristov et al., 2000).

Most of the studies of fibrolytic enzyme treatment of ruminant feeds have been done using feedstuffs grown under temperate conditions. Little is known about their effectiveness on tropical or subtropical forages which tend to be less digestible. Yet there is greater scope for improving the quality of tropical forages than there is for temperate forages due to the greater nutritive value of the latter. The aim of this series of experiments was to evaluate the effect of ammonia and proprietary fibrolytic enzyme application on the nutritive value of tropical forages and animal performance.

CHAPTER 2
LITERATURE REVIEW

Cell Wall Differences between Tropical and Temperate Forages

Even under the intensive concentrate feeding systems of ruminant animal production in developed countries, forages continue to represent the single most important feed resource; however, cell-wall concentration and digestibility limit the intake potential and energy availability from forage crops in beef and dairy production systems (Jung and Allen, 1995). Depending on the stage of maturity of the plant, cell walls represent between 30 and 80% of plant dry matter (DM) in grasses so that under some circumstances (high forage diets) the bulk of carbohydrate fermented to metabolizable volatile fatty acids (VFA) by rumen micro-organisms may be derived from cell wall polysaccharides and in senescent grasses, almost all fermentable carbohydrate arises from wall polysaccharides (Stone, 1994). Cellulose is the predominant wall polysaccharide. The cellulosic microfibrils are embedded in a matrix composed of non-cellulosic polysaccharides and some proteins. The major matrix polysaccharides in grasses are glucuronoarabinoxylans, together with smaller amounts of heteroglucans (xyloglucans) and glucans (Stone, 1994).

The main reason why the digestibility of tropical grasses is less than that of temperate perennial grasses is because of differences in cell wall composition. There are differences in polysaccharide composition in cell walls of different types and also considerable quantitative differences in components, e.g., mesophyll cells which are more abundant in temperate forages are relatively cellulose-rich (Gordon et al., 1977, cited by

Stone, 1994) and readily degraded by rumen micro-organisms; but tropical grasses are rich in lignified and secondarily thickened cells of xylem (Akin and Burdick, 1975). Differences in leaf anatomical structure between Panicoid tropical and Festucoid temperate grasses associated with the C_4 and C_3 photosynthetic pathways have been known to botanists for many years (Wilson and Hacke, 1987). These researchers determined that leaves of C_4 (tropical) grasses consistently had less mesophyll and more of the less-digestible epidermis, bundle sheath, sclerenchyma and vascular tissues than leaves of C_3 temperate and legumes grasses. Wilson and Hattersley (1989) also found that anatomical differences between the leaf structural groups were consistently expressed, with C_3 species having higher proportions of mesophyll (53-67 vs. 28-47%) and lower proportions of bundle sheath (5-20 vs. 12-33%) and vascular tissue (3-9 vs. 6-12%) than the C_4 species.

According to Wilson and Hacke (1987) the anatomy associated with either C_4 tropical or C_3 temperate grass genera clearly contributes to difference in DM digestibility between leaves. Comparisons of C_4 or C_3 leaf anatomy in a wide range of summer-growing *Panicum* species grown under the same environmental conditions determined that the C_4 anatomy of tropical grass genera causes their leaves to have lower digestibility and higher cell wall concentration than grasses with C_3 anatomy (Akin et al., 1983). Quantitative analysis of leaf anatomy of a number of grasses indicated that leaves of the tropical species had 25 percentage units more of the slowly digested cell tissues than had the temperate grasses (Akin and Burdick, 1975).

According to Buxton and Redfearn (1997), energy availability from forages is limited by fiber concentration because fiber is slowly and incompletely digested, whereas

cell soluble are almost completely digested. Thus, the proportion of fiber to cell soluble is a major determinant of energy availability in forages. Ruminants digest about 40 to 70% of grass fiber, and some fiber fragments cannot be digested no matter how long they remain in the rumen. Lignin interferes with microbial degradation of fiber polysaccharides by acting as a physical barrier and by being cross-linked to polysaccharides by ferulate bridges (Moore and Jung, 2001). Lignin and ferulate cross linkages are more abundant in C_4 than C_3 grasses. This is the chemical basis for the lower digestibility of C_4 grasses (Ramalho, 1991).

Voluntary intake of forages is a critical determinant of animal performance and cell wall concentration is negatively related to intake of ruminants consuming high-forage diets. Cell walls affect intake by contributing to ruminal fill. Cell wall concentration and rate of passage are the most critical parameters determining ruminal fill (Jung and Allen, 1995). Ruminal fill is typically greater in ruminants consuming C_4 grasses than in those consuming C_3 grasses because of the poorer digestibility of C_4 grasses.

Methods for Improving Forage Nutritive Value

Different chemical and biological treatments have been used for enhancing the nutritive value of low quality forages and roughages. The main effect of such treatments is due to modifications of cell wall components. The changes that take place when low quality roughages are treated with alkali (e.g., ammonium hydroxide, NaOH) are of a physical as well as of a chemical nature (Ramalho, 1991). It is well known that the roughages are normally softer after chemical treatment and this may be one of the reasons for the higher intake found for treated forage (Ramalho, 1991). Another important change that takes place during alkali treatment is a swelling of the plant cell wall. This is probably most pronounced for forage treated with NaOH solution (Harbers et al., 1982).

There are a number of chemical reactions taking place during alkali treatment of forages. Saponification of ester linkages between acetic acid and phenolic acids, and polysaccharides and or lignin as well as such linkages between uronic acid residues of xylans in hemicelluloses and lignin occur during the alkali treatment of straw (Harbers et al., 1982). If the temperature is high enough in the presence of alkali, lignin undergoes cleavage of other linkages between phenyl propane units and free phenolic groups are formed. As a result of the accompanying decrease in the molecular weight and cleavage of linkages to hemicellulose, an increased solubility of lignin in the alkaline solution will occur (Theander and Aman, 1984; cited by Ramalho, 1991). Sundstizil (1998) reported that the OM digestibility of alkali-treated rye straw increased from about 46 to 71%. Disruptions of ferulate bridges by ammoniation have been also associated with improving fiber digestion (Brown and Adjei, 1995, Barrios-Urdaneta and Ventura, 2002), voluntary intake (Glenn, 1990; Vagnoni et al., 1995; Lines and Weiss, 1996) and animal performance (Rasby and Ward, 1989; Brown, 1993; Brown and Pate, 1997).

Ammoniation

Ammoniation is one of the most studied chemical treatments for improving forage digestion in the past few years (Chaudhry, 1998). Chemical treatments of low quality forages, such as ammoniation, have been shown to economically extend the use of such forages into more nutritionally challenging periods of the production cycle, such as late gestation and early lactation (Wiedmeier et al., 2003). Low quality forages are treated with ammonia for the two following reasons: 1) ammonia is an effective preservative for hay containing up to 30% moisture and 2) treatment of mature grass hays and poorer quality crop residues is a cost-effective way for improving their feeding value (Weiss and Underwood, 1995). Ammoniation increases crude protein (CP) in the treated forages by

adding about 50 to 80% of the N in NH_3 to the forage. Some of the retained nitrogen is converted by microbes present on the forage into microbial protein and another fraction of the retained nitrogen is bound in an unknown manner to the forage fiber components (Weiss and Underwood, 1995). Barrios-Urdaneta and Ventura (2002) observed that their dry ammoniation method improved the CP of koroniviagrass (*Brachiaria humidicola*) from 3.2 to 8.3%. Brown (1993) observed that ammoniation (4% of DM) of stargrass (*Cynodon nlemfuensis*) hay increased ($P < 0.01$) total N concentration (from 1.0 to 1.4%). A similar increase (0.9% N, $P < 0.01$) was obtained by Lines et al. (1996) in alfalfa hay.

Ammoniation also improves forage digestibility. This is due to hydrolytic action on linkages between lignin and structural polysaccharides, thus increasing organic matter (OM) potentially available for utilization by the ruminal microorganisms (Barrios-Urdaneta and Ventura, 2002). Ammonia treatment substantially reduces the concentration of neutral detergent fiber (NDF) in forages and most of the loss of NDF is due to hydrolysis of hemicellulose and disruption of chemical linkages between lignin and hemicellulose, making the hemicellulose more digestible (Weis and Underwood, 1995). Cellulose digestibility also increases since lignified hemicellulose encases cellulose (Chaudhry, 1998). Ammoniation partially breaks down the structure of cellulose by disrupting hydrogen bonds. This reaction causes a swelling of the fiber and allows cellulase better access to the fiber for digestion (Church, 1988). Lines et al. (1996) reported lower NDF (58.8 vs. 56.2%, $P < 0.01$) and hemicellulose (13.2 vs. 9.4%, $P < 0.01$) concentrations in ammoniated alfalfa hay compared to the untreated hay. This agrees with results obtained by Brown and Adjei (1995) who found lower NDF (-5%, $P <$

0.05) and hemicellulose (-17%, $P < 0.01$) concentrations in urea-treated (6% DM) guineagrass (*Panicum maximum*) hay compared to the untreated hay**.**

Ammonia treatment also changes the physical characteristics of forages making them more pliable and increases their hydration. Hydration rate has an important role in digestion rate; the faster a forage particle is hydrated, the faster it is digested (Weiss and Underwood, 1995). Barrios-Urdaneta and Ventura (2002) showed that ammoniation increased the *in vitro* NDF digestibility (by 10.9%) of koroniviagrass. Brown (1993) observed that ammoniation increased *in vitro* OM, NDF, and ADF digestibility and decreased ($P < 0.01$) NDF concentration in stargrass hay. Vagnoni et al. (1995) showed that ammoniation of mature bermudagrass increased both the *in situ* rate ($P < 0.05$) and the potential extent ($P < 0.01$) of forage DM and NDF disappearance in lactating cows. Zorrila-Rios et al. (1991) found that the *in vitro* DM digestibility (IVDMD) of wheat straw was increased by 54% due to ammoniation. In that study, ammoniation also almost doubled the CP concentration of the straw compared to untreated straws.

Woolford and Tetlow (1984) observed that ammoniation of high-moisture hay reduced the growth of yeasts and molds, and decreased the rate of aerobic deterioration. Bates et al. (1989b) found a substantial reduction of external molding when ammonia was metered into the sealed plastic container of round bale silage; however, they observed that ammoniation was associated with undesirable fermentation characteristics, especially when direct-cut, low DM tropical forages were ensiled. Dry matter recovery and intake of ammoniated, direct-cut, bermudagrass round bale silage was very poor. Although application of ammonia to bermudagrass wilted to 40 to 50% DM improved the quality of round bale silage, these authors did not recommend this practice because of the

high level of management required for success, and because treatment of silage and hay with ammonia has, on occasion, been toxic to cattle.

Ammonia Treatment Methods

Gaseous anhydrous ammonia has been used for forage treatment in developed countries while, in developing countries, spraying ammonia solutions and dipping hay in urea solutions are preferred (Chenost and Kayouli, 1997). According to these authors, using anhydrous ammonia is more effective, but its high cost and requirement for special delivery and storage facilities have hindered its utilization by farmers. The use of a urea solution is a simple, low cost technique; however, it has not become widely accepted. The labor involved in handling the material and the appearance of molds as a consequence of the water added has limited adoption by commercial producers (Barrios-Urdaneta and Ventura, 2002). The latter researchers recently developed a method that they called "dry ammoniation" by adding water and urea into plastic containers (19-l) and suspending 1 kg hay bales 5-8 cm over the perforated cover of the container. Thereafter the hay and container were hermetically sealed with a plastic tarp and stored for 14 or 21 days. The method was found to increase CP concentration and *in vitro* NDF digestibility by 190% and 37%, respectively.

Barrios-Urdaneta and Ventura (2002) evaluated the effects of storage time (14 and 21 days), water volume (200 and 400 ml/kg DM of hay) and urea quantity (20 and 40 g/kg DM of hay) on the CP concentration and *in vitro* digestibility of NDF of koroniviagrass of 1 kg hay bales. The best increase in nutritive value was obtained when the hay was stored for 21 days and treated with 200 ml of water and 40 g of urea/kg.

According to Dolberg (1992), ammoniation treatment time may vary from one to five weeks. However, temperature and treatment time are inversely related; and

therefore, more time is required in the winter or cold weather. Simple tests of successful treatment of straw are a browning in the color of the forage, a strong smell of ammonia, and absence of rotten and molded straw (Lalman et al., 2005). The amount of anhydrous ammonia necessary to improve digestibility is between 2 and 4% of DM (Weiss and Underwood, 1995). The reaction between ammonia and fiber is dependent on temperature, so if forages are treated with ammonia during cold weather a five-week treatment period is recommended (Dolberg, 1992). After this treatment period, forage can remain covered for extended periods without problems. It is recommended that the forage be left uncovered for at least 3 to 5 days prior to feeding to allow free ammonia to escape (Lalman et al., 2005). This may not be necessary, but sometimes animal acceptance may be poor initially if ammoniated bales are not aired out prior to feeding (Weiss and Underwood, 1995).

Brown and Adjei (1995) applied a urea solution (0, 4, 6, or 8% of the forage DM) to guineagrass hay harvested at different moisture concentrations (25 or 40%) and observed that CP concentration and *in vitro* OM digestibility (IVOMD) increased linearly ($P < 0.01$), whereas concentrations of hemicellulose ($P < 0.01$) and ADL ($P < 0.05$) decreased linearly with increasing amount of urea applied. The same researchers treated guineagrass hays with urea at 0, 4, or 6% of the forage DM. The urea solution was sprayed onto the flat sides of the bales, or applied by low pressure (10 psi) injection. The greatest improvements in CP and NDF concentration and IVOMD were obtained at the 25% forage moisture concentration using the low pressure injection method.

Animal Response to Feeding Ammoniated Forages

Several reports show that ammoniation of low quality forages can improve animal performance. Weiss and Underwood (1995) stated that ammonia treatment increases the

DMI and DMD of low to medium quality grass hay by 5 to 10 percentage units. The increase in both variables results in a substantial increase in consumption of digestible energy by animals fed ammoniated forages as compared to those fed untreated forage. Consequently, ammoniated straw can provide adequate energy and protein to maintain lactating beef cows and ewes under most conditions while untreated straw can not. Vagnoni et al. (1995) fed crossbred beef steers anhydrous ammoniated (3% of hay DM) mature bermudagrass hay or supplemented their diets with urea and observed that ammoniation, unlike urea supplementation, increased ADG and DMI ($P < 0.05$), which suggests that ammoniation resulted in greater growth of ruminal microorganisms. In two digestion and growth trials, round bales of hay were sprayed with solutions of 0, 4, or 6% urea and fed to beef steers. Hay intake increased in a quadratic ($P < 0.05$) manner with increasing urea concentration. Apparent NDF and ADF digestibility increased linearly ($P < 0.05$) with increasing urea concentration and linear improvements in ADG ($P < 0.05$) and gain/feed ($P < 0.07$) were observed (Brown and Adjei, 1995).

According to Rasby and Ward (1989), when animal requirements for protein are high, as during lactation, the N needed by rumen bacteria can be supplied using ammoniated forages and by supplementing with a source of ruminally undegraded protein (RUP) but is digested in the small intestine. The latter meets the remaining protein need and may enhance animal performance because of the improved amino acid profile reaching the small intestine from dietary RUP. Because the energy requirements of lactating dairy cows are quite high, the amount of ammoniated low quality forages included in the diet should be limited (Weiss and Underwood, 1995).

The only dried forages that should be considered for 3% ammoniation are straws, mature grass hays and corn stover. No more than 1% of NH_3 should be applied to high quality forages such as alfalfa (*Medicago sativa*), immature orchardgrass (*Dactylis glomerata*), fescue (*Festuca arundinacea*), sudangrass (*Sorghum sudanense*), cereal grain hays or any moderate to early harvested grass hay (including both cool and warm season species) because the resulting product is often toxic to livestock (Weiss and Underwood, 1995). Ammoniation of high quality roughages can lead to toxicity problems known as "crazy cow syndrome" or "bovine bonkers." Symptoms include hyperexcitability, circling, convulsions, and even death. Toxicity is caused when cattle consume sufficient quantities of the toxic compound, 4-methylimidazole, which is formed when soluble sugars in the roughage react with ammonia. This compound passes through the milk to affect nursing calves, which seem to be more susceptible to the toxicity than mature animals. Mature roughages have low soluble sugar content and present little NH_3 toxicity risk (Lalman et al., 2005).

The foregoing indicates that ammonia treatment is a viable method of increasing the nutritive value of low quality forages and improving the animal performance of ruminant livestock fed such grasses. However, the use of NH_3 is limited due to the high investment in infrastructure required for delivering NH_3, treating the forages, and storing the treated forage and concerns about the hazardous nature of the alkali.

Feed Enzymes

Definition, Types, and Classification

Enzymes are naturally occurring globular protein molecules that catalyze specific chemical reactions in biological systems. Two mechanisms have been propounded to explain enzyme action. The first, the lock and key mechanism, was proposed by Emil

Fischer in the late 1800s, which postulates that enzymes accommodate substrates with specific shape that complement the enzyme active site (Scrutton, 1999). However, in 1958 Daniel Koshland proposed the induced fit theory, which postulates that the substrate can induce conformational changes in an enzyme structure to bring the catalytic groups of the enzyme into the proper alignment for binding the substrate (Koshland, 1994). Both theories are now accepted mechanisms for enzyme action.

Enzymes are involved in the digestion of complex feed molecules into their chemical constituents (e.g., glucose, amino acids) in both bacteria and the host animal. Digestive enzymes are essential to animals because complex feeds are not readily absorbed from the digestive tract unless they are degraded to simpler molecules (Kung, 2001)

Enzymes are classified broadly by the substrate on which they act and by their specificity. Commercial enzyme products are fermentation extracts of bacterial (*Bacillus* spp.) or fungal (*Trichoderma* and *Aspergillus* spp.) origin (Beauchemin et al., 2004a), and contain a unique array of enzymatic activities (Table 2.1). Enzyme activity can be assayed using *in vitro* methods by measuring end products of hydrolysis (i.e., reducing sugars, amino acids or peptides) per unit time, using a specified substrate under defined conditions. These substrates are often purified or modified to simplify measurements of activity (Kung, 2001).

Cellulose is hydrolyzed through a complex process involving cellulases. Numerous specific enzymes contribute to cellulase activity, including endocellulase, exocellulase, and ß-glucosidase. In general, endoglucanases hydrolyze cellulose chains at random to

Table 2.1 Some fibrolytic enzyme-producing microorganisms and the enzymes they produce

Microorganism	Enzymes
Aspergillus niger [1, 5]	α-amylase, endoxylanase, β -xylosidase, acetylxylan esterase, α-L-arabinofuranosidase
Aspergillus ficuum [3]	β-glucanase
Aspergillus candidus [3]	Cellulase
Aspergillus sydowi [4]	Phytase, β -D-fructofuranosidase
Microorganism	Enzymes
Aspergillus oryzae [1, 2]	α-amylase, protease
Bacillus licheniformis [3]	α-amylase
Bacillus subtilis [3]	Phytase, α-amylase
Trichoderma viridae [3]	Xylanase, β-glucanase, protease, cellulase
Saccharomyces cerevisae [1, 3]	α-galactosidase
Humicola insolens [6]	β-glucanase

[1] Beauchemin et al., 2004a
[2] Carlsen et al., 1996
[3] Hutcheson, 2001
[4] Muramatsu and Nakakuki, 1995
[5] Noel et al., 1998
[6] Schulein, 1997

produce cellulose oligomers of varying degrees of polymerization; exoglucanases hydrolyze the cellulose chain from the non-reducing end, producing cellobiose, and ß-glucosidases hydrolyze short-chain cellulose oligomers and cellobiose to glucose (Beauchemin et al., 2003).

The main enzymes involved in degrading the xylan core polymer to soluble sugars are xylanases and ß-1,4 xylosidase (Bhat and Hazlewood, 2001). The xylanases include endoxylanases, which yield xylooligomers and ß-1,4-xylosidases, which in turn yield xylose. Other hemicellulase enzymes involved primarily in the digestion of side chains include ß-mannosidase, ά-L-arabinofuranosidase, ά -D-glucuronidase, ά -D-galactosidase, acetyl xylan esterases, and ferulic acid esterase (White et al., 1993, cited by Beauchemin et al., 2003; Bhat and Hazlewood, 2001).

According to Fanutti et al. (1995), endo-β-1,4-xylanase hydrolyzes the β-1,4-linked polysaccharide backbones of xylans, which form the major hemicellulose component of forages. Some studies on the structure of xylanases have revealed that some enzymes are comprised of single catalytic domains while other xylanases are modular, consisting of single or multiple catalytic domains fused via linker sequences to noncatalytic sequences, some of which constitute cellulose binding domains (Fanutti et al., 1995). Hemicellulases derived from aerobic microorganisms do not appear to associate into multi-enzyme complexes, while anaerobic organisms often synthesize multi enzyme cellulase-hemicellulase complexes (Fanutti et al., 1995). These researchers have focused their studies on plant cell wall-degrading enzymes of anaerobic fungi that are particularly active against the more recalcitrant plant structural polysaccharides and have observed that these organisms produce cellulases and hemicellulases that associate into large molecular weight, multi-enzyme complexes and bind tightly to cellulose, exerting their cellulolytic effect.

Murashima et al. (2003) noted that plant cell walls are comprised of cellulose and hemicellulose and other polymers that are intertwined, and this complex structure presents a barrier to degradation by pure cellulases or hemicellulases. They determined the synergistic effects on corn (*Zea mays*) cell wall degradation by the action of xylanases and cellulases from *Clostridium cellulovorans*. Xylanase and cellulase were found to degrade corn cell walls synergistically but not purified substrates such as xylan and crystalline cellulose. The mixture of xylanases and cellulases at a molar ratio of 1: 2 gave the highest synergistic effect on corn cell wall degradation. The amounts both of

xylooligosaccharides and cellooligosaccharides liberated from corn cell walls were increased by the synergistic action of xylanases and cellulases.

Pectin, a minor component of grass cell walls, is digested in the rumen either by strictly pectinolytic species or by species possessing a combination of pectinases (e.g., pectin lyase, polygalacturonase, pectin methyl esterase) and xylanases (Cheng et al., 1996).

Commercial Exogenous Fibrolytic Enzymes

Commercial enzyme products are relatively concentrated and purified, and they contain specific enzyme activities (Beauchemin et al., 2004a). Use of exogenous enzymes can be beneficial when the enzyme preparation and the feed are complimentary. The use of fibrolytic enzymes as additives for ruminant diets has been the focus of considerable research recently following positive responses to enzyme supplementation in feeding trials (Beauchemin et al., 1995; Kung et al., 2000). However, in contrast to the case in non-ruminants (Bedford and Schulze, 1998), the mode of action of these enzyme additives in ruminants is not fully understood. As an alternative to costly *in vivo* trials, several *in vitro* studies have been conducted to examine the effects of enzymes on the degradation of feedstuffs, but the complexity of these feeds makes it difficult to identify which feed fractions are most influenced by enzymatic action. The use of purified xylans and cellulose can minimize this complexity and provide a more informative method of evaluating the mode of action of enzymes. However, the results of such studies may not always correlate to the enzyme effects on feedstuffs.

Several feed enzyme products that contain a blend of enzymes have been shown to be effective at enhancing the utilization of ruminant diets (Rode and Beauchemin, 1998). Nevertheless, the enzyme levels and activities that will effectively improve dietary

nutrients will vary with the diet being considered and the nature of the enzyme. Types of cellulases and hemicellulases in commercial enzyme products differ substantially, and differences in the relative proportions and activities of these individual enzymes determine the efficacy of cell wall degradation by these products (Beauchemin et al., 2003). In addition to fiber-degrading enzymes, these products also have secondary enzyme activities, including amylases, proteases, and pectinases, which contribute to their hydrolytic capacity. Various factors such as enzyme type and method of preparation and application, amount of enzyme applied and fraction of the diet targeted, and animal differences have lead to inconsistencies in results of trials in which enzymes have been added to ruminant feeds (Bowman et al., 2002).

Rode and Beauchemin (1998) evaluated commercial enzyme preparations *in vitro* using alfalfa hay or barley (*Hordeum vulgare*) silage as a substrate. Effectiveness of enzyme products differed for the two substrates, indicating that an enzyme product that elicits a positive response in one diet may not be effective if evaluated using a different diet. According to Newbold (1995, cited by McAllister et al., 2001) destruction of the multi-enzyme complexes during the extraction process may explain why enzymes from mixed ruminal microorganisms failed to release much soluble sugar from hay and straws.

Mode of Enzyme Action

The mode of action of exogenous enzymes is generally to hydrolyze some plant components that impede digestion, thereby increasing the nutritive value of the feed. A number of different mechanisms of enzyme action have been postulated, including direct hydrolysis (Sheperd and Kung, 1996b; Colombatto et al., 2003b), stimulation of microbial numbers and attachment to substrate (Morgavi et al., 2000a), improvements in palatability (Adesogan, 2005), changes in gut viscosity (Officer, 2000), and changes in

the site of digestion (Rode and Beauchemin, 1998; Hristov et al., 2000). Some of these factors increase the hydrolytic capacity of the rumen, which indirectly reduces gut fill, and hence enhances feed intake (Adesogan, 2005). Morgavi et al. (2000a) suggested that synergy between ruminal fibrolytic enzymes and added enzymes may also be responsible for improvements in animal production when ruminants are fed enzyme-supplemented feeds.

Lack of information about enzyme products used and method of providing the product to animals makes it difficult to compare the results from early studies to more recent studies. Inconsistent results seem to be caused by a number of factors including diet composition, type of enzyme preparation used, complement of enzyme activities, level of enzyme provided, enzyme stability and method of application (Rode and Beauchemin, 1998).

Factors Affecting Enzyme Action

It is essential to determine the conditions necessary for optimizing effects of supplemental fibrolytic enzymes on animal performance. When viewed across a variety of enzyme products and experimental conditions, the response to feed enzymes by ruminants has been variable. This variation can be attributed to differences in the lactation stage of cows (Lewis et al., 1999; Rode et al., 1999), enzyme type, activity and characteristics (Dawson and Tricarico, 1999), under or over-supplementation with enzymes (Beauchemin et al., 1995; Yang et al., 1999; Beauchemin et al., 2000; Kung et al., 2000), and inappropriate method of supplying the enzyme product to the animal (Bowman et al., 2002; Sutton et al., 2003). According to Beauchemin et al. (2003) animal responses to fibrolytic enzymes are also greater at times when fiber digestion is compromised and when energy is the first-limiting nutrient in the diet.

Different experiments have examined the impact of delivery method on the effectiveness of exogenous enzymes. Bowman et al. (2002) compared a Control diet to diets in which a fibrolytic enzyme product (Promote®) was applied to the concentrate (45% of TMR), or to a pelleted portion of the supplement (4% of TMR), or to a premix (0.2% of TMR). All diets that were supplemented with the enzyme product delivered about 1.0 g per cow per day. Digestibility of OM, NDF and ADF in the total tract was increased in comparison to the Control when enzymes were added to the entire concentrate. Enzyme application to smaller portions of the diet produced only numerical increases in digestibility over the Control. However, there was an increase in microbial N synthesis in cows fed the enzyme-supplemented premix. Enzyme supplementation did not affect milk production and composition, but cows receiving the enzyme-supplemented concentrate had numerically higher fat-corrected milk (FCM) production compared to the Control cows. These results indicate that the proportion of the diet to which the enzyme is applied must be maximized to ensure a beneficial response.

Lewis et al. (1996) examined the effect of a solution containing cellulases and xylanases on the digestion of a forage-based diet. Ruminally cannulated beef steers were assigned randomly to a Control diet (70:30 grass hay: barley ratio DM basis) or diets in which an enzyme was added to the forage 24 h before feeding (F-24), to the forage 0 h before feeding (F-0), to the barley 0 h before feeding (B-0), or infused ruminally 2 h after feeding (RI). Dry matter and NDF intakes were not different across treatments. *In situ* rate of NDF disappearance of the enzyme-treated barley or forage was greater ($P < 0.05$) than that of the untreated diet. Ruminal infusion of enzymes compared with F-24 and F-0 produced lower disappearance of DM and NDF at 96-h ($P < 0.05$). *In situ* rate of DM

disappearance of enzyme-treated grass tended to be greater ($P < 0.10$) in steers fed B-0 and Control than in those fed F-24 and F-0. Total tract digestibilities of DM, NDF, and ADF were greater ($P < 0.10$) in cows fed F-24 and F-0 than those fed the Control diet. Forage transit time was shorter ($P < 0.10$) for B-0 than for F-24 and F-0; however, all other contrasts for particulate passage did not differ ($P > 0.10$). Results from this study indicate that direct application of enzymes to forage is capable of improving forage digestion.

Enzyme Stability in the Digestive Tract

Several digestive enzymes have been successfully used to enhance poultry and swine performance, but they have not been used traditionally in diets fed to ruminants. The primary reason for this practice was due to the fact that enzymes are proteins and thus would be subject to degradation by microbial proteases in the rumen and/or inactivated by proteases in the small intestine (Kung, 2001). Stability in the rumen is critical for enzyme effectiveness. Considerable variation exists among fibrolytic enzymes in their ability to maintain activity in the ruminal environment. Some enzymes lose their activity rapidly when incubated in ruminal fluid due to proteolysis or adverse pH and temperature conditions that limit enzyme activity, whereas other enzymes show little or no loss in activity even after 12 hours of ruminal incubation. This is partially because enzymes also have pH and temperature optima at which they are most effective. Kopecny et al., 1994 (cited by Kung, 2001) reported that a cellulase enzyme complex was rapidly degraded by ruminal bacterial proteases and its addition to ruminal fluid had no effect on *in vitro* fiber digestion.

According to Morgavi et al. (2001) the cellulase enzyme complex from *Trichoderma* spp. has a pH and temperature optima of 4.5 and 50°C, respectively.

