USE OF EXOGENOUS FIBROLYTIC ENZYMES TO IMPROVE THE NUTRITIVE VALUE OF PRESERVED FORAGE FOR RUMINANTS

by

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ABSTRACT

This study was conducted to determine the effects of fibrolytic enzymes applied at baling, with or without ferulic acid esterase (FAE) producing bacterial inoculant, or to hay at feeding on digestibility and growth performance of lambs. Prior to starting the animal studies, two runs of replicated 24- and 48-h batch culture in vitro incubations were conducted using control alfalfa hay to select a suitable enzyme and dose. Eleven replicate bales of alfalfa-grass (93.8:6.2) hay (~500 kg) were produced with the application of one of three treatments: control (water), enzyme applied at baling (Eb; Econase RDE-L, AB Vista, Wiltshire, UK) and enzyme plus ferulic acid esterase producing bacterial additive applied at baling (EIb; 11 GFT, Pioneer HI-Bred Ltd., Chathan, ON, Canada). The mean internal bale temperature after 50 days of storage was greater ($P<0.001$) for Eb than control and EIb, as was the post-storage hemicellulose ($P<0.05$) concentration [mean bale temperature (°C), Eb = 26.8, EIb = 22.8, control = 17.8; hemicellulose concentration (g/kg dry matter), Eb = 127, control = 115, EIb = 114]. Two animal experiments using lambs were conducted after bales were stored for at least 90 days. The digestibility study was a replicated 4 × 4 Latin square design with 16 lambs and the animal performance study consisted of 32 lambs (8 per treatment) in a randomized complete block design. In both studies lambs received one of four treatments: control, Eb, EIb and enzymes added to control hay at feeding (Ef). In the digestibility study, total tract apparent organic matter (OM) ($P=0.07$) digestibility tended to be affected by treatment, with OM digestibilities greater for lambs fed Ef compared with lambs fed the other treatments, although differences were small (Ef vs. others; OM, 0.658 vs. 0.646). However, neutral detergent fiber (aNDF) and hemicellulose digestibilities were greatest ($P<0.05$) for lambs fed Eb, with no differences among the other treatments (aNDF, Eb = 0.480, control = 0.437, Ef = 0.430, EIb = 0.430; hemicellulose, Eb = 0.524, control = 0.460, Ef = 0.458, EIb = 0.446). In both studies there was no effect ($P>0.05$) of treatment on OM intakes. Average daily gain (ADG, g/d) of lambs in the performance study was greater ($P=0.048$) for EIb (233) than control (192) and Ef (202), and intermediate for Eb (206). Feed efficiency tended to be affected ($P=0.07$) by treatment; gain:feed for EIb was 18% greater than control and Eb and Ef were similar to the control. This study showed that applying enzymes to alfalfa hay at baling decreased aerobic stability, and increased fiber content and its digestibility, but ADG and gain:feed of lambs were not improved. Adding FAE producing

bacterial inoculant with enzymes at baling improved aerobic stability of hay and ADG and gain:feed of lambs were increased relative to lambs fed control and enzymes applied at feeding. Applying enzymes at feeding increased apparent OM digestibilities but not fiber digestibilities, and had no effect on animal performance. We conclude that fibrolytic enzyme application with FAE producing bacterial inoculant at baling was the most promising method for enhancing performance of lambs fed baled alfalfa hay.

Keywords: Fibrolytic enzyme, Fiber, Alfalfa, Ferulic acid esterase, Average daily gain, Apparent digestibility

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Amino acid
ACT=50 days	Mean accumulated temperature above ambient during 50 days of storage
ACT=120 days	Mean accumulated temperature above ambient during 120 days of storage
ADF	Acid detergent fiber
ADG	Average daily gain
ADICP	Acid detergent insoluble crude protein
ADIN	Acid detergent insoluble nitrogen
BW	Body weight
CF	Crude fiber
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DMD	Dry matter degradability
DOMI	Digestible organic matter intake
Eb	Enzyme added at baling
EE	Ether extract
Ef	Enzyme added at feeding
EFE	Exogenous fibrolytic enzyme
EIb	Enzyme plus FAE bacterial inoculant added at baling
ENZ1	Econase RDE-L, AB Vista, Wiltshire, UK
ENZ2	Rovabio Excel LC, Adisseo, Alpharetta, GA, USA
FAE	Ferulic acid esterase
GP	Gas production
H_2	Hydrogen gas
LAB	Lactic acid bacteria
LSD	Least significant difference
MP	Microbial protein