Colombatto et al. (2004a) found that the xylanase activity of two fibrolytic enzymes (Depol 40 and Liquicell 2500) showed optimal activity at pH of 5.6 and both products retained at least 70% of their xylanase activity after 48-h incubation at 15 or 39°C in ruminal fluid. Vicini et al. (2003) analyzed the *in vitro* activities of two commercial fibrolytic enzymes and observed that all major cellulose and hemicellulose-degrading activities were present; however, the optimal pH range was more acidic, and the optimal temperature (approximately 50°C) was greater than the normal pH and temperature in the rumen. The authors concluded that it appears that a considerable part of the potential activity of these enzyme preparations was lost due to conditions in the rumen.

Kung et al. (2002) indicated that the activity of similar fibrolytic enzymes may be optimized under different conditions. They evaluated two different xylanases (B and C) and reported that at 40°C, the activity of xylanase C was greatest at a pH of 6.5 but was substantially reduced as the pH decreased. In contrast, xylanase B showed greatest activity at pH 5 and activity of xylanase C was twice that of xylanase B at pH 5.5 and 6. Fontes et al. (1995) reported that several xylanases were resistant to several proteases but only one cellulase from a mesophilic organism was resistant to proteolytic attack.

Hristov et al. (2000) observed that increasing ruminal doses of exogenous polysaccharide-degrading enzymes in heifers increased ruminal fluid carboxymethylcellulase and xylanase activities linearly ($P < 0.01$) and that elevated levels of fibrolytic activities in the rumen resulted in increased (quadratic, $P < 0.01$) carboxymethylcellulase, xylanase and β-glucanase activities in duodenal digesta. Duodenal amylase activity and reducing sugar concentration also were increased (quadratic response, $P < 0.01$, and $P < 0.05$, respectively) by polysaccharidase enzyme

supplementation. Xylanase activity of fecal DM was increased linearly ($P < 0.05$) with increasing ruminal exogenous polysaccharidases enzyme levels.

Xylanases have been shown to be much more stable in the rumen than cellulases (Hristov et al., 1996, cited by Rode and Beauchemin, 1998). This may due to the relatively large and more complex structure of cellulases compared to xylanases. Morgavi et al. (2001) observed that polysaccharidase activities of commercial preparations from *T. longibrachiatum* incubated for up to 6-h within ruminal fluid were remarkably stable. Cellulase and cellulose 1, 4-beta-cellobiosidase activities were least stable, followed by xylanase, whereas beta-glucanase activity was not affected.

Feed enzyme supplements may exert their effect on feed digestibility in the small intestine as well as in the rumen (Rode and Beauchemin, 1998). Therefore, stability is very important if these enzymes are to remain active in the intestines as well as in the rumen. According to Fontes et al. (1995), the stability of xylanases and cellulases in the rumen may be related to glycosylation, which may protect them from inactivation from temperature and proteases. Many xylanases and cellulases from bacteria and fungal sources are glycosylated. Glycosylation involves covalent bonding of monosaccharides to specific amino acid side chains in enzymes and glycosylation has been shown to confer resistance to proteolysis in monogastrics and ruminal fluid (Fontes et al., 1995).

The survival of exogenous enzyme activities in the rumen may also depend upon the proteolytic environment of the host animal, which can be variable (Falconer and Wallace, 1998). For example, stability of exogenous enzymes varied depending upon the donor cow, and ruminal fluid obtained from cows before feeding inactivated polysaccharidases to a greater extent than ruminal fluid taken after feeding (Morgavi et

al., 2001). This was probably due to the higher ruminal feed content after feeding, which allowed the proteolytic microorganisms to colonize the feed particles and exert their enzymatic effect, thereby decreasing the residual proteolytic activity in the rumen fluid.

The activity of enzymes derived from mesophilic (e.g. *Trichoderma and Aspergillus spp*) or thermophilic (e.g., *Thermoascus aurantiacus*) sources will not be optimized when used as ruminant feed additives (Beauchemin et al., 2004a). These authors suggested that enzymes should be selected that work at lower temperatures. The organisms that produce these enzymes are psychrophilic (Beauchemin et al., 2004a), and their potential to improve the initial rate of OM degradation of corn has been demonstrated by Colombatto et al. (2004b). Cummings and Black (1999) reported that a psychrophilic, gram-negative bacterium has been isolated and has abundant xylanolytic activity. Crude enzyme activity was measured in the supernatant at temperatures ranging from -5 to 50ºC. The bacterium gave faster growth at 15ºC, however optimal enzyme temperature was observed at 37ºC. The isolation of the enzymes secreted by these microorganisms is potentially promising for improving ruminal fiber degradation.

Some researchers have suggested that feeding unprotected enzymes may be more useful in immature ruminants where ruminal microbial populations are not fully developed. For example, Baran and Kmet (cited by Kung, 2001) reported that a pectinase-cellulase enzyme additive improved ruminal fermentation in newly weaned lambs but not in adult sheep (with established ruminal microflora).

Methods of Determining Enzyme Activity

According to Beauchemin et al. (2003) fiber-degrading enzyme activities are generally determined by measuring the rate of release of reducing sugars from pure substrates, with enzyme units expressed as the quantity of reducing sugars released per

unit of time per unit of enzyme. Reducing sugars, which include monosaccharides, disaccharides and some oligosaccharides, can be measured colorimetrically using the Nelson/Somogyi copper method (Somogyi, 1952) or the dinitrosalicyclic acid method (Miller, 1959). The most commonly used substrate for measuring cellulase activity (endo-ß-1,4-glucanase activity) is carboxymethyl cellulose (Wood and Bhat, 1988). Exoglucanase activity can be measured using crystalline cellulose preparations, such as Avicel. ß-glucosidase activity is determined by measuring the release of glucose from cellobiose, or the release of *p*-nitrophenol from *p*-nitrophenyl-ß-D-glucoside (Bhat and Hazlewood, 2001). Xylanase activity is most commonly measured by determining the release of reducing sugars from prepared xylan, such as oat (*Avena sativa*) spelt or birchwood xylan. Xylanases are specific for the internal ß-1,4 linkages within the xylan backbone, and are generally considered endoxylanases (Bhat and Hazlewood, 2001). Endoxylanases can be considered to be debranching or non-debranching based on their ability to release arabinose in addition to hydrolyzing the main xylan chain. ß-xylosidase activity can be determined by using *p*-nitrophenyl derivatives (Bhat and Hazlewood, 2001).

Enzyme activity measurements must be conducted under conditions closely defined with respect to temperature, pH, ionic strength, substrate concentration, and substrate type, since all of these factors affect the enzyme activity. The optimal temperature and pH for most commercial fibrolytic enzymes is approximately 60ºC and optimal pH is between 4 and 5 (Coughlan, 1985). However, these optima are not representative of the conditions in the rumen, which are closer to a pH of 6.0 to 6.7 and 39°C (Van Soest, 1994).

Wallace and Hartnell (2001) evaluated enzymatic and tracer methods for detecting and measuring the quantity of fibrolytic enzyme preparations added to corn silage, ryegrass (*Lolium multiflorum*) silage and a total mixed ration and observed that the quantity of enzyme preparations added to the feeds could not be detected using their enzymatic activities. Glycosidase activities of soluble washed from the feed were more than an order of magnitude greater than glycosidase in the added enzymes. Carboxymethylcellulase and xylanase activity determinations which used reducing sugar release as the measurement, were subject to interference from reducing sugars present in the feed. A fluorescent tracer method, using fluorescein added at a rate of 1 g/L of feed enzymes, or 2 g/t of feed, was developed that enabled sensitive detection of liquid enzyme additions to feeds (Wallace and Hartnell, 2001).

Effect of Enzyme Treatment on Chewing Behavior

Alterations in mechanical processing (Beauchemin and Rode, 1997) and chemical properties (Beauchemin and Buchanan-Smith, 1989; cited by Rode and Beauchemin, 1998) of feeds can significantly alter chewing behavior, and consequently saliva production. Therefore, the use of exogenous fibrolytic enzymes in dairy cow diets may alter feeding behavior and saliva production. Increasing the rate of fermentation within the rumen leads to a decrease in ruminal pH, which can decrease fiber digestion (Russell and Wilson, 1996).

Supplemental fibrolytic enzymes have been shown to increase fiber digestion (Rode et al., 1999; Yang et al., 2000), and ruminal pH has been lowered in some cases (Lewis et al., 1996), but not others (Yang et al., 1999). Thus, applying fibrolytic enzymes to feed before feeding may decrease both chewing time and saliva output and increases the risk of acidosis (Bowman et al., 2003). The latter researchers investigated

the effects of enzyme supplementation on the chewing and feeding behavior, saliva secretion, and ruminal pH in lactating dairy cows fitted with ruminal cannulas. Enzyme supplementation did not alter daily time spent eating or ruminating, but increased saliva production, with no difference among enzyme application treatments. These results indicate that application of this fibrolytic enzyme product did not alter the physical structure of the feed measured by feeding and chewing variables. The increase in total saliva production observed in cows fed enzyme-supplemented diets may be attributed to a physiological response to compensate for the increase in fermentation products produced during digestion.

Beauchemin et al. (2000) evaluated two doses of a fibrolytic enzyme fed to dairy cows in a diet containing 45% forage and 50% concentrate. They observed that the time spent eating each day was similar for cows regardless of diet, even though cows fed the enzyme-treated diets ate more than the cows fed the Control diet. Thus, adding enzyme to the diet decreased the time spent eating per unit of DM, NDF, or ADF, with no difference between the low and high amount of enzyme supplementation. According to the authors, decreased time spent eating per unit of fiber suggests the enzyme mixture may have had a pre-ingestive effect on the feed that enhanced the ease of ingestive mastication, which contradicts the conclusion of Bowman et al. (2003). Beauchemin et al. (2000) also measured rumination activity and did not detect effect of an enzyme supplementation on this variable.

Effect of Enzyme Treatment on the Ruminal Microbial Population

The enzyme activities that exist in the rumen are diverse, and include those that degrade cellulases, xylanases, α -glucanases, pectinases, amylases, proteases, phytases and those that degrade specific plant toxins (e.g., tanninases) (Wang and McAllister,

2002). The variety of enzymes present in the rumen arises from the diversity of the microbial community and the multiplicity of fibrolytic enzymes produced by individual microorganisms. Efficient digestion of complex substrates in the rumen requires the coordinated activities of these enzymes. Limitations to cell wall digestion in the rumen can result from insufficient quantities or types of enzymes produced by ruminal microbes or from an inability of degradative enzyme(s) to interact with target substrates, or from an unconducive environment for optimal enzyme activity (e.g., low ruminal pH) (McAllister et al., 2001)

According to Morgavi et al. (2000), feed enzyme additives used to improve digestion in ruminants interact not only with the feed but also with ruminal microorganisms. These authors reported overall increases in the rumen microbial population due to the addition of an exogenous fibrolytic enzyme to different substrates. However, it is not clear whether this effect was due directly to microbial growth stimulation or indirectly by modifying feed structure. Morgavi et al. (2000) studied the effect of an enzyme preparation from *T. longibrachiatum* (TE) on growth of *F. succinogenes* in a medium containing cellobiose, crystalline cellulose or corn silage fiber. Fiber disappearance and fermentation products were evaluated. The growth rate of *F. succinogenes* on cellobiose was not affected by TE ($P > 0.05$), but growth on cellulose was increased by TE though substrate disappearance and gas production were unaffected. When corn silage fiber was used, the addition of TE increased NDF disappearance ($P < 0.05$) at 24 and 48-h (33 and 52% in Controls versus 53% and 65% in TE treatments, respectively). These results suggest that the *Trichoderma* enzyme preparation did not supply nutrients or growth factors to *F. succinogenes*. *Fibrobacter succinogenes* digests

cellulose efficiently and addition of exogenous cellulases did not further increase cellulose disappearance. However, TE increased corn silage fiber degradation probably by providing an enzyme(s) that limited degradation, but was not secreted by *F. succinogenes*. Enzyme additives have been shown to enhance colonization of feed by ruminal microorganisms and increase the rate of degradation in the rumen (Yang et al., 1999). Morgavi et al. (2000a) found that an enzyme product derived from *Trichoderma longibrachiatum* worked in synergy with ruminal enzymes to release sugars from corn silage, xylan, and cellulose, thereby enhancing ruminal hydrolytic activity.

Nsereko et al. (2000a) supplemented two dairy cow diets with 0, 1, 2, 5 or 10 L of enzyme per ton of DM. Incremental levels of this enzyme stimulated numbers of total viable ruminal bacteria ($P < 0.05$) by 100, 330, 390 and 250% (quadratic effect, $P < 0.05$). Of the rumen bacteria, the most notable increases in numbers were for cellobiose-utilizing ($P < 0.01$), xylanolytic ($P < 0.05$) and amylolytic ($P < 0.05$) subgroups. The numbers of cellulolytic bacteria were unaffected ($P < 0.05$). Increasing concentrations of the enzyme had a convex, quadratic effect on protozoal numbers ($P < 0.05$), and the lower protozoa numbers partially explain the increased number of bacteria. These data suggest that exogenous enzymes can enhance feed digestion at least, in part, by increasing numbers of rumen bacteria that utilize hemicellulose and secondary products of cellulose digestion.

Effect of Enzyme Treatment on Ruminal Fibrolytic Capacity

The inclusion rate of exogenous enzymes in ruminant diets is usually in the range of 0.01 to 1% of the diet, contributing about 10 to 100-times greater fibrolytic activity per gram of feed than when silage additives are used (Christensen, 1997). Based on the estimated average fibrolytic activity normally present in the fluid fraction in the rumen, it

has been estimated that supplemental enzymes may contribute up to 15% of the total fibrolytic activity (Rode and Beauchemin, 1998). However, the activity of commercial enzymes is measured at pH and temperature ranges that generally differ from that of rumen fluid. Thus, once ingested, exogenous enzymes likely contribute considerably less fibrolytic activity than calculated. Furthermore, fibrolytic enzyme activity associated with particulate matter is notably higher than in the ruminal fluid (Wang and McAllister, 2002; Rode and Beauchemin, 1998). This is probably because the attachment of the microbes to the feed particle allows the enzyme to act directly on the substrate, thereby increasing the catalytic action of the enzyme. Thus, the contribution of exogenous enzymes to ruminal fibrolytic activity is difficult to estimate and is probably less than that commonly indicated on commercial enzyme containers.

Colombatto et al. (2003a) observed that enzyme addition to rumen fluid *in vitro* increased ($P < 0.05$) the initial (up to 6-h) xylanase, endoglucanase, and α-D-glucosidase activities in the liquid fraction by an average of 85%. Xylanase and endoglucanase activities in the solid fraction also were increased ($P < 0.05$) indicating an increase in fibrolytic activity by ruminal microbes. Furthermore, incremental addition of enzyme increased ($P < 0.05$) the rate of gas production of various substrates, suggesting that fermentation of cellulose and xylan was enzyme-limited. However, adding the enzyme at levels higher than 2.55 μL/g of DM failed to further increase the rate of gas production, indicating that the maximal level of stimulation was already achieved at lower enzyme concentrations. Authors concluded that enzymes enhanced the fermentation of cellulose and xylan by a combination of pre and post-incubation effects (i.e., an increase in the release of reducing sugars during the pretreatment phase and an increase in the hydrolytic

activity of the liquid and solid fractions of the ruminal fluid), which resulted in a higher rate of fermentation.

Effect of Enzyme Treatment on Fiber Concentration Before Ingestion

Previous findings indicate that application of exogenous fibrolytic enzyme products to diets have pre-ingestive effects. The adsorption of enzyme onto the substrate is an important prerequisite for hydrolysis (Pell and Schofield, 1993). Applying exogenous enzymes directly to feeds releases reducing sugars (Hristov et al., 1998), and in some cases, partially solubilizes NDF and ADF (Krause et al., 1998). Colombatto et al. (2003a) evaluated the effects of adding a commercial enzyme product on the hydrolysis and fermentation of cellulose, xylan, and a mixture of both substrates. They reported that addition of enzyme in the absence of ruminal fluid increased ($P < 0.01$) the release of reducing sugars from xylan and the mixture after 20 h of incubation at 20°C. Hydrolysis of the fiber pre-feeding may indicate a modification of the plant cell wall structure, which could decrease the physical effectiveness of the fiber in the diet. When inadequate effective fiber is fed, chewing activity decreases, which leads to less salivary buffer secretion, resulting in a more acidic ruminal pH, altered ruminal fermentation patterns and low ratios of acetate to propionate that ultimately result in modified animal metabolism and reduced milk fat synthesis (Mertens, 1997).

Recent studies also have shown that enzyme preparations containing high ferulic acid esterase activity as well as xylanase and cellulase activity reduced the NDF and ADF concentrations and increased the digestion of hays made from 12-week regrowth of Tifton 85 bermudagrass, Coastal bermudagrass and Pensacola bahiagrass (Krueger et al., 2003). This enzyme also increased the rate and extent of *in situ* degradation of the forages and reduced the lag time before forage degradation commenced (Krueger et al.,

2004). These studies suggest that enzyme treatment can improve the nutritive value of tropical grasses.

Sheperd and Kung (1996b) observed that applying an enzyme additive containing cellulase and hemicellulase reduced NDF and ADF concentration of corn silage during the ensiling period. However, Mandevbu et al. (1999) observed that treatment of bermudagrass forages harvested after 3 or 6 wk regrowth periods with a mixture of cellulase, amylase and hemicellulase had no effect on silage fiber concentration or cell wall carbohydrate fraction. This discrepancy is probably due to differences in activity of the enzyme products used in both experiments and to differences in cell wall components of corn and bermudagrass silage.

Effect of Enzyme Treatment on DM and Fiber Digestibility Post Ingestion

There is increasing evidence that exogenous fibrolytic enzymes improve fiber digestion within the rumen, thereby increasing feed utilization in ruminants (Lewis, 1999; Rode et al., 1999). According to Beauchemin et al. (2003) the focus of most enzyme-related research for ruminants has been on plant cell-wall degrading enzymes. Cellulose and hemicellulose, the major structural polysaccharides in plants (Van Soest, 1994), are converted to soluble sugars by enzymes collectively referred to as cellulases and hemicellulases.

More than thirty years ago, various studies showed significant improvements in average daily gain (ADG) and feed conversion rate (FCR) of cattle when fed diets supplemented with enzymes containing amylolytic, proteolytic and cellulolytic activities (Rode and Beauchemin, 1998). Improvements in animal performance were due to increased DM and fiber digestibility. Christensen (1997) found an increase ($P < 0.05$) in DM digestibility when fibrolytic enzymes were added to rations of steers at feeding time

or 24 h prior to feeding. The NDF and ADF digestibilities of the rations increased numerically by approximately three percentage units. In the same study, a positive effect on rumen degradation of forage was observed, when a mixed cellulase, hemicellulase and pectinase enzyme additive was applied to alfalfa hay, grass hay, oat straw and barley silages at different rates. Christensen (1997) also observed that application of 600 IU/kg DM of xylanase had a positive effect on both *in vitro* and *in situ* degradation of both high-fiber and low-fiber forages.

Applying fibrolytic enzymes prior to feeding can alter the structure of the cell wall, thereby making it more amenable to degradation (Beauchemin et al., 2004b). Nsereko et al. (2000b) applied an enzyme product to alfalfa hay that was then autoclaved to inactivate enzyme activities and washed to remove any product of the hydrolysis, eliminating the possibility of chemotactic enhancement of digestion or synergy between microbial enzymes and exogenous enzymes. *In vitro* NDF digestibility was higher at 12 and 48-h for treated than for untreated hay and generally this effect was enhanced by longer pre-incubation with enzymes. Since these effects were observed in the absence of active ruminal enzymes and soluble hydrolysis products, the exogenous enzymes probably caused structural changes to the forages that improved digestion.

Rode et al. (1999) evaluated the effect of exogenous fibrolytic enzyme (Promote®) on DMI and digestibility in cows fed Control diets or diets in which an enzyme was added to the concentrate at a rate of 1.3 g/kg (DM basis). Enzyme addition did not affect DMI. However, total tract digestibility of nutrients as determined using Cr_2O_3, was increased by enzyme treatment (DM, 61.7 vs. 69.1%; NDF, 42.5 vs. 51.0%; ADF, 31.7 vs. 41.9%; CP, 61.7 vs. 69.8%). Nevertheless, effects of supplemental enzymes on

digestibility have been inconsistent. Enzyme products comprised mainly of xylanases and cellulases have increased digestibility (Rode et al., 1999; Yang et al., 2000), or not affected digestibility (Lewis et al., 1999). Other studies have shown that exogenous enzymes did not consistently improve animal performance, and the mechanism for improved growth was not always confirmed by digestibility trials (Rode and Beauchemin, 1998). Mandevbu et al. (1999) observed that treatment of bermudagrass forages with fibrolytic enzymes had no effect on *in vitro* or *in situ* DM or NDF disappearance of silages. Hristov et al. (2000) observed that the ruminally soluble fraction and effective degradability of feed DM *in situ* were increased (quadratic response, $P < 0.01$) by enzyme treatment in ruminally cannulated heifers, but apparent digestibility of DM, CP, and NDF were not affected.

Effect of Enzyme Treatment on Silage Fermentation

Applying cell-wall degrading enzymes during the ensiling process can increase the release of fermentable sugars from the structural polysaccharides thereby providing extra substrate for the microbial fermentation. This often increases the production of lactic acid, which reduces the risk of clostridial fermentation (Van Vuuren et al., 1989). When used as silage additives, fibrolytic enzymes predigest plant cell walls and this can increase the extent and rate of degradation of silage in the rumen, and consequently, improve digestibility and nutritive value (McHan, 1986). Rodrigues et al. (2001) reported that application of a mixture of cellulase and endoxylanase to ryegrass before ensiling reduced NDF, ADF and acetic acid ($P < 0.01$) concentration and increased lactic acid and sugar concentration ($P < 0.01$). Similar results were obtained by Clavero and Razz (2002) with dwarf elephantgrass (*Pennisetum purpureum*) silage treated with a cellulase mixture. Selmerolsen (1993) showed that the fermentation of crops with low

sugar concentration, such as tropical grasses, was improved more by enzyme addition, while that of crops with high sugar concentration were improved more by lactic acid bacteria inoculation. In agreement, recent results also have shown that treatment of bermudagrass, which is low in sugars, with fibrolytic enzymes alone or with an enzyme-inoculant blend (Adesogan et al., 2004) improved the fermentation, but contradictory results exist (Mandevbu et al., 1999). Clearly enzyme application at ensiling to forage containing low sugar contents is logical because of potential sugar release from enzyme-induced fibrolysis, but the response depends on the enzyme activities and treatment conditions (Adesogan, 2005).

Kung and Ranjit (2001) compared whole-plant barley treated with *L. buchneri* and enzymes, or a mixture of *L. plantarum, P. pentosaceus, P. freudenreichii* and enzymes or a buffered propionic acid-based additive. They observed that silages treated with *L. buchneri* and enzymes had lower pH and higher concentrations of acetic and propionic acids and improved aerobic stability when compared with untreated silage. These results indicate that enzymatic treatments can represent a viable strategy for improving the quality of silages, though they don't directly affect aerobic stability.

Effect of Enzyme Treatment on Hay Nutritive Value

Direct effects of fibrolytic enzyme treatment on chemical composition of treated hays before ingestion have not been studied previously. Instead, research has focused on the effect of such enzymes on ruminal fermentation or voluntary intake of the treated hays. Pinos-Rodriguez et al. (2002) observed that application of an exogenous fibrolytic enzyme to alfalfa or ryegrass hays increased intake of DM ($P < 0.01$), OM and CP ($P < 0.05$) in lambs; however, NDF and ADF intake were not affected. The enzyme increased apparent digestibility of CP, hemicellulose ($P < 0.05$), and NDF ($P < 0.10$) in alfalfa. The

enzyme also improved N balance ($P < 0.05$), and total VFA ($P < 0.05$) concentration in the rumen (after 3 and 6-h of incubation) for both hays. According to the authors, these results indicate that directly fed exogenous fibrolytic enzymes may change ruminal fermentation, intake, and digestibility of forages. Dawson and Tricarico (1999) showed that when fescue hay was not treated or treated with preparations high in xylanase or cellulase activity, xylanase addition increased carbohydrate utilization and VFA production, cellulase addition altered VFA proportions, and addition of a mixture of the enzymes increased carbohydrate digestion and the acetate: propionate ratio.

Novak et al. (2003) evaluated the effect of a fibrolytic enzyme containing carboxymethyl cellulase and xylanase on ruminal disappearance of DM, NDF and ADF, and intestinal DM digestibility of wheat straw. Enzyme addition had no effect ($P >0.05$) on the effective degradability of DM, NDF and ADF, but increased DM, NDF and ADF disappearance after 4 and 6-hours of incubation and decreased these measures after incubation for 12 and 24 hours. Differences in enzyme activity and stability in rumen fluid, application methods, and characteristics of rumen fluid due to donor animal diet partly explain the discrepancies between these studies. The conflicting results highlight the merits of further evaluation of the benefits of fibrolytic enzyme application to hays.

Effect of Enzyme Treatment on Animal Performance

Dry matter intake has been increased (Beauchemin et al., 2000) or unchanged (Beauchemin et al., 1999; Kung et al., 2000) by dietary supplementation with enzymes. Feed intake responses to enzyme supplementation have generally been small and inconsistent (Yang et al., 1999; Rode et al., 999; Schingoethe et al., 1999; Vicini et al., 2003) with only occasional significant ($P < 0.05$) increases (Lewis et al., 1999). Attempts to improve feed efficiency in dairy cows by the use of direct-fed fibrolytic

enzymes applied at or a few hours before feeding have yielded variable production responses (Sutton et al., 2003).

Milk production also has been increased in some studies in which dairy cow diets were supplemented with fibrolytic enzymes (Rode et al., 1999; Yang et al., 2000; Kung et al., 2002), but it has been unaffected in other studies (Sheperd and Kung, 1996b; Beauchemin et al., 2000; Vicini et al., 2003). Table 2.2 summarizes the results of supplementation with fibrolytic enzymes on milk production from several experiments. Milk yield responses have been generally positive but often not significant, while changes in milk fat and protein concentration have been both positive and negative and are often not significant (Beauchemin et al., 1999; Lewis et al., 1999; Schingoethe et al., 1999; Yang et al., 1999; 2000; Kung et al., 2000; Phipps et al., 2000; Rode et al., 1999; Vicini et al., 2003). Rode et al. (1999) used lactating Holstein cows in early lactation to investigate effects of exogenous fibrolytic enzyme (Promote®) supplementation on DMI, milk production and digestibility. Enzyme addition did not affect DMI ($P > 0.05$) but tended ($P < 0.1$) to increase milk yield (35.9 vs. 39.5 kg/d) as a consequence of increased digestibility. Percentage of milk fat was lower ($P < 0.05$) and percentage of milk protein tended to be lower ($P < 0.1$) in cows fed the enzyme-supplemented diet, such that component yields were similar ($P > 0.05$) for cows fed either diet. Energy deficiency was numerically lower ($P > 0.05$) for cows fed the enzyme-supplemented diet than for cows fed the Control diet (-3.33 vs. -3.62 Mcal/d). Consequently, the authors concluded that supplementing dairy cow diets with Promote has the potential to enhance milk yield and nutrient digestibility by cows in early lactation without changing feed intake.

Table 2.2 Effect of spraying enzymes onto feeds prior to feeding on milk production in recent studies

Study	Increase in milk production[1], kg/d	Dietary forage type	P values
Beauchemin et al., 1999	+0.3, +1.5	Barley silage + alfalfa haylage (45%)	NS
Lewis et al., 1999	+1.2, +6.3, +1.6	Alfalfa hay + alfalfa silage (41.6%)	< 0.05
Rode et al., 1999	+3.6	Corn silage + alfalfa hay (38.5%)	< 0.11
Schingoethe et al., 1999	Expt. 1: + 1.2, + 0.9, + 2.7 Expt. 2: + 1.3	Corn silage + alfalfa hay (55%)	NS < 0.01
Yang et al., 1999	+ 0.9, + 1.9, + 1.6	Barley silage + alfalfa cubes (52.8%)	<0.05
Beauchemin et al., 2000	- 0.5, - 0.5	Barley silage + alfalfa haylage (45%)	NS
Kung et al., 2000	Expt. 1: + 2.5, -0.8 Expt. 2: + 0.7, + 2.5	Corn silage + alfalfa hay (45%)	< 0.10 < 0.10
Yang et al., 2000	+ 0.1, + 2.1	Corn silage + alfalfa hay (38%)	< 0.05
Zheng et al., 2000	+ 2.0, + 4.1, + 1.5	Corn silage + alfalfa hay (50%)	< 0.07
Bowman et al., 2002	+ 0.6, - 0.6, -1.5	Barley silage + alfalfa silage (55%)	<0.10
Knowlton et al., 2002	+ 1.8, - 1.2	Corn silage + alfalfa silage (53%)	NS

[1.] The increase in milk is relative to milk production by Control cows, NS: no significant effect

Studies have shown that application of low or high amounts of enzymes to forages or diets produced different responses. Yang et al. (1999) examined the effect of two doses of a cellulase-xylanase enzyme mixture applied to the forage or concentrate component of dairy cow diets. They observed that milk production increased in cows fed a high dosage of the enzyme compared with cows fed the Control diet, but effects on milk composition were minimal. The response to enzyme supplementation was affected more by amount of enzyme applied than by the dietary component treated with the enzyme. The authors claimed that the results demonstrated the benefits of using a fibrolytic enzyme to enhance feed digestion and milk production by dairy cows.

However, Beauchemin et al. (2000) found that a high level of enzyme application was less effective than a low level at increasing total tract digestibility.