N	Nitrogen
NDF	Neutral detergent fiber
NDSC	Neutral detergent soluble carbohydrate
NDSF	Neutral detergent soluble fiber
NFE	Nitrogen-free extract
NH_3	Ammonia
NIRS	Near infra-red spectroscopy
NSC	Non-structural carbohydrates
OM	Organic matter
SD	Standard deviation
TMR	Total mixed ration
VFA	Volatile fatty acids
WSC	Water soluble carbohydrates

CHAPTER 1 – General introduction

Introduction

Preserved forage plays a significant role in ruminant production systems all over the world, especially where there is a restricted growing season, such as, during the winter or dry season. Thus, conserved forage (hay, silage, or haylage) is an essential component of ruminant diets during those periods when fresh forages are not available. Even though the primary goal of forage preservation is to maintain forage dry matter (DM) and nutrients with minimal loss, significant losses still occur, which in many cases result in reduced forage quality. Losses may occur before, during, and after storage. Average losses in haymaking are estimated to be between 24 and 28% and losses for silage production are 14 to 24%, with about half of this loss occurring during storage (Rotz and Muck, 1994). For instance, some forage crops such as alfalfa tend to be low in water soluble carbohydrates (WSC) with high buffering capacities, which limit the ability of lactic acid bacteria to reduce the pH under anaerobic conditions. As a result, alfalfa crops often conserve poorly (Nadeau, 2000).

Enzymes are proteins that are naturally needed to complete the digestion of food by humans or the feed of animals. In the case of ruminants, enzymes are produced either by the animal itself or by microbes naturally present in the gut. The microbial-enzymatic mode of digestion allows ruminants to better unlock the unavailable energy in plant cell wall components better than other non-ruminant herbivores (Van Soest, 1994). Thus ruminants have the ability to convert low nutritive plant biomass to valuable products such as milk, meat, wool and hides. Regardless of the importance of the fibrous characteristics to stimulate salivation, rumen buffering and efficient production of ruminal end products (Mertens, 1997), only 10 to 35% of the forage gross energy intake is available as net energy for maintenance or production (Varga and Kolver, 1997). The ruminant's digestive process is not 100% efficient. This is because under most feeding conditions, neutral detergent fiber (NDF) digestibility in the ruminant digestive tract is less than 65%, and ruminal NDF digestibility is often less than 50% (Beauchemin and Holtshausen, 2010).

For many decades the improvement of forage quality, evaluation, utilization and increased productive efficiency of ruminants have been milestones of forage research.

Recently, forage cell wall digestibility has undergone significant improvements through forage breeding programmes, agronomic advances (Beauchemin et al., 2003), and enzyme technology. In terms of enzyme technology, the two most popular enzyme complexes are those of the cellulase and hemicellulase families, generally known to be multicomponent enzymes that when added to forage could possibly assist in the preservation of forages, especially silage. Previous work has reported that fibrolytic or cell wall degrading enzymes applied alone, or in combination with the other additives, may enhance preservation of forage within the silo by increasing the levels of lactic acid (Nadeau, 2000). Some studies have also reported either positive or negative effects of fibrolytic enzymes, bacterial inoculants, formic acid, or surfactants on intake and apparent digestibility of forage by ruminants (McAllister et al., 2000; Nadeau, 2000; Baah, 2005). Although there can be improvements in feeding value of forage using forage preservation additives, poor forage digestibility continues to limit the energy intake of ruminants. This inevitably contributes to nutrient excretion losses (Beauchemin et al., 2003) as faeces or as methane (CH_4) gas into the environment. This is significant as ruminants contribute approximately 37% of total anthropogenic CH_4 emission (FAO, 2006; Meale, 2013). Globally, it is estimated that of the enteric sources of methane, beef and draught animals contribute 50%, dairy cows 19%, while sheep produce a modest 9% (Leng, 1993). Reducing methane gas emissions by ruminants is often at the expense of lowered digestion of the feed.

Hemicellulose and cellulose constitute most of the potentially digestible portions of forage NDF. Hemicellulose contains arabinoxylans and glucuronoarabinoxylans that may be linked to ferulic acid by esterification, which in turn may be linked directly to lignin or serve as an initiation site for lignification as the plant matures. The ferulic acid which can be linked directly to lignin is the most abundant and inhibitory phenolic acid that limits forage degradation (Yu et al., 2005). Therefore, hydrolysis of the ester linkages by ferulic acid esterase (FAE) activity may render the feruloylated polysaccharides more fragile or increase the susceptibility of cell walls to be degraded by fibrolytic enzymes.