Lewis et al. (1999) carried out two experiments to evaluate the effectiveness of adding a mixture of cellulases and xylanases to dairy cow diets. In Experiment 1, cows were assigned to diets containing forages that had or had not been treated with the enzyme between 8 and 24 h prior to feeding. They observed that cows consuming the enzyme-treated forage produced more milk (27.2 vs. 25.9 kg/d, $P < 0.05$) and digested more DM per day than did cows fed the Control forage. In Experiment 2, early lactation cows were assigned to one of four treatments for 16 wk: 1) no enzyme treatment, 2) a low (1.25 ml/kg of forage DM) enzyme treatment, 3) a medium (2.5 ml/kg of forage DM) enzyme treatment, or 4) a high (5.0 ml/ kg of forage DM) enzyme treatment. Dry matter intake was similar across enzyme treatments and intake was greater than for cows fed the Control forage. Yield of milk, 3.5% fat-corrected milk, and energy-corrected milk were greater by cows on Treatment 3 than by cows on Treatment 1. Therefore, applying fibrolytic enzymes to the forage portion of the rations improved lactational performance of early and mid-lactation cows.

Schingoethe et al. (1999) evaluated the response to a direct-fed (applied at feeding time) cellulase and xylanase enzyme mixture applied at 0, 0.7, 1.0 or 1.5 L/ton of DM to the forage portion (60% corn silage and 40% alfalfa hay) of a TMR for lactating cows just prior to feeding. Over the 12-wk trial period, milk production from cows assigned to the 1.5-L enzyme treatment increased by 10.8% relative to those in the Control (no enzyme addition) group, while fat and protein production increased by 20 and 13%, respectively. The lowest enzyme rate accounted for approximately one-half of the milk

production increases that occurred with the highest enzyme application rate. The responses to enzyme-treated forages were initially evident 2 to 4 wk after experiment started and they were maintained throughout the remainder of the experiment. Cows that started to receive enzyme-treated forage during the first 100 d postpartum produced 9 to 15% more milk and 16 to 23% more energy-corrected milk than did cows fed the Control diet. However, milk production was not increased when cows were in mid-lactation (121 d postpartum) at the start of the experiment (Schingoethe et al., 1999).

The reason for the general poor response to low levels of enzyme application is obvious, but a lack of benefit for the high levels is less apparent. Such occurrences may be attributed partly to negative feedback inhibition which is one of the classical modes of regulation of enzyme action (Adesogan, 2005). This feedback mechanism occurs when enzyme action is inhibited by production of a critical concentration of a product of the enzyme-substrate interaction. For instance, fermentation of sugars produced by cell wall hydrolysis may reduce ruminal pH to levels that inhibit cell wall digestion (Adesogan, 2005) by the negative effect of low pH on ruminal fibrolytic microorganisms. An alternative hypothesis is that excessive enzyme application blocks the binding sites for enzymes or may prevent substrate colonization (Beauchemin et al., 2003).

Sutton et al. (2003) used multiparous cows fitted with rumen and proximal duodenal cannulas in early lactation to investigate the effect of method of application of a fibrolytic enzyme product on digestive processes and milk production. The enzyme was not applied (Control), sprayed on the TMR before the morning feed (TMR-E), or on the concentrate the day before feeding (Conc-E), or infused into the rumen for 14 h/d (Rumen-E). There was no treatment effect on either feed intake or milk yield but values

were numerically higher in cows fed TMR-E than in the rest of the cows. Ruminal digestibility of DM, OM and starch were unaffected by the enzyme. Ruminal NDF digestibility was lowest in cows fed TMR-E, but these cows also had the greatest post-ruminal NDF digestibility. Total tract digestibility of starch, DM and OM were highest in cows fed TMR-E. Ruminal retention time was reduced by all enzyme treatments but postruminal transit time was increased so the decline in total tract retention time with enzymes was not significant. It was suggested that the reduction in ruminal particle retention time would reduce time available for fibrolysis to occur; and therefore, partly explain the variability in the reported responses to enzyme treatment.

Bowman et al. (2002) also investigated the effect on dairy cows (averaged 111 ± 32 DIM) of a fibrolytic enzyme (Promote®) added at 1.0 g/cow/d to the concentrate portion (45% of the dietary DM) of the TMR, to the pelleted supplement portion (4% of the dietary DM) of the TMR, or to a premix (0.2 % of the dietary DM). The effects of enzyme supplementation on milk production and composition were not significant ($P > 0.05$), but cows receiving the enzyme-supplemented concentrate had numerically higher FCM compared to the Control cows. Knowlton et al. (2002) evaluated the effect of a fibrolytic enzyme formulation on the intake, partitioning, and excretion of N and P by dairy cows in early and late lactation. Cows fed diets containing the enzyme formulation gained more weight than those fed the enzyme-free diet, particularly in early lactation. Enzyme treatment did not affect apparent digestibility, excretion of N and P, or retention of these nutrients in body tissues. Interactions observed between the effects of stage of lactation and treatment indicated that the nature of the milk yield and manure excretion responses differed between early and late-lactation cows. Milk yield, fecal output and N

excretion in cows fed enzyme-supplemented diets were greater than those of Control cows in early lactation, but lesser in late-lactation. Energy requirements of early lactation cows are higher that those in late lactation; therefore, the enzyme supplementation in early lactation is potentially more promising than in mid-lactation or late-lactation because it can improve energy balance (Jurkovich et al., 2002).

Effect of Enzyme Treatment on Blood Metabolites

Urea is the primary form of excretory N in mammals, and greater concentrations of blood urea N (BUN) have long been known to reflect inefficient utilization of dietary CP by ruminants (Broderick and Clayton, 1997). Few papers have reported the effect of fibrolytic enzymes on blood metabolites. Hristov et al. (1998) found that blood glucose and urea concentration in lactating dairy cows were not affected by enzyme treatments. Hristov et al. (2000) observed that plasma beta hydroxybutyrate (BHBA) concentration was reduced ($P < 0.01$) in cows supplemented with fibrolytic enzymes. Jurkovich et al. (2002) also found a lower incidence of hyperketonaemia and lower acetoacetic acid and non-esterified fatty acid (NEFA) concentrations in the blood of cows supplemented with a mixture of fibrolytic enzymes, which indicates that enzyme supplementation can improve energy balance in lactating cows.

Effects of Combining Enzyme and Chemical Treatments

Though chemical treatments also have been successfully used to disrupt ferulate bridges and hydrolyze cell walls in tropical forages, little is known about the effectiveness of biological treatment at achieving these objectives. Opportunities exist to improve overall utilization of lignocellulosic materials as ruminant feeds by using organisms or their secreted enzymes with the capacity to attack the most refractory fiber components that have lignin-carbohydrate bonds (Varga and Kolver, 1997). The

prospects for improved use of fibrous residues relies on enhancing the rate of fermentation of the more readily fermented cell wall constituents and increasing the extent of digestion of poorly degraded constituents.

Combinations of chemical and biological treatments have been applied to low quality forages, and the results show that they can act synergistically for improving the nutritive value of such roughages. Wang et al. (2004) carried out four experiments to study the effects of pre-treating wheat straw with alkali (5% of NaOH, wt/wt, or 3%, wt/wt of NH_3) and then spraying it with an enzyme mixture (xylanase, ß-glucanase, carboxymethylcellulase, and amylase) on *in vitro, in situ,* and *in vivo* digestibility. In Experiment 1 enzymes increased ($P < 0.01$) gas production and the incorporation of ^{15}N into microbial N at 4 h from NaOH-treated wheat straw ($P < 0.01$ for gas; $P < 0.05$ for ^{15}N) compared to untreated wheat straw. In Experiment 2, untreated and alkali-treated wheat straw were sprayed with enzymes at 0, 0.15, or 1.5 mg/g DM and incubated ruminally in nylon bags for up to 80 h to determine the *in situ* DM disappearance (ISDMD). Interactive effects ($P < 0.05$) of pretreatment and enzymes were observed on all ruminal degradation parameters. Alkali increased the rate ($P < 0.01$) and extent ($P < 0.01$) of ISDMD irrespective of enzymes. Enzyme application to untreated straw did not affect the extent of ISDMD, but increased ($P < 0.01$) that of alkali-treated straw.

In Experiment 3, substrates from Experiments 1 and 2 were incubated in acetate buffer for 24 h to measure the hydrolytic loss of DM and release of reducing sugars and phenolic compounds. Alkali pretreatment and enzymes each increased ($P < 0.01$) DM loss and the release of reducing sugars, and in combination, exerted additive effects ($P < 0.01$). Enzymes did not affect the release of phenolic compounds from the straw. In

Experiment 4, wrapped straw bales were injected with NH_3 four months before the study, and enzymes were applied immediately before feeding. Applying enzymes to ammoniated straw increased ($P < 0.05$) digestibility of DM, OM, and total N but did not affect the intake of DM or digestibility of ADF by crossbred beef cows in late gestation. According to the authors, pretreatment of straw with alkali enhanced the efficacy of exogenous enzymes, presumably by breaking esterified bonds and releasing phenolic compounds and/or by swelling the crystalline cellulose and enhancing enzyme penetration. Adogla-Bessa et al. (1999) also found that adding urea and fibrolytic enzymes to wheat silage was more effective than either treatment alone. However, using enzymes and chemicals for forage improvement is probably not economically viable. Including enzymes that mimic alkali hydrolysis (e.g., esterases) in commercial feed additives could improve substantially the effectiveness of enzyme products for ruminants.

The conflicting results on the effectiveness of fibrolytic enzymes for enhancing forage nutritive value and animal performance highlight the need for more concerted investigation of this subject. The fact that even less is known about the extent to which enzymes can improve the quality of tropical forages and enhance animal performance from such forages, emphasizes the importance of future studies in this area.

The aim of this series of experiments was to evaluate the effect of ammoniation and proprietary fibrolytic enzyme application on the nutritive value of tropical grasses and on animal performance.

The specific objectives were:

To evaluate the effect of applying ammonia or four commercial fibrolytic enzymes on the nutritive value of two C_4 grass hays (Chapter 3).

To evaluate the effect of applying different rates of four proprietary fibrolytic enzyme preparations at different rates, at ensiling, on the nutritive value of Tifton-85 bermudagrass silage (Chapter 4).

To determine the effects of applying an enzyme to bermudagrass at ensiling, or to different components of the diet at feeding 8 in feed intake, milk production and composition, blood metabolites and digestion kinetics of dairy cows (Chapter 5).

CHAPTER 3
EFFECT OF TREATMENT WITH AMMONIA OR FIBROLYTIC ENZYMES ON THE NUTRITIVE VALUE OF HAYS PRODUCED FROM TROPICAL GRASSES

Introduction

Feed enzymes have been shown to be effective in a wide range of diets containing roughages (Rode and Beauchemin, 1998). Their effectiveness is partly due to improved hydrolysis of the fiber fraction (Colombatto et al., 2003b) which increases digestibility (Christensen, 1997; Rode et al., 1999) and voluntary intake (Pinos-Rodriguez et al., 2002). Nevertheless, other studies have shown that exogenous enzymes do not consistently improve forage utilization. This inconsistency is attributable to factors such as differences in enzyme type and activity, treatment duration, application method, diet composition and level of animal performance.

Fibrolytic enzymes seem to work by increasing the rate, but not the extent of fiber digestion (Feng et al., 1996; Yang et al., 1999). This suggests that the fibrolytic enzyme products currently on the market for ruminants may not be introducing novel enzyme activities into the rumen (Wang and McAllister, 2002). One of the few studies on fibrolytic enzyme treatment of tropical grasses showed that enzyme treatment had no effect on silage fiber concentration, cell wall carbohydrate fraction and *in vitro* or *in situ* DM or NDF disappearance of silages (Mandevbu et al., 1999). Yet due to the widespread use of C_4 grasses which intrinsically have low nutritive values, it is important to determine if modifying treatment conditions will lead to enzyme-mediated enhancements in their quality.

Ammoniation is one of the most studied chemical treatments for improving forage quality. Ammoniation improves forage digestibility due to the hydrolytic action of the ammonia on linkages between lignin and structural polysaccharides, thus increasing the organic matter potentially available for utilization by the ruminal microorganisms (Barrios and Ventura, 2002). Ammoniation also increases the crude protein (CP) concentration of the treated forages, and this improvement is through fixation of the applied nitrogen (Weiss and Underwood, 1995). The objective of this experiment was to evaluate the effect of applying ammonia or four commercial fibrolytic enzymes on the nutritive value of two C_4 grass hays.

Materials and Methods

Enzyme Application

In the first of two experiments, the effects of applying NH_3 or a fibrolytic enzyme complex (Promote®, Pr) (Cargill, Minnetonka, MN) were measured on the DM and chemical composition and *in vitro* and *in situ* digestibility of two tropical grass hays. The forages tested were 12-week regrowth of Coastal bermudagrass hay (*Cynodon dactylon*) (BE) and Pensacola bahiagrass hay (*Paspalum notatum*) (BA). The ammonia was applied at 40 g/kg DM and the enzymes were applied at 0 (Control), 0.5, 1 and 2 times the rates recommended by the respective manufacturers. This was done because the optimal application rate for C_4 grass hays was unknown. The actual application rates are shown in Table 3.1. The enzymes were dissolved in 500 ml of water and applied in a fine spray to 3 replicates of 2 kg of each hay. Treated hays were stored for 3 weeks in plastic bags (30 bags for Experiment 1 and 66 bags for Experiment 2) and then chemically characterized. The manufacturer-stipulated activities of the enzymes are shown in Table 3.2. Cellulase activity was also determined at 39^{o}C and pH 5.5 using the filter paper

method (Wood and Bhat, 1988) and the values obtained for Pr, X-20, CA and A-20 were 33.7, 22, 0 and 51.3 filter paper units/g, respectively, where one unit of activity is the amount of enzyme that releases exactly 2 mg of glucose from 50 g of filter paper in 60 min. Xylanase activity was determined at 39^{o}C and pH 5.5 using the di-nitro salicylic acid procedure (Bailey et al., 1992) and the values obtained for Pr, X-20, CA and A-20 were 5190, 7025, 0 and 3530 μmol of xylose released/min/ml, respectively. In the second experiment, the effects of applying NH_3 or three fibrolytic enzymes were measured on the same variables as in the previous experiment. The enzymes studied in Experiment 2 were Biocellulase X-20® (X-20) (LodeStar, IL, USA), Cattle-Ase® (CA) (Loveland Industries Inc, Greeley, CO, USA) and Biocellulase A-20® (A-20) (LodeStar, IL, USA). Two separate experiments were conducted because the enzymes were not simultaneously available.

Laboratory Analysis

The NDF and ADF concentrations (Van Soest et al., 1991) of the samples and digested residues were determined without amylase pretreatment using an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY). Hemicellulose was calculated by difference from NDF and ADF concentrations. Water soluble carbohydrates (WSC) were determined with the anthrone reaction assay (Ministry of Agriculture Fisheries and Food, 1986). Crude protein (CP) was determined by digesting 0.5 g of sample using a micro Kjeldahl apparatus (Labconco Corporation, Kansas City, MO) and the N concentration was determined (Noel and Hambleton, 1976) using a Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA).

Table 3.1 Actual enzyme application rates used

Enzyme	Application rate		
	0.5x	1x	2x
Promote[1] (mg/kg DM)	650	1300	2600
Biocellulase X-20[2] (mg/kg DM)	7.3	14.5	29
Biocellulase A-20[2] (mg/kg DM)	7.3	14.5	29
Cattle-Ase[3] (mg/kg DM)	89	178	356

[1] Cargill, Minnentoka, MN
[2] LodeStar, Channahon, IL, USA
[3] Loveland Industries Inc, Greeley, CO, USA

Table 3.2 Manufacturer-stipulated enzyme activities.

Enzyme	Enzymatic activity			
	Cellulase (Units/g)	Xylanase (Units/g)	B-Glucanase (Units/g)	Amylase (Units/g)
Promote[1]	1,200	-	-	-
Biocellulase X-20[2]	5,700	16,000	600	1,200
Biocellulase A-20[2]	6,000	400	4,300	3,100
Cattle-Ase[3]	15,000	-	-	-

[1] Cargill, Minnentoka, MN, USA
[2] LodeStar, Channahon, IL, USA
[3] Loveland Industries Inc, Greeley, CO, USA

The *in vitro* digestibility of DM (IVDMD), NDF (IVNDFD) and ADF (IVADFD) were determined in duplicate runs after incubating forage samples in buffered rumen fluid for 6 or 48-h using two ANKOM^{II} Daisy Incubators (ANKOM Technology, Macedon, NY). The buffer was prepared according to the ANKOM Technology procedure. The rumen fluid was obtained before feeding from two, non-lactating, fistulated cows, fed 9 kg of Coastal bermudagrass hay and 400 g of soybean (*Glycine max*) meal daily.

In situ rumen degradability was measured only in hays treated with NH_3, X-20 and A-20, because these treatments were found to be more effective at increasing *in vitro* digestibility than the others. Five g of ground (4 mm screen) hay samples were weighed into nylon bags (50 µm pore size) in triplicate and placed into the two fistulated, non-

lactating Holstein cows for 0, 3, 6, 9, 12, 24, 48, 72, 96, and 120 h. At each incubation time, bags were removed and rinsed with cool water and frozen. At the end of each period, all bags were washed in a washing machine and dried for 48-h at 60 ºC. The cows used for this study were the same as those used as rumen fluid donors for the *in vitro* study. In order to avoid placing too many substrate-filled bags in the rumen, only bags for 3 treatments (Control, ammonia and X-20 or A-20) and one forage were simultaneously incubated (60 bags maximum incubated at the same time/cow).

Statistical Analysis

A completely randomized design with 3 replicates per treatment was used to quantify the effects of enzyme or NH_3 application on chemical components.

The model used was:

Yijk: $\mu + Ti + Ei$

Where:

Yij: dependent variable

μ: general mean

Ti: treatment effect (enzyme*level) and NH_3

Ei: experimental error

A completely randomized design with 3 replicates per treatment was used to quantify the effects of enzyme or NH_3 application on digestibility after 6-h and 48-h. Data from 6-h and 48-h incubations were analyzed separately.

The model used was:

Yijk: $\mu + Ti + Rj + Eij$

Where:

Yij: dependent variable

μ: general mean

Ti: treatment effect (enzyme*level) and NH_3

Rj: run effect

Eij: experimental error

Data were analyzed using the GLM procedure of SAS (1995). Orthogonal contrasts were used to compare additive treatment means, and polynomial contrasts were used to determine the effect (linear, quadratic and cubic) of increasing the amount of enzyme application. Treatment significance was declared at the 5% level and tendencies were declared at the 15% level.

The interaction treatment * forage was not included in the previous models because the *in vitro* and *in situ* trial were done separately.

The *in situ* ruminal degradation parameters were estimated using the model described by McDonald (1981):

$$P= a + b (1-e^{-c (t-L)})$$

where

P = DM degraded at time t, a = wash fraction, b = potentially degradable fraction, a+b= total degradable fraction, c = the rate at which b is degraded, t = time incubated in the rumen, and L = lag phase. The constants a, b, c, and L were estimated using the nonlinear regression (NLIN) procedure of SAS (1995) and analyzed using the GLM procedure of SAS (1995).

Results and Discussion

Chemical Composition of Tropical Hays

The chemical composition of untreated bermudagrass and bahiagrass hays is shown in Table 3.3. The low CP and high NDF, ADF, hemicellulose and lignin

concentrations are typical of mature tropical grasses. These values agree with Jung and Allen (1995) who concluded, that depending on the stage of maturity, cell walls represent between 30 and 80% of plant DM in grasses so that under most circumstances, the bulk of carbohydrates in mature grasses are from cell wall polysaccharides.

Effect of Promote and Ammoniation on Chemical Composition in Experiment 1

Enzyme treatment increased ($P < 0.05$) the NDF concentration of BE hay, suggesting that Pr also contained non-fibrolytic enzymes (Table 3.4). However, the NDF concentration of BA was decreased ($P < 0.01$) by both Pr and NH_3 treatment, though NH_3 treatment was more effective ($P < 0.01$) at hydrolyzing the NDF fraction than Pr. Bahiagrass hay had a lower ($P < 0.01$) concentration of ADF than BE. The ADF concentration of BE was decreased ($P < 0.01$) by NH_3 and Pr (linear response) treatments, but that of BA was not. Promote tended ($P = 0.052$) to be more effective at hydrolyzing the ADF of BE than NH_3. The hemicellulose concentration of BE was increased ($P < 0.01$) by enzyme (quadratic response) and NH_3 treatment, whereas that of BA was decreased ($P < 0.01$) by NH_3 treatment.

The WSC concentration of BA was increased ($P < 0.01$) by Pr and NH_3 treatment ($P < 0.01$) and NH_3 was more effective in this respect. Both treatments also numerically ($P > 0.05$) increased the WSC concentration of BE. The higher WSC concentration observed in the NH_3-treated BA hay compared to other hays, shows that the chemical treatment was more effective at hydrolyzing the fiber fraction of this forage. Enzyme treatment did not affect the CP concentration of either of the hays; however NH_3 treatment produced greater ($P < 0.01$) values than those of Control and enzyme-treated hays.

Table 3.3 Chemical composition of the untreated hays

	Forage	
Nutrient	Bahiagrass	Bermudagrass
CP, g/kg DM	66	69
NDF, g/kg DM	792	821
ADF, g/kg DM	431	485
Lignin, g/kg DM	55	67
Hemicellulose, g/kg DM	336	354
Ash, g/kg DM	57	50

CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber

These results indicate that the treatments, particularly NH_3, had different effects on cell wall components of the forages and forage type affected the response. In BE, total cell wall concentration was not reduced by additive treatment but the NDF fraction was hydrolyzed by other additives, thus increasing the ADF. Whereas in BA, the ADF fraction was unaffected by treatment, but NDF and hemicellulose fractions were hydrolyzed into sugars, which is in agreement with Colombatto et al. (2003), and Hristov et al. (1998). Promote treatment was less effective than NH_3 treatment at cell wall hydrolysis. This result agrees with those of Brown (1993), who observed that ammoniation decreased ($P < 0.01$) the NDF concentration of stargrass (*Cynodon nlemfuensis*) hay.

Effect of Promote and Ammonia Application on *in vitro* DM, NDF, and ADF Digestibility in Experiment 1

After 6-h of digestion, BA had greater ($P < 0.01$) IVDMD than BE, but this difference was no longer evident after 48-h of digestion. Promote-treatment increased ($P < 0.01$) the 6-h IVDMD of BE; however, ammoniation was more effective (Table 3.5). Only NH_3 treatment increased ($P < 0.01$) the 6-h IVDMD of BA or the 48-h IVDMD of both forages. The effectiveness of NH_3 at increasing the 6-h and 48-h digestibility of the hays concurs with the results of Zorrila-Rios et al. (1991), who observed that the

Table 3.4 Effect of Promote or ammonia treatment on chemical composition (% DM) of tropical grass hays

		NDF		ADF		Hemicellulose		WSC		CP	
Additive	Level	BE	BA	BE	BA	BE	BA	BE	BA	BE	BA
Control		82.1	79.2	48.5	43.1	33.6	35.4	2.0	0.9	6.6	6.9
Promote	0.5x	85.2	78.1	46.7	43.8	38.4	35.0	2.6	4.1	6.7	6.7
	1x	85.1	78.1	46.8	42.6	38.3	34.4	2.5	3.8	6.4	6.8
	2x	86.2	78.5	44.5	44.3	41.7	34.2	2.6	3.2	6.4	7.1
	Mean	85.5	78.2	46.0	43.6	39.5	34.5	2.6	3.7	6.5	6.9
Ammonia		83.6	75.4	46.7	43.7	37.0	31.7	2.7	4.9	17.1	12.8
s.e.m.		0.964	0.354	0.293	0.491	0.872	0.737	0.039	0.019	0.258	0.139
Contrasts		*P* values									
Polynomial effects											
Promote level		NS	NS	L**	NS	L*	NS	NS	C*	NS	NS
Promote vs. Control		0.015	<0.01	<0.01	0.452	<0.01	0.339	0.232	<0.01	0.477	0.285
Control vs. Ammonia		0.289	<0.01	<0.01	0.962	0.021	<0.01	0.234	<0.01	<0.01	<0.01
Promote vs. Ammonia		0.128	<0.01	0.052	0.485	0.593	0.098	0.785	<0.01	<0.01	<0.01

NDF: neutral detergent fiber, ADF: acid detergent fiber, WSC: water soluble carbohydrates, CP: crude protein, BE: bermudagrass, BA: bahiagrass, L: linear effect, Q: quadratic effect, C: cubic effect, NS: no significant effect, *: $P < 0.05$, **: $P < 0.01$, [1]Bahiagrass vs. bermudagrass

Table 3.5 Effect of Promote or ammonia application on the IVDMD of tropical grass hays

		DM digestibility, %			
		After 6-h		After 48-h	
Additive	Level	BE	BA	BE	BA
Control		10.1	14.2	49.8	47.9
	0.5x	10.6	14.2	49.7	48.8
Promote	1x	11.4	14.9	49.0	49.3
	2x	11.1	14.4	49.9	48.3
	Mean	11.0	14.5	49.5	48.8
Ammonia		16.5	18.7	61.4	61.7
s.e.m.		0.237	0.308	0.463	1.582
Polynomial effects		*P* values			
Promote level		NS	NS	NS	NS
Contrasts					
Promote vs. Control		<0.01	0.418	0.686	0.633
Control vs. Ammonia		<0.01	<0.01	<0.01	<0.01
Promote vs. Ammonia		<0.01	<0.01	<0.01	<0.01

DM: dry matter, BE: bermudagrass, BA: bahiagrass, NS: non significant effect

IVDMD of the wheat (*Triticum aestivum*) straw was increased by approximately 54% by ammoniation.

Dawson and Tricarico (1999) suggested that the most active period for exogenous enzyme is the first 6 – 12 h of digestion, which transpires prior to bacterial colonization of feed substrates or action of endogenous enzymes. This partly explains why Pr increased the 6-h IVDMD and not the 48-h IVDMD of BE.

The 6 and 48-h IVNDFD of both hays were similar (Table 3.6), despite the lower NDF and lignin concentrations of BA. Treatment with NH_3 increased ($P < 0.01$) the 6 and 48-h IVNDFD in both forages. Pr treatment tended ($P < 0.15$) to reduce the 6-h and 48-h IVNDFD of BE but did not affect the corresponding values for BA. In contrast to the 6-h IVADFD, the 48-h IVADFD was higher ($P < 0.01$) in BE than in BA hay

Table 3.6 Effect of Promote or ammonia application on the IVNDFD of tropical grass hays

Additive	Level	NDF digestibility, %			
		After 6-h		After 48-h	
		BE	BA	BE	BA
Control		6.4	5.5	43.6	40.7
	0.5x	5.1	4.9	44.5	42.9
Promote	1x	3.5	4.1	38.2	37.1
	2x	6.3	5.8	40.2	38.9
	Mean	5.0	4.9	41.0	39.6
Ammonia		8.1	7.9	58.4	59.4
s.e.m.		0.723	0.473	1.305	1.032
Polynomial effects		*P* values			
Promote level		Q*	NS	Q*	Q*
Contrasts					
Promote vs. Control		0.149	0.349	0.105	0.383
Control vs. Ammonia		<0.01	<0.01	<0.01	<0.01
Promote vs. Ammonia		<0.01	0.080	<0.01	<0.01

NDF: neutral detergent fiber, BE: bermudagrass, BA: bahiagrass, Q: quadratic effect, NS: no significant effect, *: $P < 0.05$, **: $P < 0.01$,

(Table 3.7). The 6-h and 48-h IVADFD were higher ($P < 0.01$) in NH_3-treated hays than the other hays for both forage types. Pr treatment reduced ($P < 0.15$) most of the 6-h and 48-h IVADFD values of the hays.

These results suggest that except for slightly increasing the 6-h IVDMD of BE, Pr treatment did not improve DM or cell wall digestion in the forages. The fact that some of these measures were decreased by Pr treatment is surprising. In contrast, NH_3 treatment increased 6-h and 48-h digestibility estimates, which reflect the rate and extent of digestion, respectively. Since intake is constrained by the rate at which the diet is digested (Romney and Gill, 2000), ammoniation is more likely to increase intake and thereby increase animal performance than Pr treatment. Incremental addition of Pr did not have consistent beneficial effects on the nutritive value of BA or BE.

Table 3.7 Effect of Promote or ammonia application on the IVADFD of tropical grass hays

Additive	Level	ADF digestibility, %			
		After 6-h		After 48-h	
		BE	BA	BE	BA
Control		3.7	5.4	48.0	41.9
	0.5x	1.5	4.2	46.7	41.8
Promote	1x	3.1	3.1	45.0	37.2
	2x	1.8	5.1	48.2	39.2
	Mean	2.1	4.1	46.6	39.4
Ammonia		9.3	7.4	65.5	58.3
s.e.m.		0.855	0.511	1.345	0.880
Polynomial effects		*P* values			
Promote level		Q*	NS	Q*	Q*
Contrasts					
Promote vs. Control		0.148	0.052	0.413	0.032
Control vs. Ammonia		<0.01	0.021	<0.01	<0.01
Promote vs. Ammonia		<0.01	<0.01	<0.01	<0.01

ADF: acid detergent fiber, BE: bermudagrass, BA: bahiagrass, Q: quadratic effect, NS: no significant effect, *: $P < 0.05$, **: $P < 0.01$

Effect of Fibrolytic Enzyme and Ammonia Application on Chemical Concentration of C_4 Forages in Experiment 2

Unlike responses with BA, treating BE with NH_3 ($P < 0.01$), X-20 ($P < 0.05$) or A-20 ($P < 0.01$) decreased the NDF concentration and the CA enzyme gave the same tendency (P=0.073) (Table 3.8). Ammonia treatment was more effective than CA treatment. The ADF concentration of BE was decreased ($P < 0.01$) by NH_3 treatment and increased ($P < 0.05$) by CA and A-20 treatment, but that of BA was only decreased by X-20 treatment ($P < 0.01$). The hemicellulose concentration of BE was decreased ($P < 0.01$) by treatment with X-20 (cubic response), CA and A-20, but not NH_3. Only X-20 treatment increased ($P < 0.01$) the hemicellulose concentration of BA, other treatments had no effect.

Table 3.8 Effect of fibrolytic enzyme or ammonia application on the NDF, ADF and hemicellulose concentrations (%) of tropical hays

		NDF		ADF		Hemicellulose	
Additive	Level	BE	BA	BE	BA	BE	BA
Control		87.1	78.7	46.5	47.9	40.6	30.9
NH_3		84.6	80.3	43.7	47.8	40.9	32.5
	0.5x	85.6	77.7	44.3	43.9	41.4	33.8
X-20	1x	85.4	78.8	47.2	44.7	38.2	34.1
	2x	85.4	78.2	51.9	44.0	38.5	34.3
	Mean	85.5	78.2	46.1	44.1	39.4	39.4
	0.5x	86.1	79.7	47.7	48.8	38.4	30.9
CA	1x	85.8	82.1	48.1	49.6	37.6	32.6
	2x	86.1	78.4	48.3	46.3	37.8	32
	Mean	86.0	80.1	48.0	48.2	37.9	37.9
	0.5x	86.1	79.8	48.7	48.7	37.4	31
A-20	1x	85.1	80.7	48.4	48.1	36.7	32.6
	2x	84.9	78.7	49.0	49.4	35.9	29.3
	Mean	85.3	79.5	48.7	48.7	36.7	36.7
s.e.m.		0.49	0.63	0.63	0.48	0.61	0.91
Polynomial effects				*P* values			
X-20 level		NS	NS	C*	NS	C*	NS
CA level		NS	Q**	NS	C**	NS	NS
A-20 level		NS	NS	NS	NS	NS	Q*
Contrasts							
Control vs. X-20		0.011	0.530	0.674	<0.01	0.096	< 0.01
Control vs. CA		0.073	0.171	0039	0.507	< 0.01	0.358
Control vs. A-20		<0.01	0.176	<0.01	0.131	< 0.01	0.892
Control vs. NH_3		<0.01	0.082	<0.01	0.997	0.791	0.219
X-20 vs. CA		0.216	<0.01	<0.01	<0.01	< 0.01	< 0.01
X-20 vs. A-20		0.662	<0.01	<0.01	<0.01	< 0.01	< 0.01
CA vs. A-20		0.099	0.512	0.242	0.219	0.016	0.269
NH_3 vs. X-20		0.131	<0.01	<0.01	<0.01	0.050	0.149
NH_3 vs. CA		0.022	0.719	<0.01	0.507	< 0.01	0.547
NH_3 vs. A-20		0.223	0.412	<0.01	0.132	< 0.01	0.172

NDF: neutral detergent fiber, ADF: acid detergent fiber, BE: bermudagrass, BA: bahiagrass, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20, L: linear effect, Q: quadratic effect, C: cubic effect, NS: no significant effect, *: $P < 0.05$, **: $P < 0.01$

The WSC concentration of BE hays was reduced by CA (tendency, P=0.053) and A-20 ($P < 0.05$) treatment and unaffected by NH_3 or X-20 treatment (Table 3.9). However, that of BA hays was increased ($P < 0.05$) by X-20 treatment.

Table 3.9 Effect of fibrolytic enzyme or ammonia application on the WSC and CP concentrations (%) of tropical hays

		WSC		CP	
Additive	Level	BE	BA	BE	BA
Control		2.81	1.21	6.5	7.0
NH_3		2.82	0.98	16.3	13.0
	0.5x	3.39	2.36	6.9	7.2
X-20	1x	2.01	1.34	6.7	7.1
	2x	1.75	1.84	6.5	7.0
	Mean	2.4	1.8	6.7	7.1
	0.5x	1.75	1.26	6.7	7.2
CA	1x	1.19	0.99	7.0	6.9
	2x	2.63	2.21	6.5	7.7
	Mean	1.9	1.5	6.7	7.3
	0.5x	1.64	0.79	6.8	7.1
A-20	1x	1.67	0.69	6.8	6.9
	2x	1.74	0.94	6.9	7.3
	Mean	1.7	0.8	6.8	7.1
s.e.m.		0.041	0.021	0.019	0.019
Polynomial effects		*P* values			
X-20 level		C*	NS	L*	NS
CA level		C*	L**	NS	NS
A-20 level		NS	NS	NS	Q*
Contrasts		BE	BA	BE	BA
Control vs. X-20		0.370	0.019	0.509	0.731
Control vs. CA		0.053	0.281	0.338	0.287
Control vs. A-20		0.025	0.127	0.163	0.286
Control vs. NH_3		0.998	0.481	<0.01	<0.01
X-20 vs. CA		0.125	0.056	0.666	0.304
X-20 vs. A-20		0.047	<0.01	0.286	0.303
CA vs. A-20		0.613	<0.01	0.518	0.998
NH_3 vs. X-20		0.370	<0.01	< 0.01	< 0.01
NH_3 vs. CA		0.053	<0.01	< 0.01	< 0.01
NH_3 vs. A-20		0.025	0.060	< 0.01	< 0.01

WSC: water soluble carbohydrates, CP: crude protein, BE: bermudagrass,
BA: bahiagrass, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20,
L: linear effect, Q: quadratic effect, C: cubic effect , NS: no significant effect, *: $P < 0.05$,
**: $P < 0.01$,

Ammoniation decreased both ADF and NDF fractions of BE and did not affect the hemicellulose fraction. All enzyme treatments decreased the NDF concentration of BE by decreasing the hemicellulose concentration whereas the only effect on BA cell walls was that X-20 treatment increased hemicellulose concentration by decreasing ADF concentration.

This reveals a forage-specific response to the treatments which is similar to that found in Experiment 1. It is interesting to note that except for X-20 effects on BA, none of the treatments that hydrolyzed forage cell walls resulted in an increase in the WSC concentration. This may be due to the relatively low WSC concentration of the forages and the conversion of hydrolyzed cell wall fragments into oligosaccharides and disaccharides that are not water soluble, and were therefore undetected in the WSC assay.

The results for BE agree with those in Experiment 1, but conflict with those of Colombatto et al. (2003), who evaluated the effects of adding a commercial enzyme product on the hydrolysis and fermentation of cellulose, xylan, and a mixture of both substrates. They observed that addition of enzyme in the absence of ruminal fluid increased ($P < 0.01$) the release of reducing sugars from xylan and the mixture. Similarly, Hristov et al. (1998) observed that enzyme treatment increased the concentration of soluble reducing sugars ($P < 0.05$) and decreased NDF concentration ($P < 0.05$) in a TMR, consisting of rolled barley grain, corn silage and soybean meal.

The results of this study indicate a species-related difference in response to enzyme treatment which was consistent across experiments. The effect of X-20 treatment and ammoniation on the respective ADF concentrations of BE and BA show that this treatment was more effective at disrupting lignocellulosic linkages. However, the reason

why enzymes were generally more effective at hydrolyzing BE despite its higher ADF and lignin concentration is unclear, and is probably related to differences in the type of phenolic cross linkages in the cell walls. In addition to differences in lignin concentration, Mandevbu et al. (1999) also showed that differences in concentration of ether-linked and ester-linked ferulic acid explained digestibility differences between Tifton-85 and coastal bermudagrass.

The CP concentration of the hays was unaffected by enzymatic treatments, but increased ($P < 0.01$) by NH_3 treatment. This agrees with Weiss and Underwood (1995), Brown and Adjei (1995) and Barrios-Urdaneta and Ventura (2002) who observed that ammoniation increased the CP in forages, due to the supplemental N provided. Brown (1993) also observed that, compared to a Control treatment, ammoniation (4% DM) increased ($P < 0.01$) total N concentration (1.0 to 1.4% vs. 1.7 to 2.8%) of stargrass (Cynodon nlemfuensis) hay, and a similar effect (3.26 vs. 4.16% N, $P < 0.01$) was obtained by Lines et al. (1996) for alfalfa hay. Although enzymes are proteins, the small amount of enzyme applied is not enough to effect forage CP concentration.

Effect of Enzyme Treatment and Ammoniation on *in vitro* DM, NDF, and ADF Digestibility in Experiment 2

All of the additives were effective to increase 6-h IVDMD of BE (Table 3.10). However, only NH_3 ($P < 0.01$) and X-20 (tendency: $P = 0.088$) increased the 6-h IVDMD of BA. Ammoniation was the most effective treatment for increasing the 6-h IVDMD ($P < 0.01$) in both grasses. The 6-h IVDMD was consistently greater ($P < 0.01$) in BA than in BE hays. This is probably due to the lower NDF concentration and presumably higher soluble fraction of BA hays and which would facilitate the initial degradation of the forage.

Enzyme X-20 increased the 48-h IVDMD of BE ($P < 0.05$) and BA ($P < 0.01$) hays, while CA and A-20 tended ($P < 0.08$) to have similar effects on only BE. However, NH_3 treatment was more effective ($P < 0.01$) than any of the enzymes at increasing the 48-h IVDMD of both hays.

These results suggest that all additive treatments can improve the 6-h and 48-h digestion of BE, but only NH_3 and X-20 had similar effects on BA. This supports the conclusion that fibrolytic enzyme application can increase the rate of digestion of forages (Wang and McAllister, 2002), but indicates that enzyme effects on rate and extent of digestion depend on the enzyme and forage being tested.

The 6-h IVNDFD was higher ($P < 0.05$) in BA than in BE hays; however, the 48-h IVNDFD was greater ($P < 0.01$) in BE than in BA hays. The 6-h and 48-h IVNDFD of both hays were unaffected by enzyme treatment except for a linear increase with increasing A-20 application to BE (Table 3.11). However, NH_3 treatment did increase 6 and 48-h IVNDFD ($P < 0.01$) of BE and 48-h IVNDFD of BA ($P < 0.01$). The 6-h IVADFD of BE hay was improved ($P < 0.01$) by X-20, A-20 and NH_3 treatment. Only NH_3 treatment increased the 6-h IVADFD of BA ($P < 0.01$); however digestibility of BA hay increased linearly ($P < 0.05$) with increasing application of X-20 (Table 3.12). Therefore, the increases in 6-h IVDMD due to X-20 and A-20 treatment were partly due to increases in 6-h IVADFD. Ammoniation was the only treatment that increased ($P < 0.01$) the 48-h IVADFD in either of the hays; though responses also occurred as the respective rates of CA (linear) and X-20 (cubic) application to BE and BA increased.

None of the enzymes increased the extent of fiber digestion in the hays. Thus the results concur with those of Mandevbu et al. (1999) who observed that treatment of

Table 3.10 Effect of fibrolytic enzyme or ammonia application on the IVDMD (%) of tropical hays

		After 6 h		After 48 h	
Additive	Level	BE	BA	BE	BA
Control		7.7	12.6	43.6	44.5
NH_3		13.7	18.1	56.8	59.9
	0.5x	10.6	14.1	49.6	47.5
X-20	1x	11.4	13.0	51.1	45.6
	2x	11.3	12.9	51.4	47.3
	Mean	11.1	13.3	50.7	46.8
	0.5x	9.0	12.1	46.5	42.3
CA	1x	9.9	12.6	47.9	41.9
	2x	10.1	12.9	47.6	45.6
	Mean	9.7	12.5	47.3	43.3
	0.5x	10.2	12.2	47.2	43.9
A-20	1x	11.6	11.9	48.1	46.8
	2x	9.4	12.2	46.9	42.4
	Mean	10.4	12.1	47.4	44.4
s.e.m.		0.761	0.361	1.789	1.067
Polynomial effects		*P* values			
X-20 level		NS	L*	NS	NS
CA level		NS	NS	NS	NS
A-20 level		NS	NS	NS	NS
Contrasts					
Control vs. X-20		<0.01	0.088	<0.01	0.064
Control vs. CA		0.028	0.854	0.076	0.336
Control vs. A-20		<0.01	0.240	0.071	0.939
Control vs. NH_3		<0.01	<0.01	<0.01	<0.01
X-20 vs. CA		0.035	<0.01	0.027	<0.01
X-20 vs. A-20		0.300	<0.01	0.026	<0.01
CA vs. A-20		0.261	0.169	0.985	0.215
NH_3 vs. X-20		<0.01	<0.01	<0.01	<0.01
NH_3 vs. CA		<0.01	<0.01	<0.01	<0.01
NH_3 vs. A-20		<0.01	<0.01	<0.01	<0.01

DM: dry matter, BE: bermudagrass, BA: bahiagrass, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20, L: linear effect, NS: no significant effect, *: P < 0.05, **: P < 0.01.

bermudagrass forages with fibrolytic enzymes had no effect on *in vitro* or *in situ* DM or NDF disappearance of silages. However, Rode et al. (1999) observed that *in vivo*

Table 3.11 Effect of fibrolytic enzyme or ammonia application on the IVNDFD (%) of tropical hays

		After 6-h		After 48-h	
Additive	Level	BE	BA	BE	BA
Control		5.0	6.2	43.1	37.3
NH_3		7.4	7.0	52.6	54.4
	0.5x	5.4	6.0	46.7	35.8
X-20	1x	5.1	5.5	47.5	35.2
	2x	5.1	5.3	43.1	37.9
	Mean	5.2	5.6	46.2	36.3
	0.5x	4.0	5.8	48.0	33.1
CA	1x	5.4	7.0	46.9	35.7
	2x	5.6	4.5	46.4	34.0
	Mean	5.0	5.8	47.1	34.2
	0.5x	4.9	6.1	47.9	36.2
A-20	1x	4.7	6.3	45.0	38.9
	2x	6.1	7.2	47.4	35.4
	Mean	5.2	6.5	47.5	36.8
s.e.m.		0.47	0.88	1.74	1.59
Polynomial effects		*P* values			
X-20 level		NS	NS	NS	C*
CA level		NS	NS	NS	NS
A-20 level		L*	NS	NS	NS
Contrasts					
Control vs. X-20		0.760	0.542	0.635	0.586
Control vs. CA		0.951	0.683	0.146	0.244
Control vs. A-20		0.714	0.780	0.827	0.312
Control vs. NH_3		<0.01	0.546	<0.01	<0.01
X-20 vs. CA		0.605	0.773	0.571	0.162
X-20 vs. A-20		0.931	0.215	0.399	0.716
CA vs. A-20		0.546	0.336	0.779	0.083
NH_3 vs. X-20		<0.01	0.185	<0.01	<0.01
NH_3 vs. CA		<0.01	0.257	0.169	<0.01
NH_3 vs. A-20		<0.01	0.645	<0.01	<0.01

NDF: neutral detergent fiber, BE: bermudagrass, BA: bahiagrass, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20, L: linear effect, Q: quadratic effect, C: cubic effect, NS: no significant effect, *: P < 0.05, **: P < 0.01

digestibility determined using Cr_2O_3 was increased by a commercial enzyme (Promote®) added to the concentrate (DM: 61.7 vs. 69.1%; NDF: 42.5 vs. 51.0%; ADF: 31.7 vs. 41.9%; and CP: 61.7 vs. 69.8%) to a dairy cow diet. This conflicting responseis

Table 3.12 Effect of fibrolytic enzyme or ammonia application on the IVADFD (% of DM) of tropical hays

		After 6 h		After 48 h	
Additive	Level	BE	BA	BE	BA
Control		3.7	5.1	36.8	35.3
NH_3		9.3	9.0	53.4	44.2
	0.5x	4.1	4.9	36.8	39.9
X-20	1x	7.3	5.3	36.0	36.2
	2x	7.2	4.0	39.3	32.7
	Mean	6.2	4.7	37.4	36.3
	0.5x	2.2	4.0	34.3	37.1
CA	1x	2.8	5.3	36.5	39.2
	2x	5.6	3.5	37.7	36.7
	Mean	3.5	4.3	36.2	37.7
	0.5x	6.0	4.8	37.0	35.9
A-20	1x	6.6	4.7	39.2	33.8
	2x	6.6	6.1	36.7	38.3
	Mean	6.4	5.2	37.6	36.0
s.e.m.		0.77	0.70	1.22	1.24
Polynomial effects		*P* values			
X-20 level		L**	NS	NS	C**
CA level		L*	NS	L*	NS
A-20 level		NS	L*	NS	NS
Contrasts					
Control vs. X-20		<0.01	0.625	0.686	0.508
Control vs. CA		0.902	0.314	0.663	0.120
Control vs. A-20		<0.01	0.902	0.560	0.631
Control vs. NH_3		<0.01	<0.01	<0.01	<0.01
X-20 vs. CA		<0.01	0.456	0.241	0.196
X-20 vs. A-20		0.741	0.391	0.799	0.795
CA vs. A-20		<0.01	0.117	0.158	0.125
NH_3 vs. X-20		<0.01	<0.01	<0.01	<0.01
NH_3 vs. CA		<0.01	<0.01	<0.01	<0.01
NH_3 vs. A-20		<0.01	<0.01	<0.01	<0.01

ADF: acid detergent fiber, BE: bermudagrass, BA: bahiagrass, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20, L: linear effect, Q: quadratic effect, C: cubic effect, NS: no significant effect, *: P < 0.05, **: P < 0.01

probably attributable to the higher nutritive value of the dairy cow diet relative to that of C_4 grasses, suggesting that higher quality diets respond more to enzyme supplementation, presumably due to lower ADF concentration.No other studies that simultaneously

compared the effectiveness of chemical and biological treatments at improving the quality of C_4 grasses were found in the literature. However, several reports have shown that ammonia treatment is very effective for improving the DM and fiber digestibility of low quality forages (Brown, 1993; Vagnoni, et al., 1995; Weiss and Underwood, 1995; Barrios-Urdaneta and Ventura, 2002). The beneficial effect of enzyme treatment on 6-h and 48-h IVDMD were not due to increases in the extent of fiber digestion. Rather they may have been attributable to increased microbial attachment (Dawson and Tricarico, 1999) and an increased rate of ADF digestion.

According to Barrios-Urdaneta and Ventura (2002), ammoniation improves forage digestibility due to the hydrolytic action on linkages between lignin and structural polysaccharides, thus increasing the OM potentially available for utilization by ruminal microorganisms. These authors observed that ammoniation increased ($P < 0.01$) the *in vitro* NDF digestibility (from 46.2 to 57.1%) of koroniviagrass. Ammonia treatment also changes the physical characteristics of forages making them more pliable and increasing their hydration rate. Hydration rate has an important role in digestion rate; the faster a forage particle is hydrated, the faster it is digested (Weiss and Underwood, 1995).

Effect of Enzyme Treatments and Ammoniation on *in situ* DM Degradation

The effect of X-20 and NH_3 on the kinetics of *in situ* DM disappearance of BE and BA is presented in Table 3.13. Treatment with X-20 (linear, $P < 0.05$) and NH_3 ($P < 0.01$) increased the wash loss (a) fraction of BE, but only NH_3 treatment increased that of BA. This result supports the findings obtained *in vitro*, where both of these treatments increased the initial phase of digestion of BE.

Table 3.13 Effect of X-20 or ammonia application on the in situ kinetics of DM disappearance of bermudagrass and bahiagrass

Forage	Treatment	Parameter a, %	b, %	a + b, %	P, %	c	L^1, h
Bermudagrass							
	Control	2.65	60.2	62.8	59.1	0.041	9.064
	NH_3	6.50	70.9	77.4	71.7	0.054	8.341
	X-20, 0.5x	2.95	57.5	60.5	57.5	0.041	7.439
	X-20, 1x	3.60	57.7	61.4	57.6	0.029	6.226
	X-20, 2x	3.85	56.8	60.6	56.0	0.053	9.326
	s.e.m.	0.21	1.95	1.99	2.34	0.01	1.00
	Contrasts	*P* values					
	Polynomial	L*	NS	NS	NS	NS	NS
	Control vs. X-20	0.019	0.267	0.425	0.514	0.997	0.287
	Control vs. NH_3	< 0.01	0.012	< 0.01	0.017	0.346	0.632
	X-20 vs. NH_3	< 0.01	< 0.01	< 0.01	< 0.01	0.260	0.595
Bahiagrass							
	Control	6.35	58.0	64.4	63.4	0.019	2.232
	NH_3	7.35	75.4	82.7	81.1	0.026	3.978
	X-20, 0.5x	7.50	59.3	66.8	65.2	0.024	2.432
	X-20, 1x	5.70	59.6	65.3	63.6	0.025	4.300
	X-20, 2x	6.20	60.6	66.8	65.1	0.025	4.158
	s.e.m.	0.27	1.04	1.15	1.26	0.003	0.89
	Contrasts	*P* values					
	Polynomial	Q*	NS	NS	NS	NS	NS
	Control vs. X-20	0.727	0.191	0.207	0.419	0.153	0.714
	Control vs. NH_3	0.049	< 0.01	< 0.01	< 0.01	0.144	0.578
	X-20 vs. NH_3	0.038	< 0.01	< 0.01	< 0.01	0.679	0.747

DM: dry matter, X-20: Biocellulase X-20, a: soluble fraction, b: insoluble but potentially degradable fraction, a+b= total degradability, P= DM degraded at time t, c: rate of constant degradation, L^1: lag phase (period when no net disappearance of substrate occurs), L: linear effect, Q: quadratic effect

Ammonia treatment was more effective than X-20 treatment at increasing ($P < 0.01$) the insoluble but potentially degradable (b) fraction, the total degradable fraction (a + b) and the degradability (P) of both forages. These results partially concur with those

Table 3.14 Effect of A-20 or ammonia application on the in situ kinetics of DM disappearance of bermudagrass and bahiagrass

		Parameter					
Forage	Treatment	a, %	b, %	a + b, %	P, %	c	L, h
Bermudagrass							
	Control	6.95	61.4	68.4	68.2	0.009	1.535
	NH_3	7.95	62.2	70.2	69.0	0.024	6.849
	A-20, 0.5x	7.75	60.0	67.7	67.1	0.015	4.316
	A-20, 1x	7.50	59.7	67.2	66.7	0.014	2.778
	A-20, 2x	7.25	59.9	67.1	66.2	0.016	3.971
	s.e.m.	0.456	0.435	0.351	0.407	0.003	1.572
	Contrasts	*P* values					
	Polynomial	NS	NS	NS	NS	NS	NS
	Control vs. A-20	0.344	0.025	0.052	0.023	0.119	0.289
	Control vs. NH_3	0.182	0.251	0.015	0.199	0.011	0.062
	A-20 vs. NH_3	0.432	< 0.01	< 0.01	< 0.01	0.032	0.142
Bahiagrass							
	Control	6.50	56.1	62.6	62.2	0.015	7.583
	NH_3	5.95	62.1	68.0	67.5	0.016	7.731
	A-20, 0.5x	7.00	59.2	66.2	65.8	0.010	7.120
	A-20, 1x	6.45	58.9	65.4	64.9	0.014	6.011
	A-20, 2x	6.45	58.5	64.9	64.5	0.014	7.366
	s.e.m.	0.541	1.644	1.458	1.424	0.003	3.373
	Contrasts	*P* values					
	Polynomial	NS	NS	NS	NS	NS	NS
	Control vs. A-20	0.839	0.209	0.149	0142	0.576	0.855
	Control vs. NH_3	0.505	0.051	0.047	0.048	0.845	0.976
	A-20 vs. NH_3	0.324	0.151	0.193	0.207	0.435	0.827

DM: dry matter, A-20: Biocellulase A-20, a: soluble fraction, b: insoluble but potentially degradable fraction, a+b= total degradability, P= DM degraded at time t, c: rate of constant degradation of b, L: lag phase (period when no net disappearance of substrate occurs)

obtained *in vitro*, where NH_3 was the most effective treatment at increasing the extent of digestion. Only the 'a' fraction of BE was affected by X-20, and neither treatment affected the degradation rate or lag phase of the forages.

The results presented in Table 3.14 show that A-20-treated BE hays had lower b, a + b and P values than Control ($P < 0.05$) and NH_3-treated hays ($P < 0.01$). The A-20

treatment also tended to increase the c value for BE and the b and a + b fraction of BA. In BE hays ammoniation increased the lag phase and the c value and a + b fraction, while in BA it increased b, a+b and P. The NH_3 effects concur with results of Vagnoni, et al. (1995), which showed that ammoniation of mature bermudagrass increased both the rate ($P < 0.05$) and the potential extent ($P < 0.01$) of ruminal forage *in situ* DM disappearance in lactating cows. The response to A-20 and X-20 treatments partly agree with Feng et al. (1996) who found that applying cellulase, xylanase and a mixture of both enzymes at different levels did not affect the *in situ* DM disappearance of cool-season grasses. Lewis et al. (1996) evaluated a 70% grass hay diet treated with fibrolytic enzymes that were applied at feeding or 24 h before feeding and observed that *in situ* DM disappearance was unaffected by enzyme treatment of samples incubated for 8, 16, and 24 h, but increased after 96-h ($P < 0.05$). The authors proposed that improved DM disappearance at 96-h of incubation in enzyme-treated grass may have resulted from enhanced colonization and digestion of the slowly degradable fiber fraction by ruminal microorganisms.

Conclusions

This work demonstrates that fibrolytic enzymes had negligible effects on in situ DM degradation of C_4 grass hays, though certain enzymes (X-20 and A-20) did increase the initial and final phases of DM digestion. Such effects were more pronounced in BE than BA. Increasing the enzyme application rate produced inconsistent effects on nutritive value. However, several key measures were increased ith increasing X-20 or A-20 application rate, suggesting that high (1x and 2x) application rates were most effective than the low (0.5x) rate. Most of the enzyme-induced enhancements in digestibility were not attributable to increased fiber digestion; therefore other mechanisms such as increased substrate colonization by ruminal microbes may have been involved.

Ammoniation was more effective than any of the enzyme treatments at improving the initial and final phases of digestion, due to increased fiber hydrolysis. Ammoniation also increased the CP concentration and in situ ruminal degradation of the C_4 grass hays.

CHAPTER 4
EFFECT OF FIBROLYTIC ENZYMES ON THE FERMENTATION CHARACTERISTICS, AEROBIC STABILITY, AND DIGESTIBILITY OF BERMUDAGRASS SILAGE

Introduction

Interest in applying fibrolytic enzymes to ruminant diets has increased recently due to enzyme-mediated increases in feed digestion *in vitro* (Lewis et al., 1996; Kung et al., 2002; Hristov et al., 2000; Bowman et al., 2002) and diet utilization *in vivo* (Yang et al., 1999; Lewis et al., 1999; Schingoethe et al., 1999). However, in certain studies (Sheperd and Kung, 1996b; Bowman et al., 2002; Vicini et al., 2003) exogenous enzyme supplementation did not consistently improve animal performance. Where improved performance was observed, the mechanism was not always confirmed by improved digestion (Mandebvu et al., 1999). These inconsistencies were due to various factoros such as enzyme type, concentration and activity, application method, substrate to which enzyme is added and animal differences (Bowman et al., 2002). Additional factors that may be implicated include prevailing temperature and pH, presence of co-factors and inhibitors, and enzyme and substrate concentration. Nevertheless, feed enzymes have been used to improve the utilization of a wide range of diets containing legumes, grasses, haylage, straw and other feedstuffs (Beauchemin et al., 2003). The mode of action of these enzymes in ruminants is not fully understood. They can enhance feed colonization by increasing the numbers of ruminal fibrolytic microbes (Morgavi et al., 2000; Nsereko et al., 2000a) and thereby increase the rate of degradation in the rumen (Yang et al., 1999). Enzymes can also partially solubilize NDF and ADF and release reducing sugars

in the process. Colombatto et al. (2003) observed that fibrolytic enzymes enhanced the fermentation of cellulose and xylan by a combination of pre- and post-incubation effects. These were evident from an increase in the release of reducing sugars during a 20 h pre-incubation phase and an increase in the hydrolytic activity of the liquid and solid fractions of the ruminal fluid 6-h after incubation, which led to a higher rate of fermentation. Most of the studies on fibrolytic enzyme treatment of ruminant feeds have been done using temperate feedstuffs. Little is known about their effectiveness on tropical or subtropical forages which tend to be poorly digested. The objective of this experiment was to evaluate the effect of four proprietary fibrolytic enzyme preparations applied at different rates, at ensiling, on the nutritive value of Tifton 85 bermudagrass (*Cynodon* spp,) silage. Most of the recent studies in this area have involved enzyme application to individual components of the ration or to the total ration just prior to feeding. There were two reasons for applying the enzymes directly to bermudagrass at the point of ensiling in this study. Firstly, we sought to determine if the sugars produced by enzymatic cell wall hydrolysis would improve the fermentation of bermudagrass which is typically poor and decrease DM losses, which are typically high for this forage. Secondly, we wanted to determine the effectiveness of the enzymes at improving the digestibility of the forage, because although bermudagrass is poorly digested, it is an important digestible fiber source in the rations of dairy cows in the Southeast.

Materials and Methods

Enzyme Application

A five-week regrowth of Tifton 85 bermudagrass silage was conserved without treatment (Control) or after treatment with four proprietary fibrolytic enzymes. The enzymes were applied at half (0.5×), exactly (1×) or twice (2×) the rates recommended

by the respective manufacturers for addition at the time of feeding. Because the enzymes were applied at ensiling rather than at feeding as recommended by the manufacturers, this study was not designed to test the effectiveness of the enzymes as used commercially, and the results should not be misconstrued as doing so. The rationale for the mode of enzyme application employed was to determine if the fermentation and digestibility of bermudagrass could be improved by enzyme addition. The enzymes used were: (a) Promote® (Cargill Corp. St. Louis, MO) applied at: 0.65, 1.3 and 2.6 g/kg DM, (b) Biocellulase X-20® (LodeStar, IL, USA) applied at: 7.3, 14.5 and 29 mg/kg DM, (c) Biocellulase A-20® (LodeStar, IL, USA) applied: at 7.3, 14.4 and 29 mg/kg DM,, and (d) Cattle-Ase (CA) applied at 89, 178 and 356 mg/kg DM. Cellulase activity was determined at 39^{o}C and a pH of 5.5 using the filter paper method (Wood and Bhat, 1988) and the values obtained for Pr, X-20, CA and A-20 were 33.7, 22, 0 and 51.3 filter paper units/g, respectively. Xylanase activity was determined at 39^{o}C and a pH of 5.5 using the di-nitro salicylic acid procedure (Bailey et al., 1992) and the values obtained for Pr, X-20, CA and A-20 were 5190, 7025, 0 and 3530 μ mol/min/ml, respectively. The units of xylanase activity are expressed as μmol xylose equivalents released ml^{-1} min^{-1} from 1% birchwood xylan (X-0502, Sigma Chemical Company, St. Louis, MO, USA).