The importance of forage and ruminant nutrition

Forage crops are important in ruminant production throughout the world, and these crops are widely distributed globally, more than any other group of crops (Cherny and

Cherny, 2003). Forage has direct benefits to the livestock industry, as well as indirect benefits, including ecological goods and services such as: erosion control, flood control, improved surface water quality, wildlife habitat, pollination services, and carbon sequestration (Yungblut, 2012). Progressive forage management compliments current worldwide trends towards more environmentally sensitive cropping systems (Cherny and Cherny, 2003). The majority of the world's ruminants (cattle, sheep, and goats) depend on forages throughout their lifetime that are often poor in quality (Leng, 1993). Ruminants are able to convert low quality feeds into food of high biological value for human beings. This is because they have evolved to utilize plant cell walls as major components of nourishment (McDonald et al., 2011). In developing countries, where ruminants sometimes serve as the only source of income for the small farmer, forages often form the major part of the diet for these animals, and in most cases, are their only source of nutrition. This is even the case in the developed world, where, in many cases, naturally occurring forages constitute the major part of the ruminant diet and can provide nutrients at a relatively low input cost (Wilkins, 2000). In Canada, the economic value of the forage industry has been estimated to be over $5 billion (Yungblut, 2012). The Canadian forage industry is also the foundation of the country's dairy and beef industries, which together contribute $11 billion in direct value to Canadian farmers, and generate over $50 billion in economic activity annually (Yungblut, 2012).

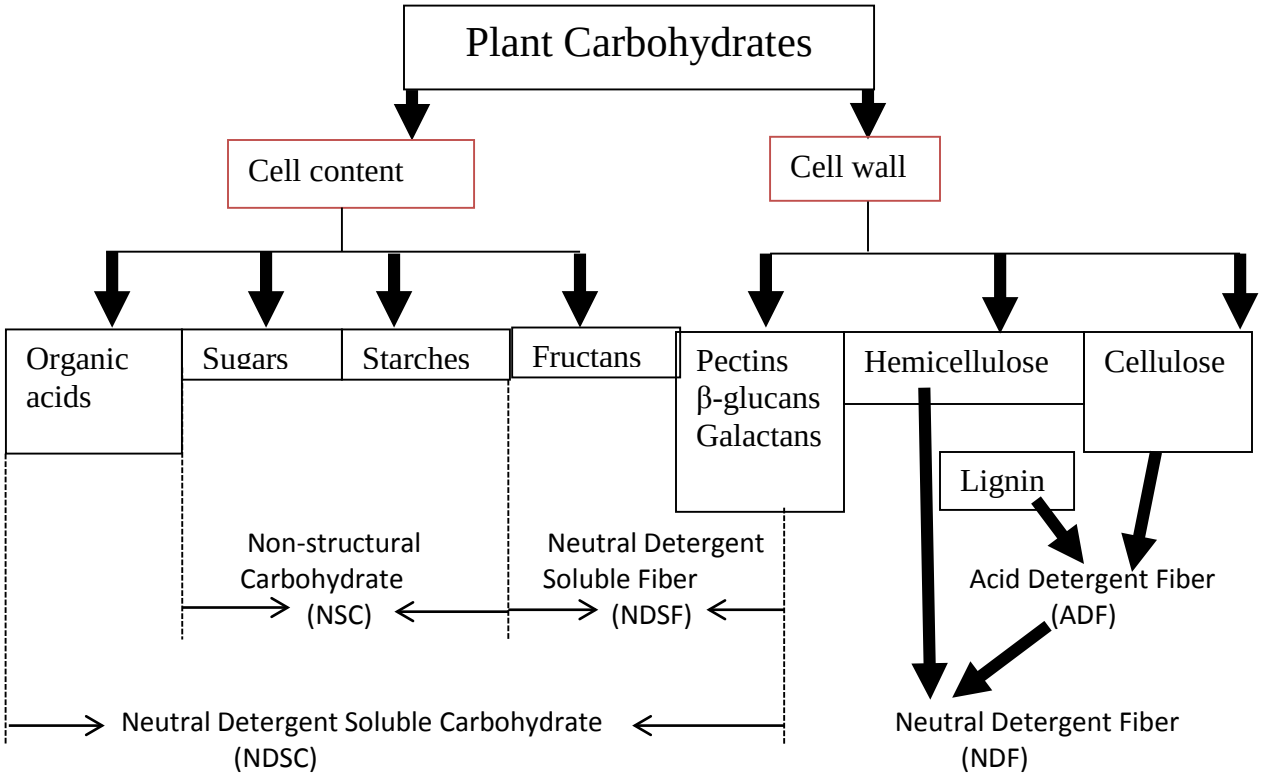
The economic implications of forage cell walls or fiber in ruminant nutrition are unavoidable. Fiber makes up the bulk of the plant nutrients, and provides an important source of energy to the microbes of the rumen and ultimately the animal itself (Yang et al., 2002). The form of the fiber provided also plays an important role in this regard, as this has a direct influence on rumen function. When insufficient coarse fibrous diets, such as those with high grain, or less forages are fed, the rumen pH declines and efficiency of digestion can be compromised. Forage, in the form of coarse fibrous particles, is essential to stimulate rumination. Rumination elevates rumen pH through buffer content in the saliva flow and cation exchange on the surface of fiber particles. Therefore, adequate daily fiber content can prevent economic losses from digestive and metabolic disorders which may lead to increased morbidity and sometimes even to death. These disorders include: erosion of rumen epithelium, abscess and inflammation of the livers, milk fat depression, metabolic changes

leading to diarrhea, acidosis causing ruminal parakeratosis and chronic laminitis, altered ruminal fermentation, and reduced energy intake (Mertens, 1997; Plaizier et al., 2009).

Chemical composition and structure of plant cell wall

The plant cell wall is a complex mix of carbohydrate polymers associated with various non-carbohydrate components (McDougall et al., 1996), which include protein matrix (extensins) and phenolic compounds in the cell networks, together with lignin (Fisher et al., 1995; Graminha et al., 2008). In ruminant nutrition, carbohydrates often represent the largest component of the diet and are vital for meeting the energy requirements of the animal while maintaining rumen health. There are two broad classifications of carbohydrates, those found in the plant cell walls and those located in the cell content of plants, which are usually more digestible than carbohydrates in the cell wall (Figure 1.1). The carbohydrates of plant cell walls include cellulose, hemicellulose, pectic substances, β-glucans, and galactans; while those of the cell content contain starches, sugars, and fructans plus organic acids for ensiled feeds (Ishler and Varga, 2001). Carbohydrates in the cell content are easily degraded by enzymes and rumen microbes, but not all carbohydrates in the cell walls (based on their individual nutritional characteristics) are easily degraded by microbes in the ruminant. Carbohydrates easily degraded by enzymes and/or rumen microbes are soluble in neutral detergent and hence referred to as neutral detergent soluble carbohydrates (NDSC). Fiber is normally characterized as cell wall structure (Jung, 2012) and it is usually insoluble in the detergent fiber analysis (Van Soest, 1967); thus measured as NDF. Though lignin is not carbohydrate, it is associated with the insoluble cell wall carbohydrates in the neutral detergent analysis, hence measured as part of NDF. On the contrary, pectins, β-glucans, and galactans, which form part of the cell wall carbohydrates, are usually soluble in neutral detergent and referred to as neutral detergent soluble fiber (NDSF). For a grass, NDF is a reasonably accurate measure of cell wall concentration, as compared to legumes where solubility of pectin in neutral detergent results in a significant underestimation of cell wall concentration by the NDF method (Theander and Westerlund, 1993; Jung, 2012). This is because legumes contain more pectins than grasses.

Figure 1.1 Schematic representation of structural and non-structural carbohydrates of forage. Modified from Ishler and Varga (2001).



The complex biochemical structures of plant cell walls (primary and secondary cell walls) surround plant cells to provide physical rigidity, allow water transport, and prevent pest attack (Paulson et al., 2008). They usually consist of about 35–50% cellulose, 20–35% hemicellulose and 10–25% lignin by dry mass (Sticklen, 2008) depending on tissue types, plant parts, and changes that occur during maturation (Paulson et al., 2008). Some plant tissues (mesophyll and phloem tissue in grasses and legume leaves) never develop thick secondary cell walls, and such tissues do not lignify, whereas many tissues (sclerenchyma, xylem fiber, and xylem vessels in grass and legume stems) develop thick secondary walls as they mature, and these walls are heavily lignified (Wilson, 1993; Paulson et al., 2008; Jung, 2012). Thus, the overall herbage cell wall concentration increases as the plant matures and this shifts the leaf-to-stem ratio towards a greater proportion of stem. These changes in concentration reflect the composition of secondary walls compared to primary walls, and the fact that stems contain more tissues that accumulate secondary wall material (Jung, 2012).

Cellulose is a water-insoluble β-glucan, consisting of a linear molecule of glucose residues linked by a β-(1→ 4) bond (Paloheimo et al., 2010), and is found predominantly in the primary cell wall (Jung, 2012). Anhydrocellobiose is the repeating unit of cellulose in which the adjacent glucose moieties are rotated 180º in relation to their immediate neighbors' bond (Figure 1.2; Paloheimo et al., 2010). The cellulose microfibrils are aligned in a parallel fashion to create crystalline regions with maximal hydrogen bonding. Other regions of the fibril are less organized and form paracrystalline (amorphous) sections.