Each enzyme was dissolved in 400 ml of water and sprayed in a fine mist using a four-liter garden sprayer, over 10 kg of chopped (5 cm) forage per treatment. The same amount of water was sprayed on the Control forages. After thorough mixing, a one-kg representative sample of the treated forage was ensiled within a polythene bag in six, replicate 2.8-L PVC cylindrical mini silos. A hydraulic press was used to compress the forage in the silo to achieve a density of 280 kg/m^3. Weights of the empty and full silos

were recorded, and silos were then stored for 145 days at ambient temperature (23–27^{o}C) in a covered barn. Representative samples (4 kg) of the freshly-treated, unensiled forages were frozen (-20^{o}C) for subsequent laboratory analysis.

Laboratory Analysis

At silo opening, final silo weights were recorded and silages from each of three silos per treatment were sub-sampled for DM determination (200 g) and silage juice extraction (200 g) or freeze-dried for chemical analysis (200 g). Each of the other three silos was sub-sampled for microbial enumeration (200 g) and aerobic stability (800 g). Samples destined for microbial analysis were heat-sealed within gas-impermeable bags (Kapak / Scotch Pak®, Kapak Corp., Minneapolis, MN), placed in an icebox and transported on the same day to the American Bacteriological & Chemical (ABC) Research Corporation, Gainesville, Florida. Serial dilutions up to 1×10^{10} were made using 25 g of silage and Butterfields' phosphate buffer. Yeast and molds were enumerated by pour plating in Standard Methods (M124) agar, to which 40 ppm of chloramphenicol and chlortetracycline were added (Tournas et al., 1999). Plates were incubated aerobically at 25^{o}C for 5 days. Aerobic stability was measured by placing thermocouple wires at the center of a bag containing 800 g of silage, within an open-top polystyrene box. The silages were covered with two layers of cheesecloth to prevent drying. The thermocouple wires were connected to data loggers (Campbell Scientific Inc. North Logan, UT) that recorded the temperature every 30 min for 30 d. Aerobic instability was denoted by the time (h) taken for a 2ºC rise in silage temperature above ambient temperature (23–27^{o}C). Dry mater losses were estimated using DM concentrations and silo weights measured before and after ensiling. Oven DM concentration was determined in a forced draft oven set at 60^{o}C for 48-h. Ash

concentration was determined in a muffle furnace at 500^{o}C for 5 h. Silage juice was obtained by blending 20 g of silage in 200 ml of distilled water for 30 s at high speed and the slurry was filtered through two layers of cheesecloth. The pH was measured using a pH meter (Corning Model 12, Corning Scientific Instruments, Medfield, MA). The filtrate was centrifuged at 4^{o}C and 21,500 × g for 20 min and the supernatant was frozen (-20^{o}C) in 20 ml vials for subsequent analysis of lactic acid, VFA, WSC, ammonia nitrogen (NH_3-N) and water soluble N (WSN).

Organic acids were measured using the method of Muck and Dickerson (1988) and a High Performance Liquid Chromatograph (Hitachi®, FL 7485, Tokyo, Japan) coupled to a UV Detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, NJ) set at 210 nm. The column used was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) with 0.015M sulfuric acid mobile phase and a flow rate of 0.7 ml/min at 45°C. Ethanol was measured by gas chromatography using the procedure of Yomano et al. (1998) with a Varian Star 3400 CX gas chromatograph (Varian, Santa Clarita, CA). The anthrone reaction assay (Ministry of Agriculture, 1986) was used to quantify WSC. Ammonia N was determined using an adaptation for the Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA) of the Noel and Hambleton (1976) procedure. Water-soluble N (WSN) was determined by digesting 10 ml of supernatant using micro Kjeldahl apparatus (Labconco Corporation, Kansas City, MO) and the N concentration was determined using a Technicon auto analyzer (Technicon, Tarrytown, NY, USA).

Freeze-dried ground (1mm) samples were analyzed for CP, *in vitro* digestibility, ADF and NDF. *In vitro* digestibility of DM, NDF and ADF was determined after

incubating forage samples in buffered, rumen fluid for 6 or 48-h using two ANKOM^{II} Daisy Incubators (ANKOM Technology, Fairport, NY). The buffer was prepared according to the ANKOM Technology procedure. The rumen fluid was obtained before feeding from two, non-lactating, fistulated cows, fed 9 kg of Coastal bermudagrass (*Cynodon dactylon*) and 400 g soybean meal daily. The NDF and ADF concentrations (Van Soest et al., 1991) of the samples and digested residues were determined without amylase pretreatment using an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY). Hemicellulose was calculated by difference from NDF and ADF concentrations.

Statistical Analysis

A completely randomized design and a 4 × 4 factorial arrangement of enzyme types and application rates with 3 replicates per treatment was used. The data were analyzed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). Polynomial contrasts were used to test the effect of increasing enzyme application rate and orthogonal contrasts were used to compare the Control and enzyme treatments.

The model used to analyze individual treatment effects was:

Yij: $\mu + Ti + Eij$

where:

μ= general mean

Ti = effect of treatment (enzyme type × enzyme rate)

Eij= experimental error.

Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$

Results and Discussion

Chemical Composition of Freshly-treated Bermudagrass before Ensiling

Table 4.1 shows the chemical composition of the bermudagrass forages prior to ensiling. The concentrations of the measured chemical components were similar for all treatments. This is probably attributable to the short duration of enzyme action due to placement of the freshly-treated samples on ice after enzyme application. The DM concentration at harvest was typical of that at the stage at which bermudagrass is ensiled in Florida, and similar to that (324 g/kg) reported for bermudagrass harvested at a similar maturity stage by Umana et al., (1991) and Adesogan et al. (2004). The low WSC and CP concentrations and high NDF and ADF concentrations are also typical of bermudagrass (Umana et al., 1991; Adesogan et al., 2004). These results indicate that the bermudagrass was representative of those used for dairy production in the southeastern US.

Chemical Composition, Microbial Counts and Aerobic Stability of Bermudagrass Silages

Neither enzyme type nor application rate affected ($P > 0.05$) the DM concentration of the silages. Dry matter values ranged between 296 and 308 g/kg (Table 4.2). The pH of Pr-treated silages was lower ($P < 0.01$) than that of all other silages, while the other enzyme- treated silages had similar pH values to Control silages. This suggests that compared to the other forages, Pr was more effective at increasing the availability of WSC for microbial fermentation, through cell wall hydrolysis. Though this is not evident from the WSC concentration of the freshly-treated forages due to the short duration allowed for enzyme action, Pr-treated silages did have greater ($P < 0.05$) residual WSC concentration

Table 4.1 Chemical composition of bermudagrass forages before ensiling (g/kg DM).

Enzyme treatment[1]	DM (g/kg)	pH	Ash	WSC[2]	NH_3-N[3]	CP	NDF	ADF	Hem[4]
Control	305	5.97	57	6.93	54	105	786	436	350
Pr	302	6.42	59	6.14	43	99	776	428	348
X-20	305	6.14	55	6.79	56	97	791	440	351
CA	303	5.67	62	5.83	37	97	786	440	346
A-20	306	6.04	57	6.67	44	97	791	438	353
P	0.836	0.795	0.762	0.717	0.758	0.504	0.264	0.236	0.547
S.E.	4.26	0.73	6.67	1.01	18.52	4.01	8.65	6.61	5.50
Contrasts	*P values*								
Control vs. Pr	0.559	0.607	0.834	0.516	0.631	0.209	0.331	0.325	0.609
Control vs. X-20	0.999	0.846	0.834	0.911	0.939	0.136	0.608	0.614	0.919
Control vs. CA	0.744	0.729	0.508	0.371	0.441	0.136	0.999	0.586	0.514
Control vs. A-20	0.896	0.933	0.999	0.828	0.642	0.110	0.630	0.833	0.614

[1] Cellulase-hemicellulase preparations: Pr, Promote, X-20, Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20, [2] WSC: water-soluble carbohydrates, [3]Ammonia-N expressed as g/total N, [4] Hemicellulose

than the other silages (Table 4.3). Silage pH also decreased ($P < 0.01$) linearly as the rate of Pr application increased.

The Pr-treated silages had pH values that were similar to or lower than that which is required for achieving stability during the fermentation (Bates et al., 1989a). Similar reductions in pH were obtained when fibrolytic enzymes were applied to wheat silage (Adogla-Bessa et al., 1999), corn silage (Sheperd and Kung, 1996a; Colombatto et al., 2004) or orchardgrass and alfalfa silages (Nadeau et al., 2000).

Ammonia-N levels were lower in the Pr-treated silages ($P < 0.01$) than in the other silages. This reveals that less proteolysis occurred during ensiling in Pr-treated silages than in other silages, and this was probably due to a faster pH decline in Pr-treated silages. The lower ammonia-N concentration of Pr-treated silages contrasts with

Table 4.2 Effect of fibrolytic enzymes on pH, concentrations of DM (g/kg) and ammonia-N (g/kg total N), DM losses (%), microbial counts (log cfu /g) and aerobic stability (h) of bermudagrass silage.

Enzyme Treatment[1]	Application rate	pH	DM	DM loss	Ammonia-N	Yeasts	Molds	Aerobic stability
Control		4.40	305	8.6	32	1.65	3.24	96
Pr	0.5×	4.28	299	4.0	26	2.83	3.70	103
Pr	1×	3.93	308	5.9	25	1.50	3.01	102
Pr	2×	3.87	299	2.6	24	2.31	2.18	229
Pr	*Mean*	4.03	302	4.2	25	2.21	2.96	138
Pr	*Rate effect*	L **	NS	NS	NS	NS	NS	L**
X-20	0.5×	4.40	305	7.3	31	1.42	2.37	196
X-20	1×	4.49	303	5.8	38	1.59	4.83	210
X-20	2×	4.32	307	7.7	38	3.57	4.49	96
X-20	*Mean*	4.40	305	7.0	35	2.19	3.90	162
X-20	*Rate effect*	NS	NS	NS	L*	NS	NS	L**
CA	0.5×	4.00	306	8.8	33	2.05	1.93	96
CA	1×	4.46	308	7.4	24	1.00	3.37	203
CA	2×	4.41	296	5.7	32	2.17	3.12	205
CA	*Mean*	4.40	304	7.3	30	1.74	2.81	168
CA	*Rate effect*	NS	NS	NS	Q**	NS	NS	L**
A-20	0.5×	4.54	306	9.5	39	1.54	3.00	96
A-20	1×	4.32	305	6.4	36	1.42	4.63	232
A-20	2×	4.12	306	3.6	25	1.00	3.50	261
A-20	*Mean*	4.33	306	6.5	33	1.32	3.71	196
A-20	*Rate effect*	NS	NS	L **	C**	NS	NS	L**
S.E.		0.09	7.74	1.12	0.03	0.89	0.77	38.00
Contrasts					*P* values			
Control vs. Pr		0.002	0.703	< 0.01	< 0.01	0.573	0.671	0.011
Control vs. X-20		0.975	0.961	0.218	0.126	0.649	0.472	< 0.01
Control vs. CA		0.992	0.844	0.316	0.248	0.925	0.633	< 0.01
Control vs. A-20		0.516	0.908	0.120	0.630	0.752	0.609	< 0.01
Pr vs. X-20		<0.01	0.639	0.005	< 0.01	0.878	0.121	0.096
Pr vs. CA		<0.01	0.794	0.002	< 0.01	0.508	0.939	0.110
Pr vs. A-20		0.005	0.555	0.017	< 0.01	0.225	0.198	< 0.01
X-20 vs. CA		0.977	0.835	0.737	< 0.01	0.609	0.106	0.974
X-20 vs. A-20		0.338	0.903	0.631	0.135	0.285	0.766	0.043
CA vs. A-20		0.353	0.741	0.416	0.026	0.564	0.175	0.059

L: linear effect, Q: quadratic effect, C: cubic effect, NS: Not significant, * $P < 0.05$, ** $P < 0.01$.
[1] Cellulase-hemicellulase enzyme preparations: Pr: Promote, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20.

Table 4.3 Effect of fibrolytic enzymes on the chemical composition of bermudagrass silage (g/kg DM).

Enzyme Treatment[1]	Application rate	CP	Ash	NDF	ADF	Hem.[2]	WSC[3]	WSN[4]
Control		97	53	753	431	323	4.5	0.84
Pr	0.5×	96	49	723	408	315	8.2	0.73
Pr	1×	92	50	725	408	317	12.6	0.88
Pr	2×	93	54	728	408	319	15.9	0.73
Pr	Mean	94	51	725	408	317	12.2	0.78
Pr	Rate effect	NS	NS	NS	NS	NS	L **	NS
X-20	0.5×	98	61	744	420	324	6.1	0.71
X-20	1×	96	53	738	426	313	6.1	0.70
X-20	2×	97	51	747	426	321	5.9	0.89
X-20	Mean	97	55	743	424	319	6.0	0.77
X-20	Rate effect	NS	NS	NS	NS	NS	NS	NS
CA	0.5×	99	53	750	422	327	5.3	0.86
CA	1×	105	53	743	444	299	7.1	0.62
CA	2×	89	54	736	442	295	8.5	0.70
CA	Mean	98	53	743	436	307	6.9	0.73
CA	Rate effect	Q **	NS	L **	L **	L *	L *	NS
A-20	0.5×	96	54	761	448	313	5.3	0.76
A-20	1×	95	54	757	438	319	5.3	0.67
A-20	2×	97	57	741	428	312	5.2	0.57
A-20	Mean	96	55	753	438	315	5.3	0.67
A-20	Rate effect	NS	NS	L **	C **	NS	NS	NS
S.E.		1.64	2.29	3.36	2.70	3.84	0.81	0.07
Contrasts					*P values*			
Control vs. Pr		0.125	0.386	< 0.01	< 0.01	0.204	< 0.01	0.466
Control vs. X-20		0.954	0.590	0.016	< 0.01	0.444	0.109	0.350
Control vs. CA		0.602	0.934	0.015	0.105	0.002	0.014	0.170
Control vs. A-20		0.816	0.508	0.977	0.022	0.078	0.407	0.039
Pr vs. X-20		0.028	0.054	< 0.01	< 0.01	0.463	< 0.01	0.766
Pr vs. CA		0.006	0.269	< 0.01	< 0.01	0.004	< 0.01	0.351
Pr vs. A-20		0.067	0.071	< 0.01	< 0.01	0.463	< 0.01	0.053
X-20 vs. CA		0.513	0.381	0.968	< 0.01	0.001	0.179	0.522
X-20 vs. A-20		0.682	0.860	< 0.01	< 0.01	0.149	0.260	0.097
CA vs. A-20		0.291	0.295	< 0.01	0.299	0.022	0.018	0.293

L: linear effect, Q: quadratic effect, C: cubic effect, NS: Not significant, * $P < 0.05$, ** $P < 0.01$.
[1]Cellulase-hemicellulase enzyme preparations: Pr: Promote, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20, Biocellulase A-20. [2] Hem: Hemicellulose. [3] WSC: water-soluble carbohydrates.
[4] WSN: water-soluble nitrogen.

previous studies in which ammonia-N concentration of silages was unaffected by fibrolytic enzyme application (Sheperd and Kung, 1996a; Adogla-Bessa et al., 1999).

Yeast and mold counts were unaffected by enzyme type or rate and the numbers found were less than those (5.0 cfu/g) that predispose to rapid deterioration in silage (Kung, 2004). These low yeast and mold counts reflect the antimycotic properties of the VFA produced during the ensiling process (Table 4.4). Yeasts usually initiate aerobic deterioration of silages, while molds continue the deterioration process because yeast grow faster but tolerate less heat than do molds (Higginbotham et al., 1998).

Aerobic stability was increased ($P < 0.05$) by enzyme treatment and such increases were linear ($P < 0.05$) as the rate of enzyme application increased except in X-20-treated silages. Silages treated with A-20 enzyme tended ($P < 0.06$) to be more stable than other additive-treated silages. Nevertheless, all the forages were stable for at least four days, such that all of them would be adequately preserved in the feedbunk for several days. This observation is typical of bermudagrass silages which usually undergo heterolactic fermentation, resulting in the production of antimycotic acids like acetic acid that ensure the stability of the silage (Bates et al., 1989a; Adesogan et al., 2004).

Dry matter losses were lower in the Pr-treated silages than in the other silages ($P < 0.05$). Though there was no effect of increasing Pr application on DM lost, this work demonstrates that Pr can be used to reduce the losses of DM that usually occur when bermudagrass is conserved as silage. Although DM losses decreased linearly ($P < 0.05$) as the rate of A-20 application increased, the mean DM loss for A-20-treated silages was similar to that of Control silages.

Neither enzyme type nor application rate affected ($P > 0.05$) the ash concentration of the silages (Table 4.3). Compared to Control silages, NDF concentration was reduced by Pr ($P < 0.01$), X-20 and CA ($P < 0.05$). However the lowest NDF values ($P < 0.01$) were observed in the Pr-treated silages ($P < 0.01$), indicating that this treatment was the most effective at reducing the total fiber fraction. Silages treated with CA had lower ($P < 0.05$) hemicellulose concentrations than other silages. As the rate of CA application increased, hemicellulose concentration decreased linearly ($P < 0.01$) while ADF concentration increased linearly ($P < 0.01$). This suggests that CA hydrolyzed the digestible fiber fraction in the silage but did not affect the less digestible ADF fraction. Silages treated with Pr had lower ($P < 0.01$) ADF concentrations than Control silages and other enzyme-treated silages, suggesting that this treatment was particularly effective at reducing the concentration of the ADF fraction which is usually poorly digested by ruminants. Treatment with Pr; therefore, reduced the total and less digestible fiber fractions, and could potentially result in improved utilization of the fiber fraction in dairy cows fed bermudagrass silage. Although CA treatment reduced the total fiber fraction, it also reduced the digestible fiber concentration, which is an important source of slowly-released energy for cattle.

The reduction in NDF and ADF concentration by Pr and X-20 treatment, and NDF and hemicellulose concentration by CA treatment, contradicts the findings of Mandebvu et al. (1999) on bermudagrass but concurs with previous observations on enzyme-treated wheat silage (Adogla-Bessa et al., 1999), corn silage (Sheperd and Kung, 1996a; Sheperd and Kung, 1996b; Colombatto et al., 2004) and orchardgrass or alfalfa silages (Nadeau et al., 2000). Differences between the effects of enzymes on cell wall hydrolysis in

bermudagrass silage in this study and that of Mandebvu et al. (1999) may be due to differences in enzyme activity. These results; therefore, provide new evidence that fibrolytic enzymes can enhance cell wall hydrolysis in C_4 grasses, as is the case in C_3 grasses.

Silages treated with Pr ($P < 0.01$) and CA ($P < 0.05$) had greater residual WSC concentration than Control silages. As the rate of application of both of these enzymes increased, residual WSC concentration increased linearly ($P < 0.05$). However, these enzymes increased residual WSC concentration by hydrolyzing different fiber fractions. While Pr hydrolyzed the less digestible fiber fraction, CA reduced the digestible fiber fraction. Therefore, both of these enzymes were effective in increasing the availability of fermentation substrates, but Pr was potentially more beneficial at improving the digestibility of the silages.

The WSC values obtained in the Pr-treated silages are also higher than those reported by Adesogan et al. (2004), probably because of greater cellulase and xylanase activity in Pr than in the enzyme included in the inoculant used by Adesogan et al. (2004). The increase in WSC concentration of enzyme treated silages agrees with results obtained by Sheperd and Kung (1996a); Adogla-Bessa et al. (1999) and Nadeau et al. (2000). Colombatto et al. (2003) also observed that addition of fibrolytic enzymes increased ($P < 0.01$) release of reducing sugars from fibrous fractions of forage during a 20-h pre-incubation phase.

The concentration of CP was similar in enzyme-treated and Control silages (Table 4.3). However, CP concentration was lower in Pr-treated silages than silages treated with X-20 ($P < 0.05$) and CA ($P < 0.01$). This numerically small difference in CP did not

result from greater proteolysis in Pr-treated silages since they had lower ($P < 0.05$) ammonia-N concentrations than the other silages. Rather, it may have been due to the higher WSC concentration of Pr – treated silages.

Organic Acid Concentration of Bermudagrass Silages

The lactic acid concentrations (Table 4.4) of the Control and enzyme treated silages were similar ($P > 0.05$). These results agree with those obtained of Mandebvu et al. (1999) who found that though fibrolytic enzyme treatment of bermudagrass did not increase lactic acid concentration, values for enzyme-treated forages were 5.4 % higher than those for untreated forage. Acetic acid concentration was lower ($P < 0.05$) in enzyme-treated silages than in Control silages. Promote-treated silages had the lowest ($P < 0.01$) acetic acid concentrations and unlike other enzymes increasing the rate of Pr application decreased ($P < 0.05$) acetic acid concentration linearly. These results are in contrast with those obtained by Sheperd and Kung (1996a) and Mandebvu et al. (1999)

These factors are partly responsible for the lower ($P < 0.05$) DM losses in the Pr- who found no effect of fibrolytic enzyme treatment on acetic acid concentration of silages. The lower acetic acid concentration ($P < 0.01$) and numerically higher lactic acid concentration in Pr-treated silages suggest that this enzyme enhanced homofermentative processes during ensiling, which reduce CO_2 formation; and therefore, minimize DM and energy losses.treated silages relative to those in the other silages. However, the tendency towards greater homofermentative processes in enzyme-treated forages conflicts with their greater aerobic stability as homofermentative silages are often more susceptible to aerobic spoilage. The reason for this anomaly is not clear. Nevertheless, the extent of enzyme-induced homofermentation was not sufficient to override the inherent heterofermentation and aerobic stability of bermudagrass silage.

Table 4.4 Effect of fibrolytic enzymes on the organic acid concentration (g/kg DM)of bermudagrass silage.

Enzyme Treatment[1]	Application rate	Lactic acid	Acetic acid	Isobutyric acid	Butyric acid	Isovaleric acid
Control		50	46	54	7.1	11
Pr	0.5×	83	29	63	7.7	17
Pr	1×	54	13	37	0.57	8
Pr	2×	60	15	46	0	10
Pr	Mean	66	19	48	2.8	11
Pr	Rate effect	NS	L **	NS	L **	L *
X-20	0.5×	32	22	34	3.9	8
X-20	1×	49	38	42	5.9	9
X-20	2×	64	38	53	5.9	13
X-20	Mean	48	33	43	5.3	10
X-20	Rate effect	L *	L **	NS	NS	NS
CA	0.5×	57	38	46	5.8	11
CA	1×	54	36	68	0	18
CA	2×	57	32	58	4.9	14
CA	Mean	56	35	57	3.6	14
CA	Rate effect	NS	NS	NS	Q *	Q *
A-20	0.5×	44	42	54	8.1	12
A-20	1×	62	35	59	9.2	14
A-20	2×	64	33	76	0	14
A-20	*Mean*	56	37	63	5.7	13
A-20	*Rate effect*	NS	NS	L *	C **	NS
S.E.		10.61	3.36	7.41	1.96	1.97
Contrasts				*P values*		
Control vs. Pr		0.211	< 0.01	0.504	0.064	0.735
Control vs. X-20		0.899	< 0.01	0.213	0.414	0.915
Control vs. CA		0.635	0.012	0.690	0.129	0.126
Control vs. A-20		0.612	0.028	0.287	0.551	0.210
Pr vs. X-20		0.057	< 0.01	0.372	0.130	0.531
Pr vs. CA		0.267	< 0.01	0.154	0.607	0.091
Pr vs. A-20		0.287	< 0.01	0.022	0.071	0.194
X-20 vs. CA		0.398	0.366	0.025	0.307	0.024
X-20 vs. A-20		0.373	0.159	0.003	0.752	0.060
CA vs. A-20		0.963	0.600	0.342	0.185	0.677

L: linear effect, Q: quadratic effect, C: cubic effect, NS: Not significant, * P < 0.05, ** P < 0.01.
[1] Cellulase-hemicellulase enzyme preparations: Pr: Promote, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20, Biocellulase A-20

Butyric acid was found in all the silages except those treated with Pr and A-20 at twice the recommended rate and CA at the recommended rate. Butyric acid concentration decreased ($P < 0.05$) with increasing application of Pr (linear), A-20 (cubic effect), and CA (quadratic effect). However, only Pr treatment produced butyric acid concentrations that tended to be less ($P = 0.064$) than those in Control silages. The decrease in butyric acid concentration following Pr treatment supports the observations of Adogla-Bessa et al. (1999) for wheat silage, but contradicts those of Mandebvu et al. (1999) for bermudagrass silage. This discrepancy is attributable to the high cellulase and xylanase activities in Pr which resulted in substantial hydrolysis of cell walls into WSC. When the concentration of such WSC is adequate, and moisture is not excessive, homofermentative lactic acid bacteria proliferate rather than heterofermenters and clostridia, such that lactic acid accumulates instead of butyric acid.

The isobutyric acid concentrations of the treated and untreated silages were similar ($P > 0.05$). Neither propionic acid nor ethanol was found in the silages. The absence of ethanol in the silages may be explained by the low yeast counts and relatively low WSC concentrations in the silages, because yeasts are primarily responsible for ethanol production from the fermentation of sugars in silages.

In vitro DM and Fiber Digestibility of Bermudagrass Silages

Unlike silages treated with the other enzymes, Pr-treated silages had greater ($P < 0.05$) 6-h and 48-h IVDMD values as well as greater 48-h IVNDFD and IVADFD than Control silages (Table 4.5). Silages treated with Pr consistently had greater 6-h and 48-h IVDMD, and 48-h IVNDF values than those treated with the other enzymes. The increase in IVDMD at 6-h and 48-h by Pr treatment suggests that application of this enzyme can increase both the rate and extent of digestion of bermudagrass silage.

Table 4.5 Effect of fibrolytic enzymes on in vitro digestibility of DM (g/kg), NDF, ADF and hemicellulose (Hem) in bermudagrass silage after 6 or 48-h of digestion (g/kg DM).

Enzyme Treatment[1]	Application rate	DM 6-h	DM 48h	NDF 48h	ADF 48h	Hem 48h
Control		200	501	402	427	368
Pr	0.5×	227	561	461	489	425
Pr	1×	223	546	442	457	423
Pr	2×	225	554	448	442	456
Pr	*Mean*	225	554	450	462	435
Pr	*Rate effect*	NS	NS	NS	C *	NS
X-20	0.5×	208	498	393	414	365
X-20	1×	195	498	370	426	344
X-20	2×	209	495	385	405	360
X-20	*Mean*	204	497	383	415	356
X-20	*Rate effect*	Q *	NS	NS	NS	Q **
CA	0.5×	198	487	385	401	363
CA	1×	199	536	447	512	342
CA	2×	205	521	416	479	321
CA	*Mean*	201	515	416	464	342
CA	*Rate effect*	NS	L **	Q **	L **	NS
A-20	0.5×	183	502	424	462	369
A-20	1×	190	491	399	436	349
A-20	2×	184	538	464	504	411
A-20	Mean	186	510	429	467	376
A-20	*Rate effect*	NS	C **	C **	Q **	C*
S.E.		4.398	8.290	13.102	13.295	13.134
Contrasts				*P values*		
Control vs. Pr		< 0.01	<0.01	0.004	0.032	0.007
Control vs. X-20		0.401	0.638	0.215	0.425	0.206
Control vs. CA		0.897	0.176	0.371	0.026	0.264
Control vs. A-20		0.009	0.350	0.085	0.017	0.724
Pr vs. X-20		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Pr vs. CA		< 0.01	< 0.01	0.003	0.895	< 0.01
Pr vs. A-20		< 0.01	< 0.01	0.059	0.686	< 0.01
X-20 vs. CA		0.317	0.014	0.005	< 0.01	0.826
X-20 vs. A-20		< 0.01	0.054	0.000	< 0.01	0.027
CA vs. A-20		< 0.01	0.538	0.224	0.785	0.044

L: linear effect, Q: quadratic effect, C: cubic effect, NS: Not significant, * P < 0.05, ** P < 0.01,
[1] Cellulase-hemicellulase enzyme preparations: Pr: Promote, X-20: Biocellulase X-20, CA: Cattle-Ase, A-20: Biocellulase A-20.

Furthermore, Pr treatment was effective at improving the digestibility of the total, digestible and less digestible cell wall fractions, corroborating the earlier suggestion that Pr treatment will increase the utilization of bermudagrass silage by ruminants. Previous studies did not detect such benefits of enzyme treatment on the *in vitro* digestibility of DM and NDF of bermudagrass silage (Mandebvu et al., 1999) or the *in vivo* digestibility of orchardgrass silage (Nadeau et al., 2000). The IVADFD was greater in the Pr, CA and A-20 -treated silages compared to Control silages ($P < 0.05$) and X-20 ($P < 0.01$). Therefore, these enzymes increased the susceptibility of the typically indigestible cell wall fraction to ruminal digestion. Hemicellulose digestibility was also greater in the Pr-treated silages ($P < 0.01$) than Control silages or other enzyme-treated silages, while A-20 showed higher values ($P < 0.05$) than X-20.