Hemicellulose consists of a complex and diverse group of polymers that are heterogeneous in their composition, having branched chains, and consisting of various sugar units. Hemicelluloses include xylans, xyloglucans, and mannans (Åman, 1993) and their proportion is greater in the secondary cell wall than in the primary cell wall (Jung, 2012). Xylan is the major component of hemicellulose and is, after cellulose, the second most abundant polysaccharide in nature (Paloheimo et al., 2010). The main chain of xylan is composed of 1,4-β-linked D-xylopyranose units (Figure 1.3a). Xylan is normally linked by a subunit α-L-arabinofuranose at position 2 or 3 of the main unit (xylose) in most annual plants, cereal grains, and grasses and therefore referred to as arabinoxylan (Figure 1.3b; Wilkie, 1979; Paloheimo et al., 2010).

Lignin is a polymer composed of phenylpropanoid monolignol units (Lapierre, 1993). All forages contain both guaiacyl- and syringyl-type lignin, and small amounts of *p*-hydroxyphenyl-type lignin (Jung, 2012). The hydroxycinnamic acids, ferulic and *p*-coumaric, are the other phenylpropanoid components of grass cell walls but are virtually absent from legume cell walls (Hartley and Ford, 1989; Jung, 2012). Ferulic acid is esterified to arabinose molecules of arabinoxylans (Figure 1.3c) or may serve as the initiation site for lignification. Some *p*-coumaric acid is similarly esterified to arabinoxylan, but most is esterified to syringyl-type lignin. Arabinoxylan polymers are cross-linked through diferulates in grasses (Ralph et al., 1994). Chemically, the cell wall of dicotyledonous plants such as legumes or oilseeds are a far more complex group than those of monocotyledons, and their chemical structure is still not well defined (Paloheimo et al., 2010).

Figure 1.2 Schematic representation of cellulose structure (courtesy of the US Department of Energy Genome Program, available at http://genomics.energy.gov.

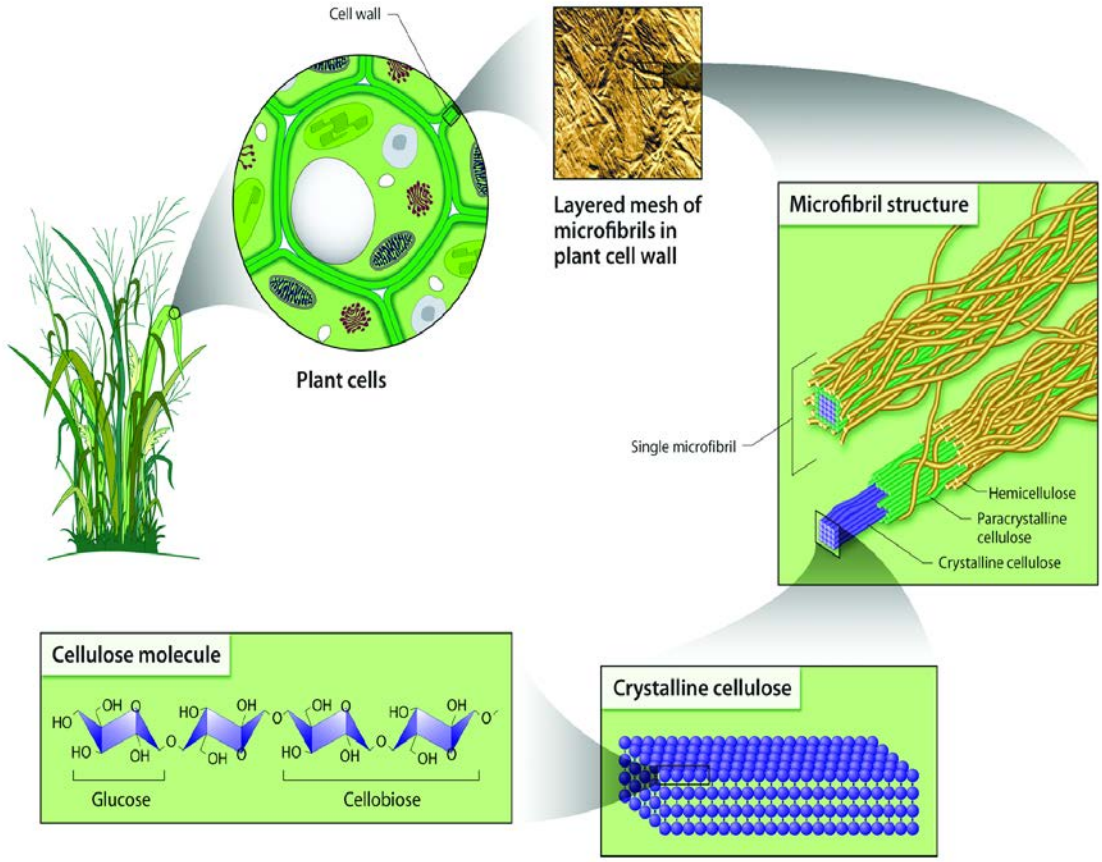
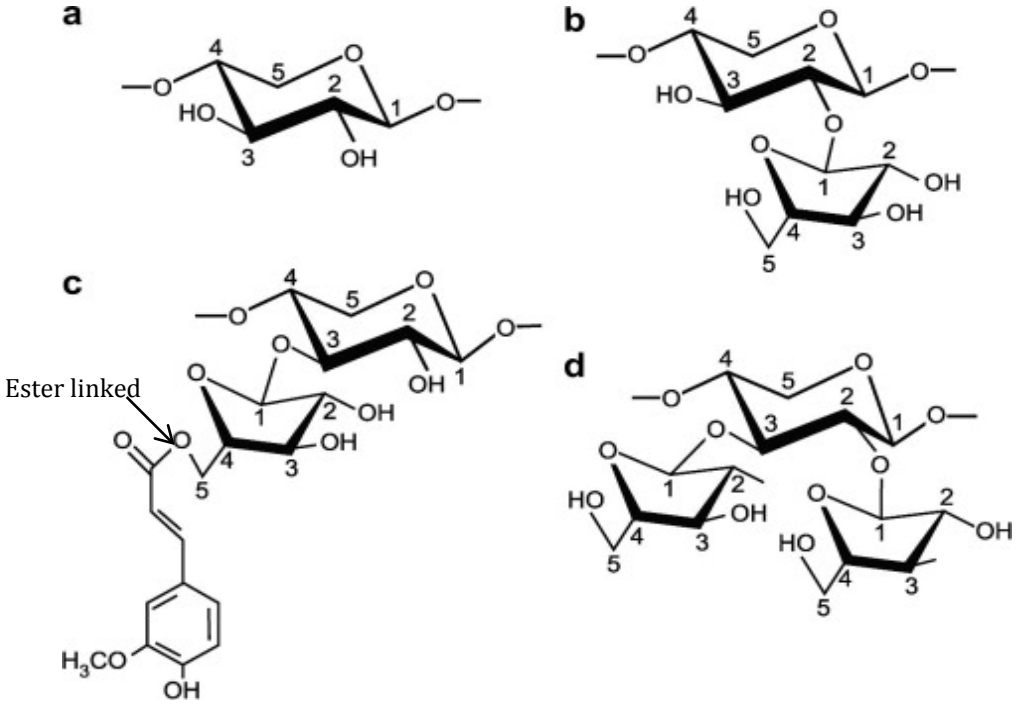


Figure 1.3 Schematic representation of arabinoxylan structural units: (a) unsubstituted D-xylopyranose, Xyl p; (b) monosubstituted Xyl p with L-arabinofuranosidase, Ara f at O-2; (c) monosubstituted Xyl p at O-3 with ferulic acid residue esterified to Ara f and (d) disubstituted Xyl p with Ara f at O-2,3. Modified from Izydorczyk and Dexter (2008).



Digestion and metabolism of forage in ruminants

The ruminant foregut or stomach has evolved into three pre-gastric fermentation chambers (rumen, reticulum, and omasum) of which the rumen is by far the largest. The symbiosis between ruminants and mixed, microbial culture in the rumen allows for a cooperative system in which both benefit (McSweeney and Mackie, 2012). Ingested plant material is hydrolyzed and fermented by microorganisms in the rumen to produce volatile fatty acids (VFA), ammonia (NH_3), microbial protein (MP), carbon dioxide (CO_2), and methane (CH_4) (Krause et al., 2003). The rumen is generally an anaerobic and highly reduced environment, with an average temperature and pH of 39.5ºC and 6.4, respectively (McSweeney and Mackie, 2012).