The superior effect of Pr treatment in this study is partly attributable to the fact that in accordance with the each of the enzyme manufacturer's guidelines, a greater amount of enzyme was applied to the forages in the Pr treatment, than in the other enzyme treatments. This is partly because Pr is supplied in liquid form, while the other enzymes were supplied in solid form. Though Pr did not have the highest xylanase or cellulase activity, it had greater combined cellulase and xylanase activity than any of the other enzymes. The synergistic, complementary effects of these enzymes in Pr, probably accounted for its' superior effects on the silages. Though no cellulase or xylanase activity was detected in CA, the results of this study indicate that it had fibrolytic activity. It probably; therefore, contained different enzymes to those that were analyzed.

Conclusions

This study shows that the nutritive value and fermentation of bermudagrass silage can be improved by treating it with fibrolytic enzymes. Compared to Control silages,

enzyme-treated silages had greater WSC concentration due to hydrolysis of different cell wall fractions. However, Pr was the most promising enzyme for increasing residual WSC concentration enhancing homofermentation, and reducing the pH, DM losses and proteolysis.

CHAPTER 5
EFFECT OF METHOD OF DIETARY ADDITION OF A FIBROLYTIC ENZYME ON THE PERFORMANCE OF LACTATING DAIRY COWS

Introduction

One of the main problems limiting livestock production in the southeastern United States is the seasonal variation in forage quality and quantity. Dairy producers have reduced the winter forage deficiency by harvesting and conserving forages during their peak growth periods in the summer for feeding in the winter. However since the nutritive value of the tropical forages is usually low due to their high indigestible fiber concentration, ideal methods of conservation should also aim to improve forage quality. Several recent studies have evaluated the potential of improving forage quality and diet utilization with fibrolytic enzymes (Rode and Beauchemin 1998; Colombatto et al., 2003a). Milk yield has been increased in some studies in which dairy cow diets were supplemented with exogenous fibrolytic enzymes (Rode et al., 1999; Yang et al., 2000, Kung et al., 2002), but not in others (Sheperd and Kung, 1996; Lewis et al., 1999; Beauchemin et al., 2000; Vicini et al., 2003). Dry matter intake (DMI) either increased (Beauchemin et al., 2000) or was unaffected (Beauchemin et al., 1999; Kung et al., 2000) when enzymes were added to the diet. Feed intake increases have been generally small and inconsistent (Yang et al., 1999 and 2000; Rode et al., 1999; Schingoethe et al., 1999; Vicini et al., 2003).

Similarly, effects of supplemental enzymes on digestibility have been inconsistent. Use of enzyme products comprised mainly of xylanases and cellulases have increased

digestibility (Rode et al., 1999; Yang et al., 2000) or did not affect digestibility (Lewis et al., 1999). Attempts to improve feed efficiency for milk production in dairy cows by the use of direct-fed fibrolytic enzymes applied at or shortly before feeding have also yielded variable production responses (Sutton et al., 2003). Changes in milk fat and protein also have been inconsistent (Beauchemin et al., 1999; Lewis et al., 1999; Schingoethe et al., 1999; Rode et al., 1999; Yang et al., 1999; 2000; Kung et al., 2000; Vicini et al., 2003).

Although a few studies demonstrated that enzyme application to bermudagrass silage improved DM recovery (Dean et al., 2005) and feed intake by beef cows (Bates et al., 1989b), there has been little concerted effort aimed at determining the efficacy of using commercial enzymes to improve milk production in cows fed enzyme-supplemented tropical grasses. The objective of this study was to determine the effects of applying an enzyme to bermudagrass at ensiling, or to different components of the diet at feeding on feed intake, milk production and composition, digestion kinetics and blood metabolites of lactating dairy cows. The enzyme selected for this study was found to be more effective than three other commercial enzymes at reducing DM losses and fiber concentration, increasing water soluble carbohydrate concentration, promoting a more homolactic fermentation and increasing fiber digestibility in bermudagrass silage (Dean et al., 2005).

Material and Methods

Two experiments were carried out at the Dairy Research Unit of the University of Florida from November 2004 to March 2005. In the first experiment, 30 lactating Holstein cows (peimiparous and multiparous) in mid-lactation (129 ± 6 days in milk) were allocated randomly to 5 dietary treatments for two, 28-d periods. Each period consisted of 14 d for adaptation to a new diet and 14 d for sample collection. A cellulase,

xylanase enzyme complex (Promote®, Cargill, Minnetonka, MN, USA) was applied to different fractions of the diet. The manufacturer-stipulated main activity of this product is 1200 cellulase units/g of the product, where one unit is the amount of enzyme that releases 1 μmol of glucose from cellulose in 1 min at 40ºC. Cellulase activity also was determined at 39^{o}C and pH 5.5 using the filter paper method (Wood and Bhat, 1988). The activity was found to be 38.4 filter paper units/g, where one unit of activity is the amount of enzyme that releases exactly 2 mg of glucose from 50 g of filter paper in 60 min.

Diets

For both experiments, the diets contained Tifton 85 bermudagrass (*Cynodon* spp.) silage (BS), corn silage (CS) and concentrate mixed at 35, 10 and 55% of dietary DM, respectively (Table 5.1). The dietary treatments evaluated were the following: 1) no enzyme added (Control), 2) enzyme applied to the concentrate (EC), 3) enzyme applied to the TMR at feeding (ETMR), 4) enzyme applied to the forage at feeding (EF) and 5) enzyme applied at a rate of 1.3 g/kg of DM to bermudagrass at ensiling (TS). Each cow in Treatments 2, 3 and 4 received four g of enzyme/per d. Cows were individually fed twice daily (at 0700 and 1430 h), using Calan gates (American Calan Inc., Northwood, NH). Feed refusals were collected daily at 0600 h. Cows were trained to use calan gates for 10 days before the beginning of the trial**.** Diets were mixed prior feeding using 250 kg Calan Data Rangers (American Calan Inc., Northwood, NH). Three data rangers were used for mixing the diets. One was reserved for the Control diet, another for the TS diet and a third for diets to which enzyme application occurred just before feeding. The latter was washed between feeding Treatments 2, 3 and 4 to avoid cross contamination.

Table 5.1 Ingredient and chemical composition of the basal untreated diet.

Ingredient composition	% Diet DM
Bermudagrass silage	35.0
Corn silage	10.0
Ground corn	27.0
Citrus pulp	5.1
Whole Cottonseed	2.8
Mineral mix[1]	4.4
Biophos (Calcium phosphate)[2]	0.4
SoyPlus[3]	6.6
Soybean meal	8.6
Chemical composition	
DM, %	46.4
CP, % of DM	16.1
ADF, % of DM	27.2
NDF, % of DM	46.5
TDN, % of DM	66.0
NEL, mcal/kg of DM	1.57

[1] Mineral mix contained 26.4% CP, 10.2 Ca, 0.9% P, 3.1% Mg, 1.5% S, 5.1% K, 8.6% Na, 11698 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 2231 mg/kg of Mn, 31 mg/kg of Co, 26 mg/kg of I,
7.9 mg/kg of Se, 147,75 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis).
[2] Biophos contained 15.9% Ca, 21.2% P.
[3] West Central Soy, Ralston, IA.

Tifton 85 bermudagrass (T-85) was mowed after 35 d of regrowth using a CLAAS Disco 3000 TC forage mower (CLAAS of America, Omaha, NE). After wilting for 2 h, the forage was chopped (5-cm particle size) using a CLAAS Jaguar 9000 (CLAAS of America, Omaha, NE) forage harvester. For the TS treatment, the enzyme was applied to bermudagrass as it was packed at a rate of 3 ton/min into an Ag Bag (AG Bag International, Warrenton, OR) using a Versa Bagger (Versa Corporation, Astoria, OR) model ID 1012. Two 62-ton untreated bags were prepared followed by one 46-ton enzyme-treated bag. The forage was ensiled for 35 days before the experiments commenced.

The enzyme-treated concentrate was prepared weekly by dissolving the enzyme in water (1:5 ratio v/v) and spraying the solution on 140 kg of ground corn using a 3.75-l hand sprayer, while the corn was being mixed in a Marion Mixer (Rapids Machinery Co., Marion, IA). The rest of the concentrate ingredients were subsequently mixed with the treated corn in a 900 kg New Holland 355 mixer. Untreated and treated concentrates were stored in metal grain bins (5.5 ton capacity). For the EF and ETMR treatments, the enzyme was dissolved in water (1:10 ratio v/v) and sprayed on the forage and the TMR, respectively, while loaded in a Calan Data Ranger. The enzyme-treated feedstuff was subsequently mixed for 5 min to ensure proper distribution. For EF, the enzyme was applied in the morning to the entire untreated bermudagrass silage that was to be offered during that day.

Sample Collection and Analysis

In Experiment 1, cows were balanced for parity, milk production and DIM and assigned to each treatment at the beginning of the first period. At the end of Period 1, cows were randomly assigned to another treatment with the requirement that no treatment follow the same treatment. Cows were milked thrice daily at 0200, 1000 and 1800 h and milk production (MP) was measured on the last 14 d of each period. Milk samples were collected twice daily on two days during each week in the last 14 d of each period, preserved with potassium dichromate and stored at 4ºC. Milk samples were analyzed by Southeast Milk lab (Belleview, FL) for concentration of fat (MCF), true protein (MCP) and somatic cell counts (SCC) using a Bentley 2000 NIR analyzer (Bentley Instruments Inc., Chaska, MN). Feed efficiency was calculated based on milk production and DMI (kg milk/kg DM fed). Body weight and BCS ranging from 1 (thin) to 5 (obese) were measured on three consecutive days after the 1000 h milking at the beginning and end of

each period. Blood samples (10 ml) were taken using vacutainers by caudal arterio-venipuncture on the last day of each period. Samples were centrifuged at 2500 × g for 20 min and the plasma was frozen at -20 ºC. Concentration of plasma glucose (Glc) was determined using a Technicon Autoanalyzer II (Bran-Luebbe, Elinsford, NY) and method modified from Gochman and Schmitz (1972). In this modification the specificity of glucose oxidase is combined with the peroxidase indication couple (3-methyl-2-benzothiazolinone hydrazone-HCl, MBTH and N-N-dimethylaniline, DMA**,** method N-38). Glucose oxidase initiates reactions which generate hydrogen peroxide that reacts with the peroxidase indicator to form an intensely-colored indamine dye. Blood urea nitrogen (BUN) was determined using an autoanalyzer method (Technicon Industrial systems Autoanalyzer II; Industrial method # 339-01; Tarrytown, NY), which is a modification of the carbamido-diacetyl reaction, described by Coulombe and Favreau (1963). Plasma concentration of β-hydroxybutyrate acid (BHBA) was determined using the procedure described by Williamson et al. (1962).

Chromic oxide ($Cr_2 O_3$) was used for determination of apparent digestibility. Chromium oxide powder weighed into gelatin capsules and was dosed twice daily via balling gun (10 g/dose at 0700 and 1900 h) for 10 consecutive days in each experimental period. Fecal samples (approximately 100 g) were collected during the last 5 days of each period at the time of dosing. Feces were dried to constant weight at 55ºC in a convection oven, ground to pass through a 1-mm screen and a composite sample was made from all ten fecal samples per cow per period. Chromium concentration in feces was determined using a Perkin Elmer 5000 (Wellesley, MA) Atomic Absorption Spectrometer, according to the procedure described by Williams et al. (1962).

Quadruplicate samples of the concentrate, forages and untreated TMR collected during each week of each collection period were composited, sub-sampled and sent to the Dairy One Forage Testing Laboratory (Ithaca, NY) for CP, NDF and ADF analysis. In addition silages were analyzed for non-fiber carbohydrates (NFC), NH_3-N, lactic acid, VFA, pH and TDN.

In Experiment 2, five ruminally fistulated cows were used to evaluate the effect of the dietary treatments on ruminal pH, VFA and ammonia-N concentration and *in situ* rumen degradation, during three consecutive 15-d periods. Each period consisted of 12 d of adaptation to a new diet, 2 days of *in situ* rumen degradability measurements, and 1 d of rumen fluid collection. Rumen fluid was collected (200 ml) by aspiration and filtered through two layers of cheesecloth at 0, 2, 4, 6, 8 and 10 h after feeding on the last day of each period. The pH was measured within 20 min of rumen fluid collection using a pH meter (Accumet, model HP-71, Fisher Scientific, Pittsburgh, PA). The rumen fluid was acidified with 3 ml/sample of H_2SO_4 (50% v/v). Samples were centrifuged at 11,500 × g for 20 min, after which the supernatant was collected and frozen (-20^{o}C) in 20-ml vials. Volatile fatty acids were measured using the method of Muck and Dickerson (1988) and a High Performance Liquid Chromatograph (Hitachi®, FL 7485, Tokyo, Japan) coupled to a UV Detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, NJ) set at 210 nm. The column used was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) column with 0.015M sulfuric acid mobile phase and a flow rate of 0.7 ml/min at 45°C. Ammonia N was determined with a Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA) and adaptation of the Noel and Hambleton (1976) procedure.

During the second and third periods, the rumen degradation kinetics of the experimental diets were measured *in situ* by incubating TMR samples within nylon (50 µm pore size) bags for 0, 2, 4, 6, 8, 24, and 48 h. Dried (65ºC for 48 h), ground (4 mm) diet samples were weighed (5 g as is) into nylon bags in triplicate and incubated in cows fed the same diet during d 13 and 14 of each period. At each incubation time, bags were removed from the rumen and rinsed with cool water and frozen. At the end of each period, all bags were thawed and washed trough a rinse cycle without soap in a washing machine and dried for 48 h at 60ºC. The *in situ* degradation parameters were described with the method of McDonald (1981):

$$P = a + b\ (1-e^{-c\ (t-L)})$$

where

P= DM degraded at time t, a = wash loss of DM at time zero, b = potentially degradable DM fraction, a+b = total degradable fraction, c = the rate at which b is degraded, t = ruminal incubation duration, and L = lag time in hours. The constants a, b, c, and L were estimated using the nonlinear regression procedures of SAS (1995) and analyzed using the GLM procedure (SAS, 1995).

Statistical Analysis

Both experiments involved cross-over designs and the data were analyzed with Proc Mixed (SAS, 1995). The model used for analyzing the results from Experiment 1 and the *in situ* degradability results in Experiment 2 was:

$$Yijk = \mu + Ti + Pj + Ck + Rl + Eijkl$$

Where;

µ: general mean

Ti: treatment effect (fixed effect)

Pj: period effect (fixed)

Ck: cow effect (random effect)

Rl: residual effect

Eijkl: experimental error

For analysis of milk production, each cow's pretrial milk production (129 days in milk) was used as a covariate. Orthogonal contrasts were used to compare each of the enzyme treatments to the Control.

The model used for analyzing rumen VFA and ammonia data in Experiment 2 was:

$Yijk = \mu + Ti + Pj + Hk + Cl + Eijkl$

where

μ: general mean

Ti: treatment effect (fixed effect)

Pj: period effect (fixed)

Hk: time effect (repeated measurement)

Cl: cow effect (random effect)

Eijkl: experimental error

The covariance structure that was used was AR(1), and a slice statement was used to detect differences among treatments at each incubation time. Significance was declared at $P < 0.05$ and tendencies at $P < 0.15$.

Results and Discussion

Chemical Composition of the Dietary Ingredients

The chemical composition of the enzyme-treated and untreated forages and concentrate are shown in Table 5.2. Crude protein, NFC, TDN and organic acid concentrations were greater and fiber and NH3-N concentrations were lower in

enzyme-treated than in untreated bermudagrass silage. This suggests that the enzyme improved the fermentation indices of the bermudagrass silage

Voluntary Intake

Promote addition did not affect the intake of DM, NDF (NDFI) or CP (CPI) (Table 5.3). The DMI response is consistent with the results of Rode et al. (1999) who fed Promote-supplemented diets to lactating cows in early lactation. The lack of effect enzyme supplementation on DMI also concurs with those observed by Kung et al. (2000), Yang et al. (2000), Kung et al. (2002) and Sutton et al. (2003). Nevertheless, Beauchemin et al. (2000) observed that adding a low or high amount of an enzyme supplement to the diet increased ($P < 0.01$) DMI, and OM digestibility, and also tended to increase intake of NDF ($P = 0.17$) and ADF ($P = 0.14$). These increases were relatively small (20 and 8% for NDF and ADF, respectively) and they may be attributable to increased palatability or rate of passage. More conclusive results were obtained by Lewis et al. (1999) who detected increased ($P < 0.05$) DMI due to increasing amounts of enzyme supplementation. Knowlton et al. (2002) found that DMI was numerically higher in enzyme-supplemented cows in early lactation but not in late-lactation cows. *In vivo* Digestibility of DM, NDF, and CP

Apparent total tract digestibilities of DM, NDF and CP were unaffected by enzyme treatment (Table 5.3). Digestibility is one of the main determinants of voluntary intake; therefore, the ineffectiveness of the enzyme at improving digestibility helps explain the lack of enzyme effect on voluntary intake. These results agree with those obtained by Hristov et al. (1998) and Lewis et al. (1999). Knowlton et al. (2002) also observed that digestibility of DM was not different between Control and enzyme-supplemented diets fed to cows in early or late lactation. Beauchemin et al. (2000) found

that digestion of the TMR measured *in situ* was not affected by enzyme supplementation. However, Rode et al. (1999) observed that digestibility of a TMR consisting of corn silage (24%), alfalfa hay (15%) and concentrate (47%), determined using Cr_2O_3, was increased dramatically by enzyme treatment (DM, 61.7 vs. 69.1%; NDF, 42.5 vs. 51.0%; ADF, 31.7 vs. 41.9%; CP, 61.7 vs. 69.8%). Similarly Beauchemin et al. (1999) reported that applying enzymes to the TMR before feeding increased digestibility in the total tract due to greater post-ruminal digestion. Sutton et al. (2003) observed that rumen digestibility of DM and OM were unaffected by the enzyme addition; however, total tract digestibility of DM and OM were higher when enzyme was applied to the TMR. In the latter study, enzyme addition reduced NDF digestibility in the rumen but increased it postruminally, and did not affect total tract NDF digestibility. This was probably because the enzymes were not degraded in the rumen and therefore they exerted their fibrolytic action in the small intestine. The NDF and lignin concentrations of bermudagrass silage are greater than that of the corn silage or the temperate forages used in the studies cited above. The negative effect of lignin on the digestibility of the fiber fraction may partly explain ineffectiveness of the enzyme at improving fiber digestion in this experiment. Some fiber particles cannot be ruminally digested irrespective of rumen retention time due to physical barriers to digestion imposed by ferulate cross linkages between lignin and polysaccharides (Buxton and Redfern, 1997).

Effect of Promote on Milk Production and Composition

Production of milk and 4% FCM were not different among diets, though cows fed the Control diet tended ($P < 0.15$) to produce more milk than those fed EC or EF (Table 5.4). This result agrees with that of Beauchemin et al. (1999). However, others have reported a tendency for greater milk production when fibrolytic enzymes were

Table 5.2 Chemical composition of the enzyme-treated and untreated forages and concentrate (% DM) (n= 4 replicates per mean)

	Chemical composition										
Item	DM, %	CP	NH_3-N[1]	NDF	ADF	NFC	TDN	pH	Lactic Acid	Acetic Acid	Butyric Acid
Corn silage	28.1	8.8	12.0	45.2	27.0	38.1	79.0	4.8	3.08	6.68	0.07
Pre-ensiled bermudagrass	23.4	12.7	-	76.1	45.3	7.85	43.0	-	-	-	-
Untreated bermudagrass silage	29.9	9.3	38.0	81.8	49.9	5.3	52.7	8.4	0.10	0.05	0
Treated bermudagrass silage	29.6	11.4	13.7	76.2	45.2	8.6	55.0	4.6	1.77	3.08	0.18
Untreated concentrate	88.3	21.9	-	15.8	8.55	-	84.5	-	-	-	-
Treated concentrate	88.4	21.5	-	15.6	8.15	-	84.0	-	-	-	-

[1] As percentage of total N,

Table 5.3 Effect of method of enzyme addition on diet digestibility and voluntary intake

	Variable							
Treatment	DMI, kg/d	DMI, % BW	NDFI, kg/d	CPI, kg/d	DMD, %	NDFD, %	CPD, %	FE, kg milk/kg DMI
Control	20.9	3.35	9.7	3.4	66.4	50.7	65.6	1.64
EC	21.6	3.46	9.9	3.5	64.2	51.0	65.7	1.46
ETMR	22.4	3.65	10.0	3.5	66.3	50.4	66.9	1.42
EF	19.9	3.18	9.0	3.1	64.3	51.6	65.7	1.64
TS	21.8	3.41	9.5	3.3	68.3	48.7	67.4	1.59
s.e	0.92	0.18	0.61	0.21	1.37	2.29	2.11	0.13
	P values							
Treatment effect	0.298	0.397	0.369	0.370	0.139	0.924	0.866	0.256
Contrasts								
Control vs. EC	0.560	0.632	0.621	0.621	0.930	0.918	0.970	0.153
Control vs. ETMR	0.223	0.209	0.215	0.215	0.777	0.926	0.551	0.082
Control vs. EF	0.404	0.481	0.440	0.440	0.797	0.756	0.959	0.962
Control vs. TS	0.447	0.797	0.717	0.717	0.498	0.545	0.402	0.697

EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, TS: enzyme-treated silage: DMD: dry matter digestibility, NDFD: neutral detergent fiber digestibility, CPD: crude protein digestibility, DMI: dry matter digestibility, RIN: relative dry matter intake, FE: feed efficiency.

applied to the concentrate (Yang et al., 2000) or to the TMR (Sutton et al., 2003). Kung et al. (2002) observed that milk production was unaffected ($P < 0.05$) by enzyme treatment of a diet consisting of corn silage (30%), alfalfa hay (15%) ans a concentrate (55%), but cows fed forage treated with a mixture of cellulase and xylanase produced 2.5 kg more 3.5% FCM ($P < 0.12$) than those fed untreated forage. Lewis et al. (1999) found that cows assigned to an enzyme supplemented diet produced more ($P < 0.01$) milk than did cows fed the Control diet (27.2 vs. 25.9 kg/d), indicating that enzyme treatment increased nutrient availability for milk production.

Table 5.4 Effect of method of enzyme addition on milk production and composition

Treatment	Variable						
	Milk, kg/d	4% FCM, kg/d	Milk fat, %	Milk fat, kg/d	Milk protein %	Milk protein, kg/ d	SCC 10^3 cells/ml
Control	33.1	31.8	3.67	1.23	2.91	0.956	339
EC	30.9	29.9	3.78	1.16	3.07	0.948	488
ETMR	32.3	32.4	3.99	1.29	3.07	0.997	581
EF	31.2	30.0	3.77	1.64	3.03	0.933	817
TS	32.3	30.6	3.72	1.19	2.90	0.923	458
s.e	1.282	1.387	0.124	0.064	0.092	0.034	263
	P values						
Treatment effect	0.432	0.265	0.417	0.195	0.257	0.240	0.531
Contrasts							
Control vs. EC	0.102	0.171	0.533	0.282	0.076	0.821	0.264
Control vs. ETMR	0.521	0.677	0.073	0.330	0.081	0.235	0.530
Control vs. EF	0.131	0.175	0.587	0.273	0.187	0.496	0.101
Control vs. TS	0.512	0.404	0.787	0.503	0.917	0.345	0.581

EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, TS: enzyme-treated silage, SCC: somatic cell counts.

Furthermore, Jurkovich et al. (2002) obtained an increase of between 5 to 10% in milk production when lactating cows in early lactation were supplemented with a lignolytic enzyme. Schingoethe et al. (1999) also found that production of milk ($P = 0.12$) and FCM ($P < 0.01$) increased in enzyme supplemented cows, and the responses to enzyme-treated forages occurred 2 to 4 wk after the cows started to consume the treated forages. In the same study, cows that started to receive enzyme-treated forage during the first 100 d postpartum produced 9 to 15% more milk than cows fed the untreated diet in the same time frame. However, milk production was unaffected when cows were in mid-lactation at the start of the experiment. Therefore, the duration of the adaptation period (2 wk) and the stage of lactation (129 DIM) of cows in this study may have affected the milk response to enzyme addition. Efficiency of feed conversion into milk tended ($P = 0.082$) to be lower in cows fed ETMR compared to those fed the Control diet. Kung et al.

(2000) observed that treatment with enzymes did not improve feed efficiency relative to cows fed the Control diet.

Milk fat and true protein yields were unaffected ($P > 0.05$) by enzyme supplementation, however, cows fed ETMR tended ($P < 0.09$) to have greater milk fat and protein concentration than cows fed the Control diet. Cows fed EC also tended ($P = 0.076$) to have greater concentration of milk protein than those fed the Control diet. Tendencies for lower milk fat percentage from cows fed enzyme-treated diets have been reported by Rode et al. (1999). In contrast, Lewis et al. (1999), Zheng et al. (2000), Jurkovich et al. (2002), and Kung et al. (2002) observed that milk composition was similar between Control and enzyme-supplemented cows. Sutton et al. (2003) observed that compared to values in cows fed a Control diet, milk protein concentration was greater ($P < 0.05$) when enzyme was applied to the concentrate, whereas milk protein yield was greater when the enzyme was applied to the TMR or to the concentrate. Schingoethe et al. (1999) found that milk production was 10.8% greater ($P=0.12$) in enzyme supplemented cow diets relative to Control diets and production of milk fat and protein increased by 20 and 13%, respectively. The tendency for greater milk constituent concentrations in cows fed ETMR vs. Control cows, suggests that this mode of enzyme addition is promising and warrants further study. Somatic cell counts were unaffected ($P > 0.05$) by enzyme treatment, in agreement with results of Schingoethe et al. (1999).

Body Weight Gain and Body Condition Score

The BWG and BCS of the cows were unaffected by enzyme treatment (Table 5.5). Knowlton et al. (2002) found that enzyme addition did not affect BW, but noted a tendency ($P < 0.07$) for an interaction between the effects of stage of lactation and enzyme treatment. This tendency was due to a numerical increase in BW in early

lactation cows fed diets containing the enzyme compared to those fed the Control diet, whereas BW in late lactation cows was unaffected by enzyme treatment. Kung et al. (2002) observed that BW and ADG over a 12-wk treatment period were unaffected by treatment of diet with mixtures of xylanases and cellulases. However, Jurkovich et al. (2002) observed that body condition loss in cows fed enzyme-treated diets was lower than in those fed Control diets. According to these researchers, this was probably due to the enhanced ruminal VFA concentration of cows supplemented with fibrolityc enzymes which implies greater energy availability.

Cows assigned to the EC treatment tended ($P < 0.015$) to have greater BW gain than Control, probably because they partitioned more nutrients away from MP to BWG. However this did not result in improved BCS.

Blood Glucose, Urea-N and β-Hydroxybutyrate

Plasma glucose concentration (Table 5.5) was similar across treatments, in agreement with the results of Hristov et al. (1998) and (2000). The mean glucose concentration of the cows in this experiment was similar to the basal concentration reported by Lemosquet et al. (1996) in Holstein cows in mid-late lactation that were producing 34 kg of milk daily. However, cows fed EF and TS had lower ($P < 0.05$) BUN concentrations than those fed the Control diet and cows fed ETMR had a similar tendency ($P = 0.123$). This suggests that there was greater ruminally fermentable metabolizable energy availability from these diets, leading to improved microbial efficiency of N utilization.

Enzyme treatment tended ($P < 0.112$) to reduce BHBA concentration. The concentration of this blood metabolite was particularly lower ($P < 0.01$) in cows fed ETMR, which indicates that this treatment decreased fat mobilization in the cows.

Table 5.5 Effect of method of enzyme addition on body weight and condition score, and blood metabolites

	Variable					
Treatment	BW, kg	BW gain, kg/d	BCS	BUN, mg/dl	Glc, mg/dl	BHBA, mg/dl
Control	633	0.198	2.82	16.9	64.5	0.931
EC	635	0.638	2.61	16.0	62.9	0.877
ETMR	624	0.421	2.77	15.6	64.6	0.732
EF	618	0.205	2.64	15.2	64.5	0.826
TS	623	0.310	2.83	15.2	64.5	0.833
s.e	21.47	0.204	0.157	0.625	1.096	0.074
			P values			
Treatment effect	0.961	0.462	0.477	0.232	0.774	0.112
Contrasts						
Control vs. EC	0.938	0.138	0.175	0.282	0.294	0.467
Control vs. ETMR	0.723	0.451	0.737	0.123	0.967	0.010
Control vs. EF	0.565	0.981	0.251	0.049	0.994	0.154
Control vs. TS	0.716	0.696	0.962	0.047	0.982	0.195

EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, TS: enzyme-treated silage, BW: body weight, BCS: body condition score, BUN: blood urea nitrogen, Glc: blood glucose, BHBA: beta hydroxy butyrate.

This result concurs with that obtained by Hristov et al. (2000) who observed that concentration of plasma BHBA was reduced ($P < 0.01$) in cows supplemented with enzymes. Jurkovich et al. (2002) also found a lower incidence of hyperketonaemia, and lower concentrations of acetoacetic acid and non-esterified fatty acid (NEFA) in the blood of cows supplemented with a mixture of fibrolytic enzymes.

Ruminal pH and Concentration of VFA and NH_3-N

Mean ruminal pH was more acidic in cows fed EC ($P < 0.01$) than in those fed the Control diet and a similar tendency ($P < 0.08$) was evident in cows fed EF or TS. The pH decreased ($P < 0.01$) after feeding (Figure 5.1 and Table 5.6), and the lowest ($P < 0.05$) values were observed at 8 and 10 h after feeding in all cows. This result concurs with that of Hristov et al. (2000), who observed that ruminal pH decreased linearly ($P < 0.01$)

to a greater degree due to enzyme supplementation and the response depended on time of sampling**.** However, Bowman et al. (2003) and Sutton et al. (2003) did not detect enzyme effects on daily mean pH, though Sutton et al. (2003) found that pH was numerically lower in cows fed enzyme-treated diets. Ruminal pH fell below 6 within 6-h of feeding in cows fed EC and remained at 5.5 between 8 and 10 h after feeding. Values for cows fed EF and TS also fell below 6 by 7 h after feeding and dropped to a nadir of 5.75 10 h after feeding. A pH of 5 - 5.8 is used often to indicate sub-clinical ruminal acidosis in dairy cows (Oetzel et al., 1999). Therefore, cows fed EC and to a lesser extent FF and EC were most likely to have experienced sub-clinical ruminal acidosis. This partially explains the tendency for lower MP of cows fed EC and EF.