Particular microbial groups that regulate important aspects of rumen fermentation are broadly divided into four main taxa: bacteria, protozoa, fungi, and methanogens. Rumen bacteria represent possibly the most diverse microbial group, and have traditionally been classified in accordance with their main metabolic activity (Belanche et al., 2012). The major

fibrolytic (cellulolytic and xylanolytic) bacteria that are normally cultured are the gram-negative *Fibrobacter succinogenes*, and two species of gram-positive bacteria, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Krause et al., 2003). A number of less well characterized cellulolytic bacteria, such as *Eubacterium cellulosolvens* and highly xylanolytic gram-positive bacteria such as *Butyrivibrio fibrisolvens*, which have a central role in fiber digestion, also inhabit the rumen (Krause et al., 2003). *Prevotella* spp. are proteolytic (Belanche et al., 2012), hence not regarded as highly cellulolytic bacteria, but do produce a range of xylanases (Krause et al., 2003). The others include amylolytic (e.g., *Selenomonas ruminantium*, *Streptococcus bovis*), lipolytic (e.g., *Anaerovibrio lipolytica*), lactate producers (e.g., *S. bovis* and *S. ruminantium*), and lactate consumers (e.g., *Megasphaera elsdenii*) (Belanche et al., 2012). There is also increasing evidence that the rumen protozoa may have the capacity to digest fiber (Firkins et al., 2007). Anaerobic rumen fungi are generally considered somewhat important in fiber digestion and one of the best studied fungi is *Neocallimastix* spp., with earlier studies showing that rumen fungi are more effective in fiber digestion than cellulolytic bacteria (Wood et al., 1986; Krause et al., 2003). Methanogens belong to the domain archaea and produce CH_4 using hydrogen gas (H_2) as the main electron donor and CO_2 as a terminal electron acceptor (Morgavi et al., 2010; Belanche et al., 2012).

Because of their greater population in the rumen, bacteria are the most important group in terms of fiber digestion, although indirect estimates suggest that protozoa may be responsible for 30–40% of overall fiber digestion under certain conditions, while the role of fungi is unclear (McSweeney and Mackie, 2012). Genomics offers substantial opportunity to relate rumen microbial population structure and their hosts. Despite the wealth of knowledge that has been gained in recent years, interactions among these microbial groups, rumen fermentation, and the hosts' metabolism are not yet fully understood (Belanche et al., 2012).

Fiber-degrading anaerobic bacteria and fungi usually colonize and adhere to the surface of plant cell walls within the rumen before secreting fibrolytic enzymes for digestion to occur (Krause et al., 2003). The intimate association of anaerobic microbes with plant cell walls has evolved through the synthesis of a sophisticated molecular structure, the cellulosome, which facilitates the adherence process and fiber digestion (Flint et al., 2008). However, the degree of microbial colonization and their specific mode of adhesion on feed

particles differ between species in the rumen (McAllister et al., 1994). Miron et al. (2001) described four main phases by which bacteria and possibly fungi in the rumen may adhere to plant cell wall: (1) transport of the non-motile bacterium to the plant substrate; (2) nonspecific adhesion of bacteria to available sites on the plant cell wall; (3) specific adhesion via adhesions or ligand formation with the substrate, that may be facilitated by structures such as cellulosome complexes, fimbrial connections, and cellulose-binding domain; and (4) proliferation of the attached bacteria on potentially digestible plant tissues. Flint et al. (2008) described a cellulosome as a discrete, extracellular, multi-component, and multi-enzyme complex that is found in anaerobic cellulolytic bacteria, which provides enhanced synergistic activity among the different resident enzymes to efficiently deconstruct the intractable cellulosic and hemicellulosic substrates of the plant cell wall. They illustrated the cellulosome of *R. flavefaciens* and noted that it comprises a set of multi-modular components, some of which are structural (scaffoldin) and some of which are enzymic. The scaffoldin is a pivotal, non-catalytic subunit, which secures the various enzymic subunits into the complex through an intermodular cohesin–dockerin interaction (Figure 1.4; Flint et al., 2008).

Cellulose is the most challenging material to degrade within the plant cell walls; therefore numerous fibrolytic enzymes secreted by the rumen microbes contribute to its degradation (Paloheimo et al., 2010; Glass et al., 2013). The classical scheme for cellulose degradation includes endo-1,4-β-glucanases that cleave internal bonds or the amorphous regions of the cellulose chain and release substrates for the exo-1,4-β-glucanases or cellobiohydrolases. The latter produces disaccharide cellobiose molecules from either the reducing or non-reducing ends of a cellulose chain, which are then, hydrolyzed into two glucose molecules by β-glucosidases (Figure 1.5a; Paloheimo et al., 2010; Glass et al., 2013).