Generally, NH_3-N concentration increased after feeding and subsequently decreases progressively, presumably due to N uptake by ruminal microbes for microbial protein synthesis (Figure 5.2 and Table 5.7). Ruminal NH_3-N concentration was lower in cows fed ETMR ($P < 0.05$) than in cows fed the Control diet. The reduced ruminal NH_3-N concentration in cows fed ETMR suggests that there was enhanced uptake of NH_3-N by the ruminal microbes probably due to greater fermentable metabolisable energy availability. Hristov et al. (2000) reported that ruminal ammonia N was below 25 mg/L in their study and responded quadratically ($P < 0.01$) to enzyme supplementation.

Ammonia-N concentration decreased ($P < 0.01$) after feeding in the study of Sutton et al. (2003) but was numerically greater in cows fed enzyme supplemented diets instead of Control diets.

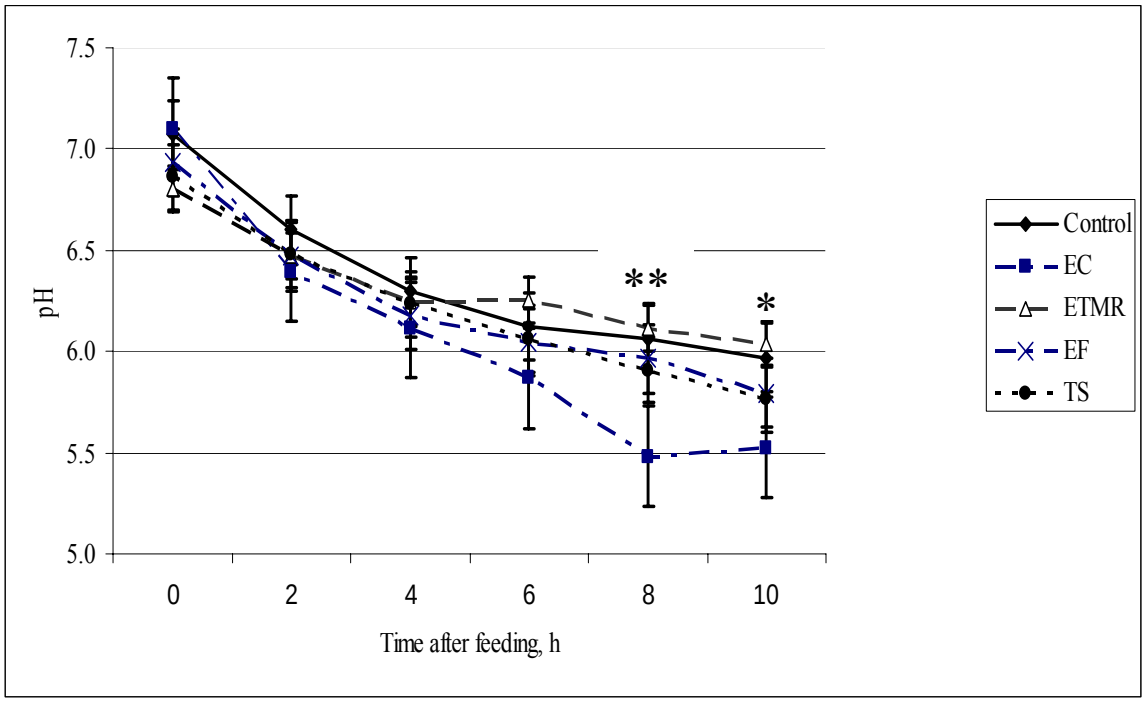

Figure 5.1 Effect of method of enzyme addition on ruminal fluid pH

** P < 0.01
* P < 0.05

Table 5.6 Effect of method of enzyme addition on ruminal fluid pH

Treatment	Mean
Control	6.35
EC	6.08
ETMR	6.32
EF	6.23
TS	6.22
	P value
Treatment effect	< 0.01
Time effect	< 0.01
Contrasts	
Control vs. EC	< 0.01
Control vs. ETMR	0.514
Control vs. EF	0.075
Control vs. TS	0.052

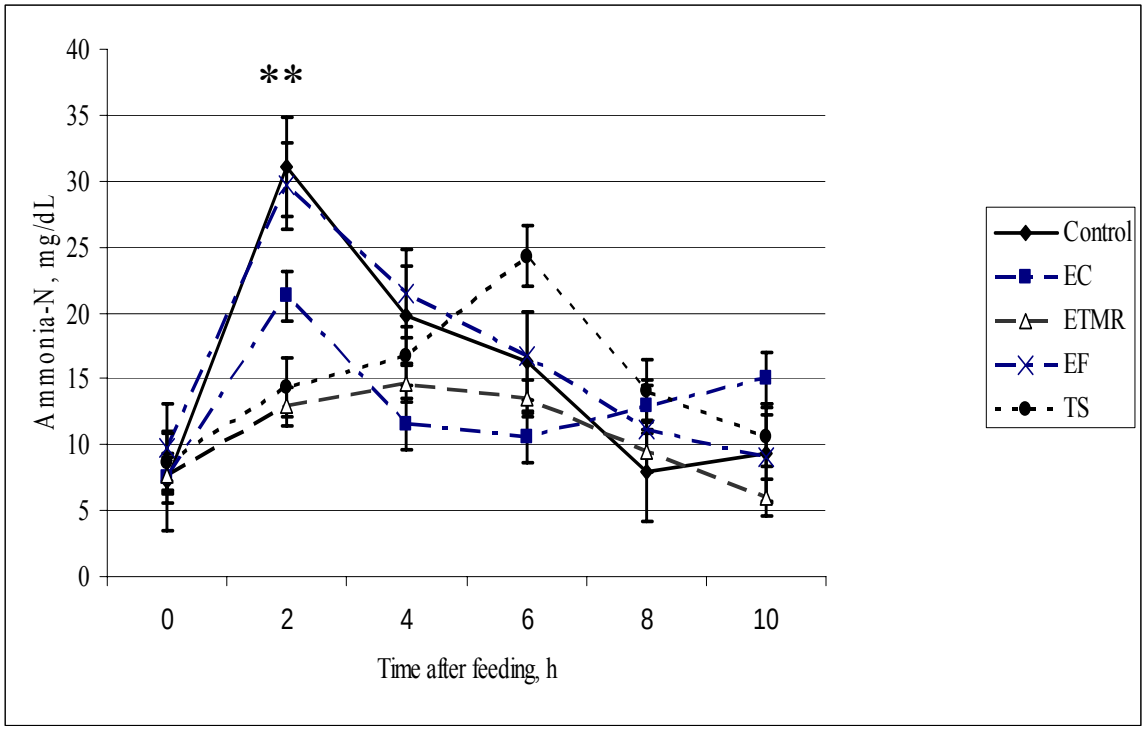

Figure 5.2 Effect of method of enzyme addition on ruminal NH3-N concentration

** P < 0.01

Table 5.7 Effect of method of enzyme addition on ruminal NH_3-N concentration

Treatment	Mean
Control	15.4
EC	13.3
ETMR	10.7
EF	16.3
TS	14.8
	P value
Treatment effect	0.041
Time effect	< 0.01
Contrasts	
Control vs. EC	0.356
Control vs. ETMR	0.023
Control vs. EF	0.615
Control vs. TS	0.814

Mean ruminal concentration of acetic acid (Figure 5.3 and Table 5.8) was lower in cows fed ETMR ($P < 0.05$) and EF ($P < 0.01$) than in Control cows. However, propionic (Figure 5.4 and Table 5.9) and butyric acids (Figure 5.5 and Table 5.10) concentrations were unaffected ($P > 0.05$) by dietary treatment or time after feeding. Ruminal fluid acetate:propionate ratio was lower in cows fed ETMR diets ($P < 0.01$) rather than the Control diet (Figure 5.6 and Table 5.11), which indicates that the ETMR diet promoted a more efficient fermentation in the rumen, which probably was due to a higher release of soluble carbohydrates by the ETMR treatment. Rapidly fermentable carbohydrates yield relatively higher ruminal propionate as compared to acetate, and thereby lower the acetate:propionate ratio. The highest ($P < 0.01$) acetate:propionate ratio was observed in Control diets at 10 h. This result disagrees with that of Dawson and Tricarico (1999) who found that treating tall fescue hay with preparations high in either xylanase or cellulase activity increased the acetate:propionate ratio.

Mean total VFA concentration was lower ($P < 0.05$) in cows fed the ETMR or in TS diets rather than the Control diet (Figure 5.7 and Table 5.12). This result contradicts that of Dawson and Tricarico (1999) and Pinos-Rodriguez et al. (2002), who found that enzyme supplementation increased ruminal VFA concentration. Hristov et al. (2000) found that enzyme supplementation produced a cubic effect on acetate and total VFA concentrations and these measures were elevated ($P < 0.01$) after feeding, while Sutton et al. (2003) did not detect treatment effects on total VFA concentration.

Jurkovich et al. (2002) found that enzyme supplementation increased VFA concentration in the rumen from about 32 days after calving leading to improved milk

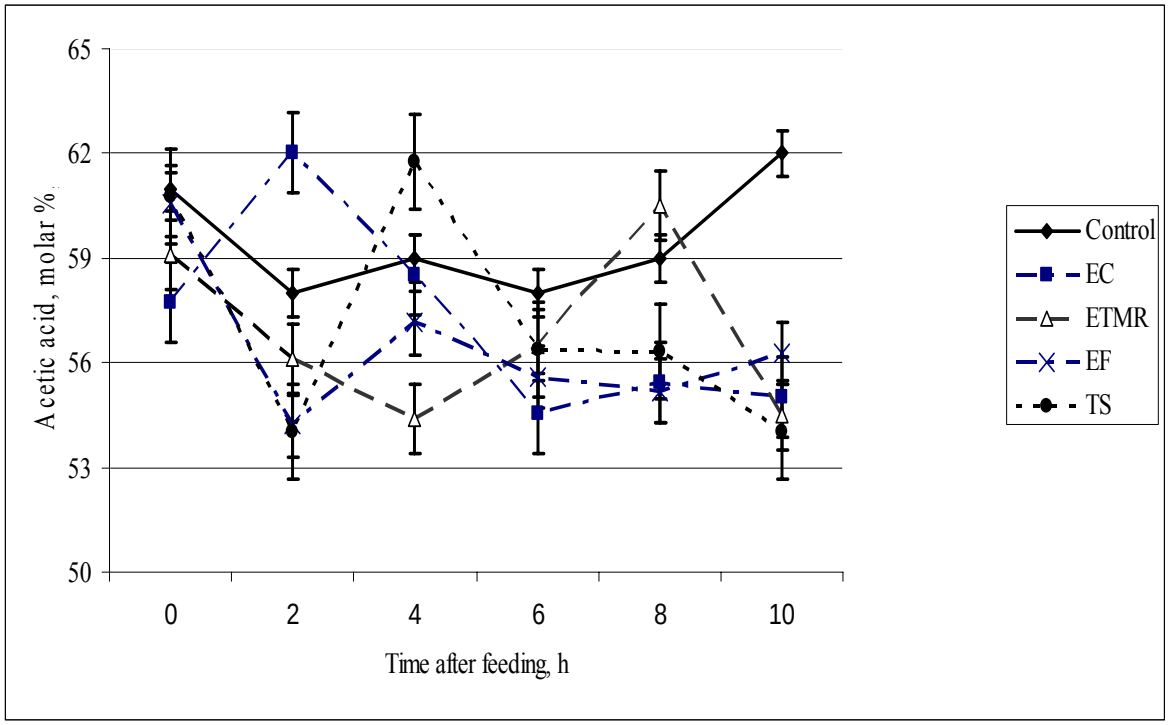

Figure 5.3 Effect of method of enzyme addition on ruminal acetic acid molar percentage

Table 5.8 Effect of method of enzyme addition on ruminal acetic acid molar percentage

Treatment	Mean
Control	58.2
EC	57.3
ETMR	54.8
EF	54.2
TS	57.8
	P value
Treatment effect	< 0.01
Time effect	< 0.05
Contrasts	
Control vs. EC	0.398
Control vs. ETMR	0.017
Control vs. EF	0.004
Control vs. TS	0.792

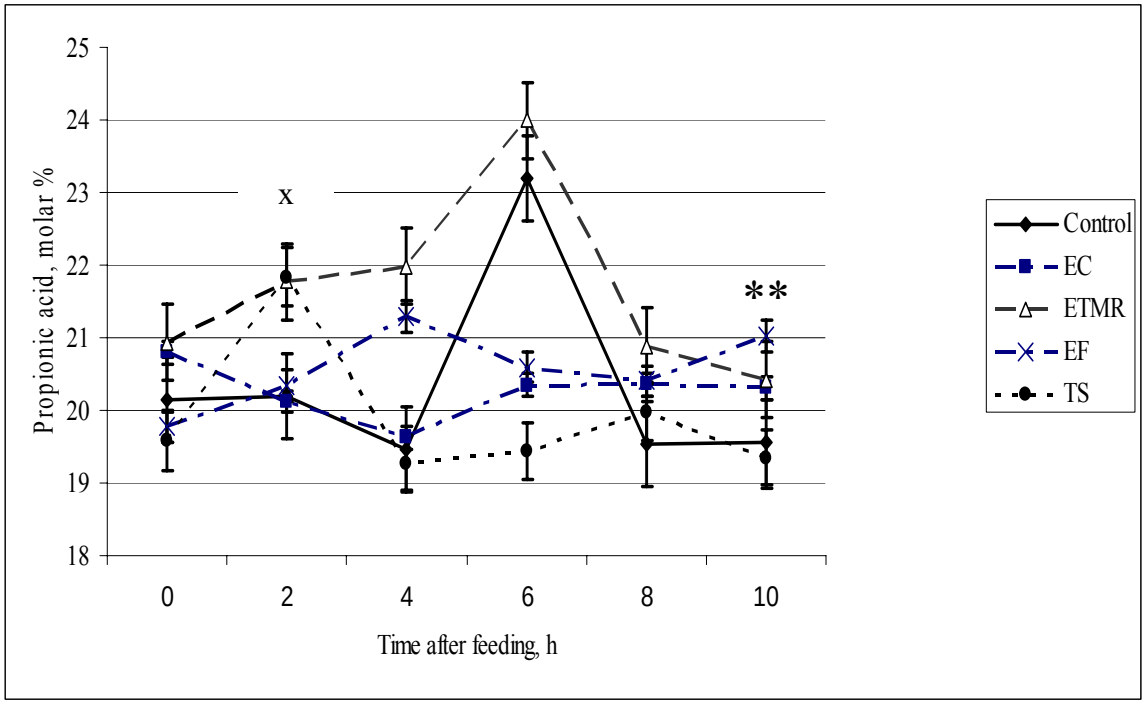

Figure 5.4 Effect of method of enzyme addition on ruminal propionic acid molar percentage

x: P = 0.071,
** P < 0.01

Table 5.9 Effect of method of enzyme addition on ruminal propionic acid molar percentage

Treatment	Mean
Control	20.4
EC	20.3
ETMR	21.7
EF	20.6
TS	19.9
	P value
Treatment effect	0.534
Time effect	0.626
Contrasts	
Control vs. EC	0.970
Control vs. ETMR	0.170
Control vs. EF	0.506
Control vs. TS	0.922

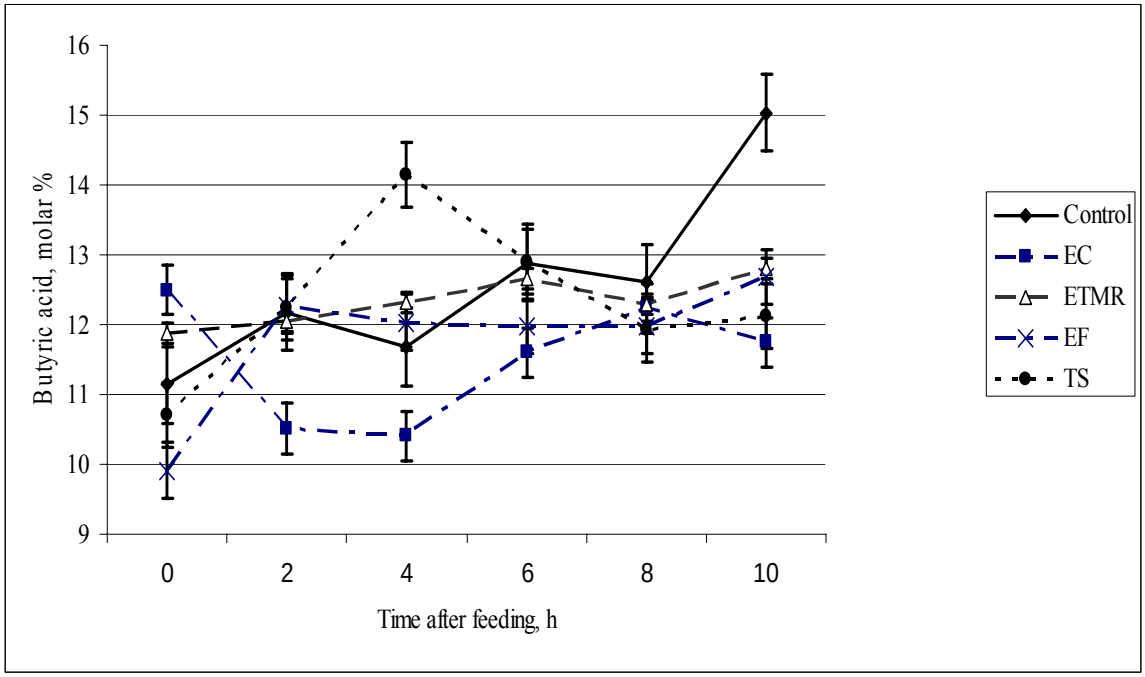

Figure 5.5 Effect of method of enzyme addition on ruminal butyric acid molar percentage

Table 5.10 Effect of method of enzyme addition on ruminal butyric acid molar percentage

Treatment	Mean
Control	12.8
EC	11.5
ETMR	12.3
EF	11.8
TS	12.3
	P value
Treatment effect	0.164
Time effect	0.069
Contrasts	
Control vs. EC	0.278
Control vs. ETMR	0.866
Control vs. EF	0.956
Control vs. TS	0.960

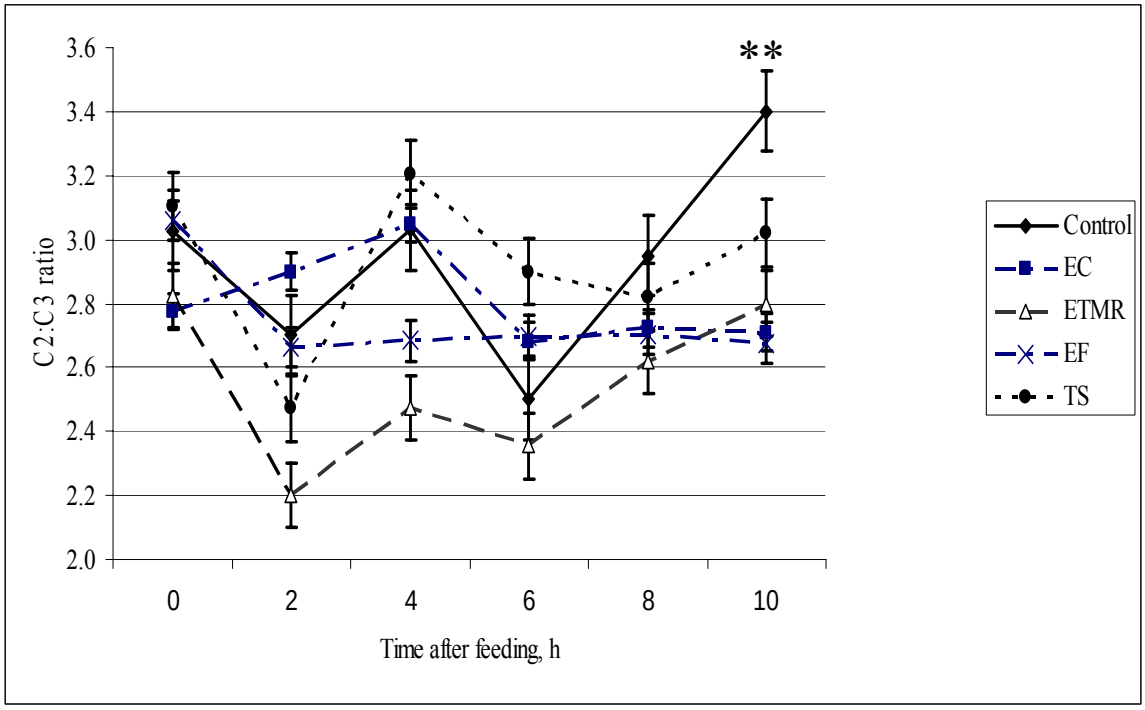

Figure 5.6 Effect of method of enzyme addition on ruminal acetic:propionic acid ratio

** P < 0.01

Table 5.11 Effect of method of enzyme addition on ruminal acetic:propionic acid ratio

Treatment	Mean
Control	2.9
EC	2.8
ETMR	2.5
EF	2.8
TS	2.9
	P value
Treatment effect	< 0.01
Time effect	0.106
Contrasts	
Control vs. EC	0.796
Control vs. ETMR	<0.01
Control vs. EF	0.025
Control vs. TS	0.984

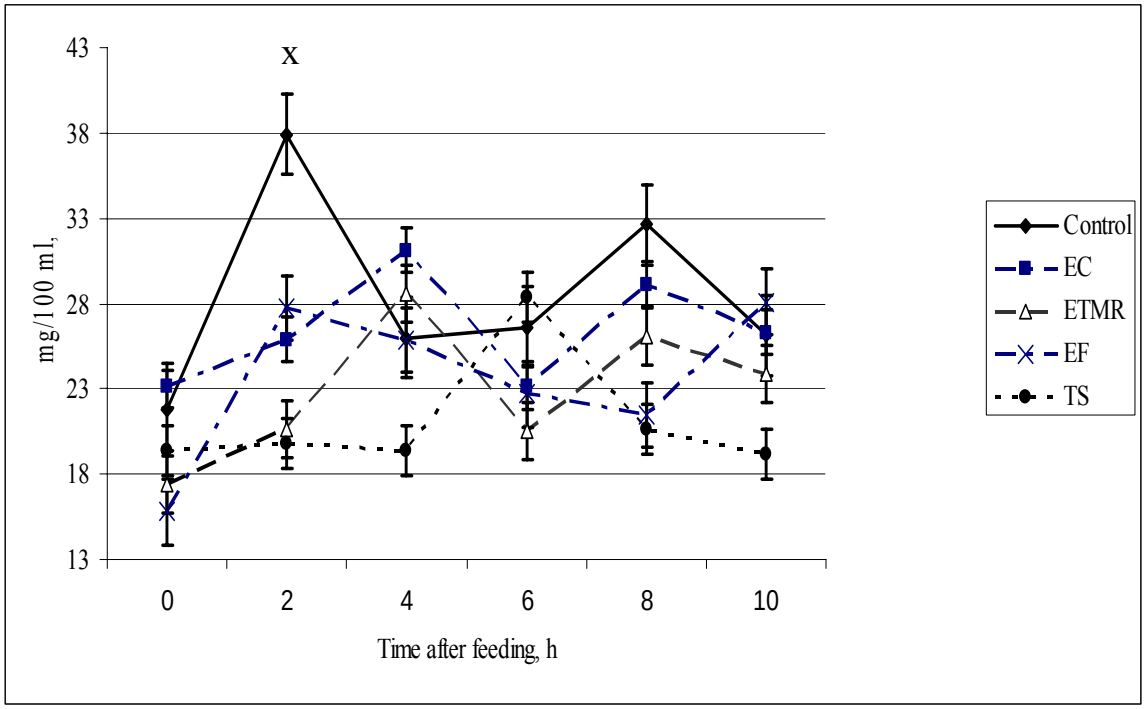

Figure 5.7 Effect of method of enzyme addition on total VFA concentration

X: $P < 0.10$

Table 5.12 Effect of method of enzyme addition on total VFA concentration

Treatment	Mean
Control	28.5
EC	26.4
ETMR	22.8
EF	23.6
TS	21.1
	P value
Treatment effect	0.139
Time effect	< 0.05
Contrasts	
Control vs. EC	0.466
Control vs. ETMR	0.041
Control vs. EF	0.192
Control vs. TS	0.410

yield and butterfat concentrations. This was because the greater VFA concentration improved energy balance in the experimental cows as indicated by lower incidence of hyperketonaemia and acetoacetic acid and lower non-esterified fatty acid (NEFA) concentration in the blood. In this study, the lower acetate:propionate ratio in cows fed ETMR, partly explains their lower BHBA values and higher ($P < 0.15$) milk fat concentrations relative to cows fed the Control diets. The lower total VFA ($P > 0.05$) concentration of cows fed ETMR is also attributable to lower acetate production and similar or greater propionate production than that in cows fed the Control diet. Branched-chain VFA concentrations were unaffected ($P > 0.05$) by enzyme treatment, except for greater isovaleric acid concentrations in cows fed EC, TS or EF rather than the Control diet (Figures 5.8 and 5.9, and Tables 5.13 and 5.14, respectively).

In situ DM disappearance

The kinetics of *in situ* feed DM disappearance of the experimental diets were unaffected

($P > 0.05$) by enzyme supplementation (Table 5.16) though the rate of degradation of TS tended to be greater ($P = 0.107$) than that of the Control diets. Feng et al. (1996) observed that *in situ* disappearance of DM of cool-season grasses was not altered by treatment with cellulase, xylanase and a mixture of both enzymes. Similarly, Adesogan et al. (2005) observed that increasing the application rate of an esterase enzyme to bermudagrass hays did not affect the fermentation rate of the hays using the gas technique, but enzyme treatment linearly increased ($P < 0.05$) the lag phase. Hristov et al. (1998) observed that the soluble, readily degradable fraction of DM was greater

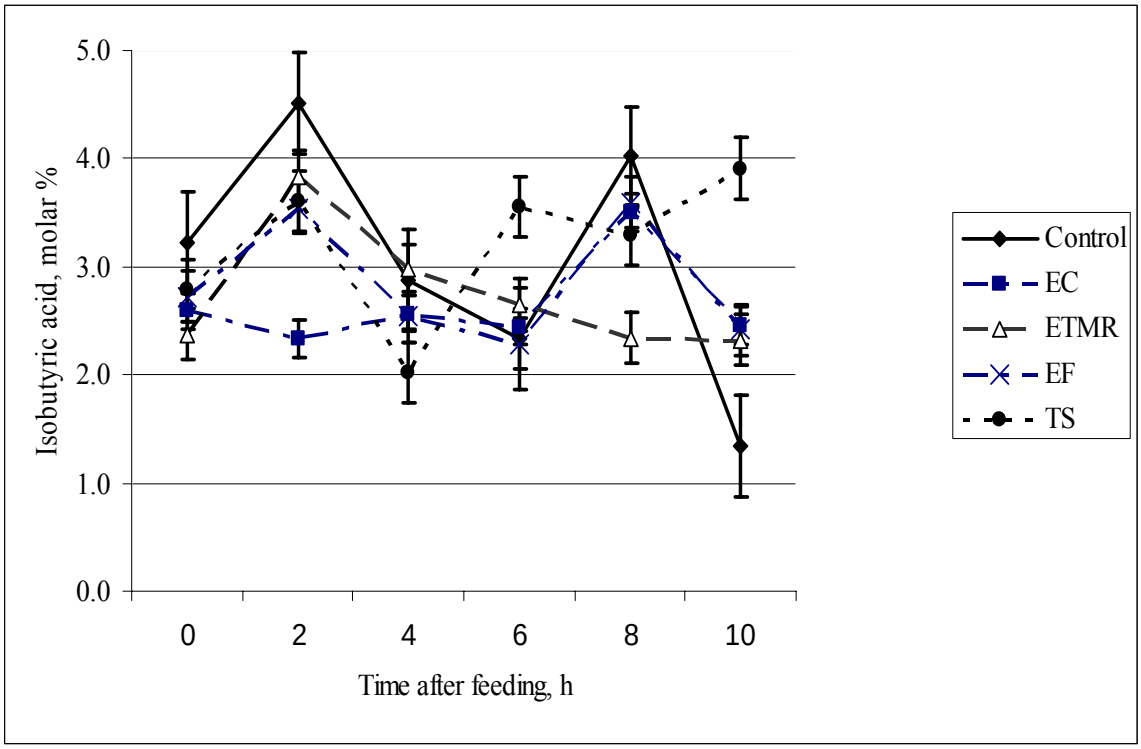

Figure 5.8 Effect of method of enzyme addition on ruminal isobutyric acid molar proportion

Table 5.13 Effect of method of enzyme addition on ruminal isobutyric acid molar proportion

Treatment	Mean
Control	3.06
EC	2.66
ETMR	2.75
EF	2.85
TS	3.19
	P value
Treatment effect	0.827
Time effect	0.123
Contrasts	
Control vs. EC	0.449
Control vs. ETMR	0.418
Control vs. EF	0.317
Control vs. TS	0.212

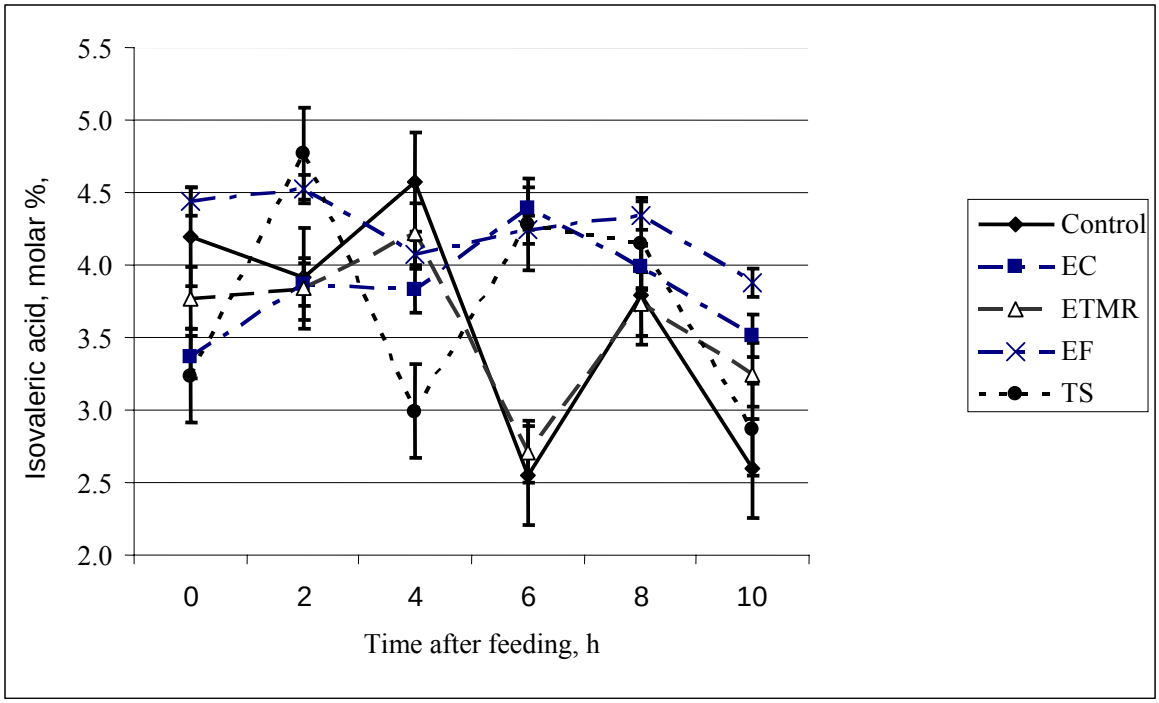

Figure 5.9 Effect of method of enzyme addition on ruminal isovaleric acid molar percentage

Table 5.14 Effect of method of enzyme addition on ruminal isovaleric acid molar percentage

Treatment	Mean
Control	2.99
EC	3.22
ETMR	3.13
EF	4.25
TS	3.71
	P value
Treatment effect	0.067
Time effect	0.057
Contrasts	
Control vs. EC	< 0.05
Control vs. ETMR	0.192
Control vs. EF	< 0.01
Control vs. TS	< 0.05

Table 5.15 Effect of method of enzyme addition on kinetics of in situ feed DM disappearance in lactating Holstein Cows

Treatments	Parameters					
	a, %	b, %	a + b, %	P, %	c, per h	L, h
Control	40.3	38.9	79.2	74.7	0.061	2.6
EC	36.2	38.5	74.7	72.5	0.058	3.6
ETMR	39.3	38.4	77.7	73.2	0.063	2.3
EF	36.2	36.6	72.8	59.0	0.079	4.2
TS	36.4	40.9	77.3	68.3	0.112	5.6
s.e.m.						
			P values			
Treatment effect	0.544	0.936	0.721	0.685	0.336	0.589
Contrasts						
Control vs. EC	0.307	0.987	0.486	0.854	0.930	0.647
Control vs. ETMR	0.723	0.947	0.765	0.899	0.924	0.907
Control vs. EF	0.318	0.578	0.236	0.236	0.507	0.494
Control vs. TS	0.161	0.829	0.751	0.606	0.107	0.216

EC: enzyme applied to concentrate, ETMR: enzyme applied to the total mixed ration, EF: enzyme applied to forage at feeding, TS: enzyme-treated silage, DM: dry matter, a: soluble fraction, b: insoluble but potentially degradable fraction, a + b= total degradability, P: DM degraded at time t, c: rate of constant degradation, L: lag phase

($P < 0.05$) in an enzyme-treated TMR, than in the untreated TMR (29.6 vs. 24.0%), but the insoluble potentially degradable fraction was similar in both diets (56.6 vs. 56.2%). These results contradict those of Colombatto et al. (2001) who observed that Promote treatment did not increase the *in vitro* DM digestibility of corn silage that was incubated for durations of 0 to 30 h in buffered rumen fluid, but did increase DM digestibility after 48-h of incubation.

The values for the soluble, readily degradable and potentially degradable fractions in this study are higher than those of Hristov et al. (1998) probably due to differences in dietary composition. Promote supplementation was not effective at improving the degradation of the bermudagrass silage. However, the numerical improvements in degradation rate and lag phase of ETMR vs. Control are consistent with tendencies for lower

acetate:propionate ratio, BHBA and BUN concentrations, and greater milk fat and protein concentrations in cows fed ETMR instead of the Control diet.

According to Russell and Wilson (1996) when ruminal pH falls below 6, fiber digestion declines for two reasons: firstly, the enzymes necessary for fiber degradation do not function effectively, and secondly, the growth rate of fibrolytic bacteria declines markedly. Therefore the occurrence of periods of low (< 6) pH in cows on EC, EF and TS partly explain the lack of degradation responses to enzyme addition.

Conclusions

Enzyme supplementation was ineffective ($P > 0.05$) at improving rumen function, in situ degradability and voluntary intake of the diets. Therefore, milk production and composition, FCM, BW gain, BCS and blood glucose and urea-N, were unaffected by enzyme supplementation. However, the ETMR treatment merits further study because compared to the Control treatment it resulted in numerically greater FCM and tendencies for greater concentrations of milk fat and milk protein, lower BHBA, BUN and lower acetate:propionate ratios.

CHAPTER 6
GENERAL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Many studies have shown that the nutritive value of tropical grasses is less than that of temperate perennial grasses, mainly due to differences in composition of cell wall polysaccharides and anatomy. Different chemical and biological treatments have been used to try to enhance the nutritive value of tropical forages. Biological treatments have been less successful than chemical treatments at increasing cell wall hydrolysis and improving nutritive value, but the hazards involved have limited the adoption of chemical treatment methods. The aim of these experiments was to determine whether fibrolytic enzyme application can improve the nutritive value of tropical forages, and therefore enhance the performance of livestock fed such forages. Five experiments were conducted in the study.

The objective of Experiments 1 and 2 was to evaluate the effect of applying ammonia or four fibrolytic enzymes at different rates on the nutritive value of two C_4 grass hays. In the first experiment, NH_3 or a fibrolytic enzyme (Promote) were evaluated, and in the second experiment, ammonia or three fibrolytic enzymes (Biocellulase X-20, Cattle-Ase and Biocellulase A-20) were evaluated. The effects of these treatments on chemical composition and DM, NDF and ADF digestibility of 12 week-regrowths of Coastal bermudagrass (*Cynodon dactylon*) hay (BE) and Pensacola bahiagrass (*Paspalum notatum*) hay (BA) were determined. The ammonia was applied at 40 g/kg DM and the enzymes were applied at 0 (Control) 0.5, 1 and 2 times the rates

recommended by the respective manufacturers. The hays were stored for three weeks after enzyme application.

The forages had low CP and high NDF, ADF, hemicellulose and lignin concentrations as usual in mature tropical grasses. Ammonia and Promote application decreased the NDF concentration of BA, however Promote treatment increased the NDF concentration of BE, probably because this product contained other non-fibrolytic enzymatic activities. The reason why both treatments reduced the NDF fraction of BA but not BE is probably due to lower ADF and lignin concentrations of BA, as well as anatomical differences between the two grasses. The hemicellulose concentration of BE was increased by Promote and NH_3 treatment, while that of BA was decreased by NH_3 treatment. Concentration of WSC was increased by Pr and NH_3 treatment in BE hay, and NH_3 treatment in BA hay. Therefore the response to treatment was dependent on forage type. Ammoniation was more effective than enzyme treatment at hydrolyzing the fiber fraction of the forages. In both forages, CP concentration was unaffected by enzyme treatment, but higher values were observed in the NH_3-treated hays, due to fixation of the supplemental N from the NH_3. Promote and NH_3 treatments increased the 6-h IVDMD, but only NH_3 increased the 48-h IVDMD of both forages. Treatment with NH_3 increased the 6 and 48-h IVNDFD and IVADFD of both forages. Promote treatment reduced most of the 6 and 48-h IVADFD values of the hays.

These results indicate that the treatments had different effects on cell wall components. In BE, total cell wall content was unaffected by treatment, but the NDF fraction was hydrolyzed, thus increasing the digestible fiber fraction. Whereas in BA, the NDF fraction was unaffected by treatment, but the total and digestible cell wall fractions

were hydrolyzed into sugars. Ammoniation increased 6 and 48-h DM and fiber digestibility but Promote treatment did not.

In Experiment 2, ammoniation decreased both ADF and NDF concentrations of BE and hence did not affect the hemicellulose concentration. All enzyme treatments decreased the NDF concentration of BE by decreasing the hemicellulose concentration whereas the only effect on BA cell walls was that X-20 treatment increased the hemicellulose concentration by decreasing ADF concentration. This confirms the forage specific-response to the treatments that was observed in Experiment 1.

The WSC concentration of BE hays had a tendency to be reduced by CA and A-20 treatment and unaffected by NH_3 or X-20 treatment. However, that of BA hays was increased by X-20 treatment. None of the treatments that hydrolyzed BE cell walls resulted in an increase in WSC concentration. This may be due to the relatively low WSC concentration of the forages and the conversion of hydrolyzed cell wall fragments into oligosaccharides and disaccharides that are not water soluble, and were therefore undetected in the WSC assay. Unlike enzyme treatment, ammoniation increased CP concentration of both hays as in Experiment 1.

All treatments increased 6-h IVDMD of BE but only ammoniation and X-20 treatment tended to increase the 6-h IVDMD of BA. Enzyme X-20 increased the 48-h IVDMD of BE and BA hays, while CA and A-20 tended to have a similar effect. However, NH_3 treatment was more effective than any of the enzymes at increasing the 48-h IVDMD of both hays. These results suggest that all additive treatments can improve the initial and final phases of DM digestion in BE, but only NH_3 and X-20 had similar effects on BA. The 6-h IVADFD of BE hay was improved by X-20, A-20 and

NH_3 treatment, but only NH_3 treatment increased the 6-h IVADFD of BA. Therefore, the increases in 6-h IVDMD due to X-20 and A-20 treatment were partly due to increases in 6-h IVADFD. Ammoniation was the only treatment that increased the 48-h IVADFD in either of the hays.

Ammoniation and X-20 treatments were therefore more effective at disrupting lignocellulosic linkages in the forages than the other enzyme treatments. Nevertheless, treatment effects varied with forage type probably because of differences in the type and concentration of phenolic cross linkages in the cell walls.

Treatment with X-20 and NH_3 increased the wash loss (a) fraction of BE, but only NH_3 treatment increased that of BA. Ammoniation was more effective than X-20 at increasing the insoluble but potentially degradable (b) fraction, the total degradable fraction (a + b) and the degradability (P) of both forages. The A-20-treated BE hays had lower b, a + b and P values than Control ($P < 0.05$) and NH_3-treated hays ($P < 0.01$). In BE hays ammoniation increased the lag phase and the c value and a + b fraction, while in BA it increased b, a + b and P.

Therefore, Experiments 1 and 2 demonstrate that fibrolytic enzymes had negligible effects on in situ DM degradation of C_4 grass hays, though certain enzymes (X-20 and A-20) did increase the initial and final phases of *in vitro* DM digestion. Such effects were more pronounced in BE than BA for reasons that are not clear. Most of the enzyme-induced enhancements in DM digestibility were not attributable to increased fiber digestion; therefore other mechanisms such as increased substrate colonization by ruminal microbes may have been involved. Ammoniation was more effective than any of the enzyme treatments at improving the initial and final phases of digestion, and these

effects were due to increased fiber hydrolysis. Ammoniation also increased the CP concentration and in situ ruminal degradation of the C_4 grass hays. Therefore although ammoniation is hazardous, it is a more effective method of improving the nutritive value of the hays than enzyme application. Further studies need to be done to determine if enzyme application is more effective on less mature forages, which contain less lignin and ferulic acid cross linkages. Furthermore, potentially more potent enzymes such as ferulic acid esterases should also be investigated as these are more likely to hydrolyze phenolic cross linkages that impede digestion.

The objective of the Experiment 3 was to determine the effectiveness of the fibrolytic enzymes examined in Experiments 1 and 2 on nutritive value of bermudagrass silage, because although bermudagrass is poorly digested, it is an important digestible fiber source in the rations of dairy cows in the southeastern US. A five-week regrowth of Tifton 85 bermudagrass was conserved for 145 days in mini-silos without treatment (Control), or after treatment with the same fibrolytic enzymes evaluated in Experiments 1 and 2. The resulting silage was analyzed for chemical composition, *in vitro* digestibility, fermentation products and aerobic stability.

The Tifton-85 bermudagrass used had low WSC and CP concentrations and high NDF and ADF concentration, at harvest. Therefore it was representative of bermudagrass used for dairy production in the southeast. Promote was more effective than the rest of the enzymes at reducing the pH of the silage. This was a consequence of improved cell wall hydrolysis in Pr-treated silages, which increased the availability of sugars that are used as fermentation substrates by silage bacteria. Promote was also more effective at decreasing the ammonia-N levels in the silage than the other enzymes, which reveals that

less proteolysis occurred during ensiling in Pr-treated silages than in other silages. This was probably due to a faster pH decline in Pr-treated silages, which probably reduced undesirable microbial activity during ensiling.

Although yeast and mold counts were unaffected by enzyme type or rate, the numbers found were less than those (1×10^5 cfu/g) that predispose to rapid deterioration in silage. Except for X-20, all the other enzymes increased the aerobic stability of the silages, and A-20 proved to be the most effective enzyme at improving the stability of the silage. Treatment with Promote and to a lesser extent X-20, was more effective at reducing DM losses and fiber concentration than the other enzymes. Therefore both products could potentially result in improved fiber utilization in dairy cows fed bermudagrass silage. Promote treatment linearly reduced acetic acid concentration and numerically increased lactic acid concentration in treated silages, suggesting that this enzyme enhanced homolactic fermentation which is more efficient than the typical heterolactic fermentation of bermudagrass silage.

The Promote-treated silages also had greater 6-h and 48-h IVDMD values as well as greater 48-h IVNDFD and IVADFD than Control silages. They also had greater 6-h and 48-h IVDMD, and 48-h IVNDF values than silages treated with the other enzymes. These results indicate that Promote was the most promising enzyme. Promote application reduced proteolysis during ensiling, improved the fermentation of bermudagrass and increased the nutritive value of the silage. Therefore feeding Promote-treated bermudagrass silage to dairy cows may improve their performance.

The objective of Experiments 4 and 5 was to determine the effects of applying Promote to bermudagrass at ensiling, or to different components of dairy cow diets at

feeding on feed intake, milk production and composition, blood metabolites and ruminal fermentation parameters. A ration consisting of Tifton 85 bermudagrass silage, corn silage, and concentrate (35, 10 and 55% DM basis respectively) was fed *ad libitum* as a total mixed ration (TMR), twice daily. Cows were randomly assigned to the following five treatments: 1) Control (no enzyme addition), 2) enzyme applied to the concentrate at feeding (EC), 3) enzyme applied to the TMR at feeding (ETMR), 4) enzyme applied to bermudagrass silage at feeding (EF), and 5) enzyme applied to bermudagrass at ensiling (TS). Cows received approximately 4 g enzyme/cow per day when added at feeding and 1.3 g/kg DM when added at ensiling. In Experiment 4, thirty Holstein cows (129 days in milk, DIM) were used in an experiment with a partially balanced, completely randomized design consisting of two-28 d periods, with 14 d for adaptation and 14 d for sample collection. In Experiment 5, five fistulated cows were fed the five same diets as in Experiment 4, for three consecutive 15-day periods. A completely randomized design consisting of 12 d for adaptation, 1 d for rumen fluid sampling and 2 d for *in situ* degradability analysis was used.

Promote application at ensiling improved the nutritive value of the treated silage, by increasing CP and non-fiber carbohydrate concentration and reducing pH and fiber and ammonia-N concentration in agreement with the results of Experiment 3. Promote treatment also increased TDN concentration revealing the positive effect of this enzyme on the energy concentration of the silage.

Enzyme treatment did not affect DMI. The digestibility of the feed fractions were unaffected by Promote treatment; however, the DM digestibility of TS was approximately 3, 6, 3 and 6% higher than Control, EC, ETMR and EF diets, respectively.

Milk production was also unaffected by treatment. Cows fed ETMR and EC rather than the Control diet tended to have higher milk protein concentrations. Cow BW gain, BCS and plasma glucose concentration were unaffected by enzyme treatment. However, concentrations of BHBA and BUN were lower in cows fed ETMR instead of the Control diet. This indicates that the TMR treatment decreased fat mobilization and increased the efficiency of protein utilization in the cows.

In Experiment 5, ruminal pH was decreased by EC diet, and EF and TS diets had a similar tendency. Ruminal pH fell below 6 after 6-h of feeding in cows fed EC, EF and TS diets, This indicates that these cows may have experienced subclinical ruminal acidosis, which probably compromised their performance. Ruminal NH_3-N, propionic acid and butyric acid concentrations were unaffected by enzyme treatment, but ruminal acetic acid concentration was lower in cows fed ETMR and EF than those fed Control diets. Consequently, acetate:propionate ratio was lower in cows fed ETMR diets rather than Control diets. This indicates that the ETMR diet promoted a more efficient fermentation in the rumen, which was probably due to a better balance in ruminal supply of readily fermentable carbohydrates and rumen degradable protein.

Enzyme supplementation did not affect the kinetics of *in situ* DM disappearance of the experimental diets. However the rate of degradation of TS diet tended to be greater than that of the Control diets. The ETMR diet also had a numerically greater degradation rate and numerically shorter lag phase than the Control diet, which support the numerically higher DM intake of the cows fed the ETMR diet. Compared to the Control diet, the numerical improvements in degradation rate and lag phase of ETMR are consistent with numerical improvements in DMI and FCM, lower BHBA and BUN

concentrations and greater milk fat and milk protein concentrations in cows fed ETMR instead of the Control diet.

Therefore this study shows that enzyme supplementation did not improve voluntary intake, BW gain, BCS, blood glucose, milk production and *in situ* degradability. However cows fed the ETMR diet had numerically greater DMI, FCM, tended to have greater milk fat and protein concentrations and lower BHBA, BUN and rumen acetate to propionate ratios. Therefore the ETMR treatment was more effective than any of the other treatments. Future experiments should validate the potential of this mode of Promote application with early lactation cows which have greater energy requirements and are therefore more likely to respond to enzyme supplementation than cows used in this study. Such experiments should be conducted for the entire lactation to establish the stage of the lactation that benefit the most from enzyme application. Furthermore, a greater number of cows per treatment should be used to facilitate identification of treatment effects and to allow more definitive conclusions to be drawn.

Fibrolytic enzyme application to mature tropical grass hays did not prove to be effective at increasing nutritive value. This is probably due to the high lignin concentration of the grasses, and the inability of the enzymes to hydrolyze the ferulate linkages in the hays. Although Promote application was effective at improving the nutritive value of bermudagrass ensiled in mini silos it had only a few beneficial effects on the performance of cows fed diets containing the enzyme. Therefore these experiments don't support the use of commercial fibrolytic enzymes for enhancing the performance of dairy cows.

APPENDIX A
ABSTRACT FOR CHAPTER 3

In the first of two experiments, the effect of applying NH_3 or a fibrolytic enzyme complex (Promote®, Pr, Cargil, Minnentoka, MN) on the chemical composition, DM, NDF, ADF and hemicellulose concentrations and digestibility of two tropical grass hays was measured. In the second experiment, the effects of applying ammonia or three fibrolytic enzymes (Biocellulase X-20® (X-20) (LodeStar, IL, USA), Cattle-Ase® (CA) (Loveland Industries Inc, Greeley, CO, USA) and Biocellulase A-20® (A-20) (LodeStar, IL, USA) on the same variables as in the previous experiment were measured. The forages were 12 week-regrowth of Coastal bermudagrass hay (*Cynodon dactylon*) (BE) and Pensacola bahiagrass hay (*Paspalum notatum*) (BA). The ammonia was applied at 40 g/kg DM and the enzymes were applied at 0 (Control) 0.5, 1 and 2 times the rates recommended by the respective manufacturers. The hays were stored for three weeks after enzyme application. Calculations of IVDMD, IVNDFD, and IVADFD were made after digesting the hays in buffered rumen fluid for 6 or 48-h in two ANKOM^{II} Daisy Incubators. Treatments were analyzed using a 2 x 4 factorial design with 3 replicates per treatment for each digestion period. Low CP and high NDF, ADF, hemicellulose and lignin concentrations were observed in both forages. Bahiagrass had lower ($P < 0.01$) NDF, ADF and lignin concentration than BE. In Experiment 1, Pr and NH_3 decreased ($P < 0.01$) the NDF concentration of BA. Both treatments decreased ($P < 0.01$) ADF concentration of BE but not BA. The hemicellulose concentration of BE was increased ($P < 0.01$) by enzyme (quadratic) and NH_3 treatment, while that of BA was decreased (P

< 0.01) by NH_3 treatment. Concentration of WSC was greater (P < 0.01) in BA than in BE hays and that of BA was increased (P < 0.01) by Pr and NH_3 treatment (P < 0.01). The CP concentration of ammoniated hays was consistently greater (P < 0.01) than those of enzyme treated or Control hays. Promote and NH_3 treatments increased the 6-h IVDMD of both hays, but only NH_3 increased their 48-h IVDMD. Treatment with NH_3 increased the 6-h and 48-h IVNDFD and IVADFD in both forages. Pr treatment reduced most of the 6 and 48-h IVADFD values of the hays. In Experiment 2, NH_3 treatment decreased both ADF and NDF fractions of BE and hence did not affect the hemicellulose fraction. All enzyme treatments decreased the NDF concentration of BE. The WSC concentration of BA hays was increased (P < 0.05) by X-20 treatment. All treatments increased 6-h IVDMD of BE but only NH_3 (P < 0.01) increased the 6-h IVDMD of BA. Enzyme X-20 increased the 48-h IVDMD of BE (P < 0.05) and BA (P < 0.01) hays, while CA and A-20 tended (P < 0.08) to have similar effects. Ammoniation increased the 6 and 48-h IVNDFD (P < 0.01) of BE and 48-h IVNDFD of BA (P < 0.01). The 6-h IVADFD of BE hay was improved (P < 0.01) by X-20, A-20 and NH_3 treatment. Treatment with X-20 (linear, P < 0.05) and NH_3 (P < 0.01) increased the wash loss (a) fraction of BE, but only NH_3 treatment increased that of BA. Ammoniation was more effective than X-20 at increasing (P < 0.01) the insoluble but potentially degradable (b) fraction, the total degradable fraction (a + b) and the degradability (P) of both forages. The A-20-treated BE hays had lower b, a + b and P values than Control (P < 0.05) and NH_3-treated hays (P < 0.01). In BE hays, ammoniation increased the lag phase and the c value and a + b fraction, while in BA it increased b, a + b and P. This work demonstrates that fibrolytic enzymes had negligible effects on in situ DM degradation of C_4 grass hays,

though certain enzymes (X-20 and A-20) did increase the initial and final phases of *in vitro* DM digestion. Ammoniation was more effective than any of the enzyme treatments at improving the initial and final phases of digestion, due to increased fiber hydrolysis.

APPENDIX B
ABSTRACT FOR CHAPTER 4

The aim of this study was to determine if the nutritive value and aerobic stability of bermudagrass (*Cynodon dactylon*) silage can be improved by addition of proprietary, exogenous cellulase/hemicellulase enzyme preparations at ensiling. A five-week regrowth of Tifton 85 bermudagrass was conserved without treatment (Control), or after treatment with exogenous fibrolytic enzymes including Promote® NET (Cargill Corp. St. Louis, MO), Biocellulase X-20® (LodeStar, IL, USA), Biocellulase A-20® (LodeStar, IL, USA), and Enzyme CA. The respective enzymes were applied at half the recommended rate, the recommended rate or twice the recommended rate corresponding to 0.65, 1.3 and 2.6 g/kg DM, 7.3, 14.5 and 29 mg/kg DM, at 7.3, 14.4 and 29 mg/kg DM 89, 178 and 356 mg/kg DM, for Promote, X-20, A-20 and CA, respectively. The enzymes were sprayed on the bermudagrass at ensiling, and not added at feeding as suggested by the manufacturers in order to test the objectives of the study. Six replicates of 1 kg of chopped (5 cm) forage were ensiled for 145 days in 2.8 L mini silos. Three silos per treatment were used for chemical analysis and three for aerobic stability monitoring. The silage juice was analyzed for organic acids, pH, water soluble carbohydrates (WSC), ammonia-N and soluble N. Freeze-dried samples were analyzed for crude protein (CP), NDF and ADF. *In vitro* digestibility of DM (IVDMD), NDF (IVNDFD) and ADF (IVADFD) were determined after digesting the silages in buffered-rumen fluid for 6 or 48-h in two ANKOM^{II} Daisy Incubators. Compared to the other silages, those treated with Pr had lower DM losses, and lower pH and ammonia-N

concentration than Control silages. Residual WSC concentration was greater in Pr ($P < 0.01$) and CA ($P < 0.05$) - treated silages than in Control silages, and greater ($P < 0.01$) in Pr-treated silages than CA - treated silages. Compared to Control silages, NDF concentration was lower in silages treated with Pr ($P < 0.01$), X-20 ($P < 0.05$), and CA ($P < 0.05$) while ADF concentration was lower ($P < 0.05$) in silages treated with Pr, X-20 and A-20. Nevertheless, Pr-treated silages contained lower ($P < 0.01$) ADF and NDF concentrations than silages treated with the other enzymes. Enzyme-treated silages contained less ($P < 0.05$) acetic acid than Control silages, and Pr-treated silages had the lowest concentrations of acetic acid. Aerobic stability (AS) and microbial counts were unaffected by treatment. The 6-h IVDMD was increased ($P < 0.01$) by treatment with Pr and A-20, however only Pr increased ($P < 0.01$) the IVDMD and IVNDFD at 48-h. The 48-h IVADFD ($P < 0.05$) was also increased by treatment with Pr, CA and A-20. These results show that when applied at ensiling, certain fibrolytic enzymes, particularly Promote can improve the digestibility, fermentation and aerobic stability of bermudagrass silage.

APPENDIX C
ABSTRACT FOR CHAPTER 5

Two experiments were carried out to investigate the effect of applying a cellulase enzyme (Promote ®; Cargill; Minnetonka, MN) on the performance of lactating dairy cows. A ration consisting of Tifton 85 bermudagrass silage, corn silage, and concentrate (35, 10 and 55% DM basis respectively) was fed *ad libitum* as a total mixed ration (TMR) twice daily. Cows were randomly assigned to the following five treatments: 1) Control (no enzyme addition), 2) enzyme applied to the concentrate at feeding (EC), 3) enzyme applied to the TMR at feeding (ETMR), 4) enzyme applied to bermudagrass silage at feeding (EF), and 5) enzyme applied to bermudagrass at ensiling (TS). Cows received approximately 4 g enzyme/cow per day when added at feeding and the application rate at ensiling was 1.3 g/kg DM. In Experiment 1, thirty Holstein cows (129 days in milk, DIM) were used in a partially balanced, completely randomized design consisting of two-28 d periods, with 14 d for adaptation and 14 d for sample collection. Voluntary DMI, digestibility of DM, NDF and CP, milk production and composition and blood glucose were not affected ($P>0.05$) by enzyme supplementation. Cows fed ETMR had lower ($P <0.01$) blood β-hydroxybutyrate concentration and tended to have greater milk fat ($P=.073$) and protein ($P=0.081$) concentrations and lower blood urea-N concentration ($P=0.123$) than cows fed the Control diet. In Experiment 2, five fistulated cows were fed the five same diets as in Experiment 1, for three consecutive 5-day periods. A completely randomized design consisting of 12 d for adaptation, 1 d for rumen fluid sampling and 2 d for *in situ* degradability analysis was used. Ruminal pH,

acetic, propionic and butyric acid molar proportions were unaffected by enzyme treatments. The kinetics of *in situ* DM disappearance were also unaffected by enzyme treatment. Therefore applying the enzyme to the TMR reduced fat mobilization and tended to increase milk fat and protein contents and decreased BUN concentration and ruminal acetate:propionate ratio. However, other animal performance and ruminal fermentation parameters were unaffected by fibrolytic enzyme supplementation.

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BIOGRAPHICAL SKETCH

The author was born in Ciudad Ojeda, Venezuela. He got his B.S. in Animal Science from the University Rafael Urdaneta, in Maracaibo, Venezuela. After working for two years as a Farms Supervisor for a dairy company and as Director and Technical Consultant for beef and dairy farmers for two more years, he pursued an M.S. program in Animal Nutrition at the University of Zulia. During the last two years of his M.S. program and one year afterwards he was employed as an Assistant Professor in the Maracaibo Technological College. Subsequently he joined Protinal, as Manager of Nutrition and almost two years later, he became employed as an Associate Professor in the Veterinary School of the University of Zulia. This University is his sponsor and they have covered all of the expenses for his Ph.D. program in Ruminant Nutrition in the Animal Science Department of the University of Florida.