Hemicellulose degradation is also very complex due to the different side chains and compounds linked to the hemicellulose backbone. Xylan, the most abundant hemicellulose, is hydrolyzed by a variety of enzymes (Figure 1.5b) including endo-β-1,4 xylanases, α-L-arabinofuranosidases, β-xylosidases, acetylxylan esterase, and ferulic acid esterases (Paloheimo et al., 2010; Glass et al., 2013).

Figure 1.4 Schematic representation of fiber and its component cellulose, microfibrils, hemicellulose, and lignin that are degraded via the cellulosome complex. The cellulosomes are associated with the microbial cell surface, mediate cell attachment to the insoluble substrate, and degrade it to soluble products that are then absorbed. It comprises a set of multi-modular components, some of which are structural (scaffoldin) and some of which are enzymic to interact with each other and with the cellulosic substrate. The scaffoldin subunit selectively integrates the various cellulases and xylanase subunits into the cohesive complex, by combining its 'cohesin' domains with a 'dockerin' domain present on each of the subunit enzymes. The cellulose-binding domain attaches the cellulosome to the cellulose surface for degradation to occur. Sourced from Krause et al. (2003); Flint et al. (2008).

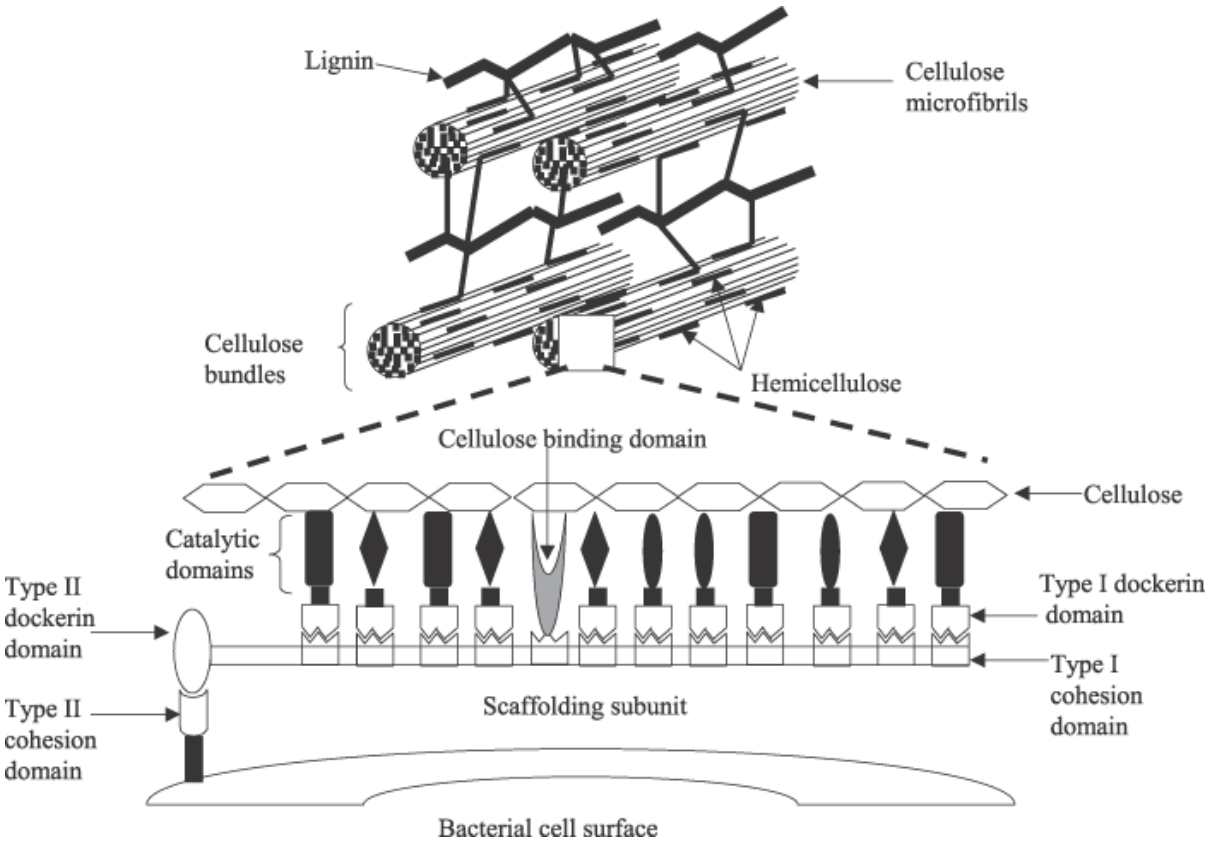
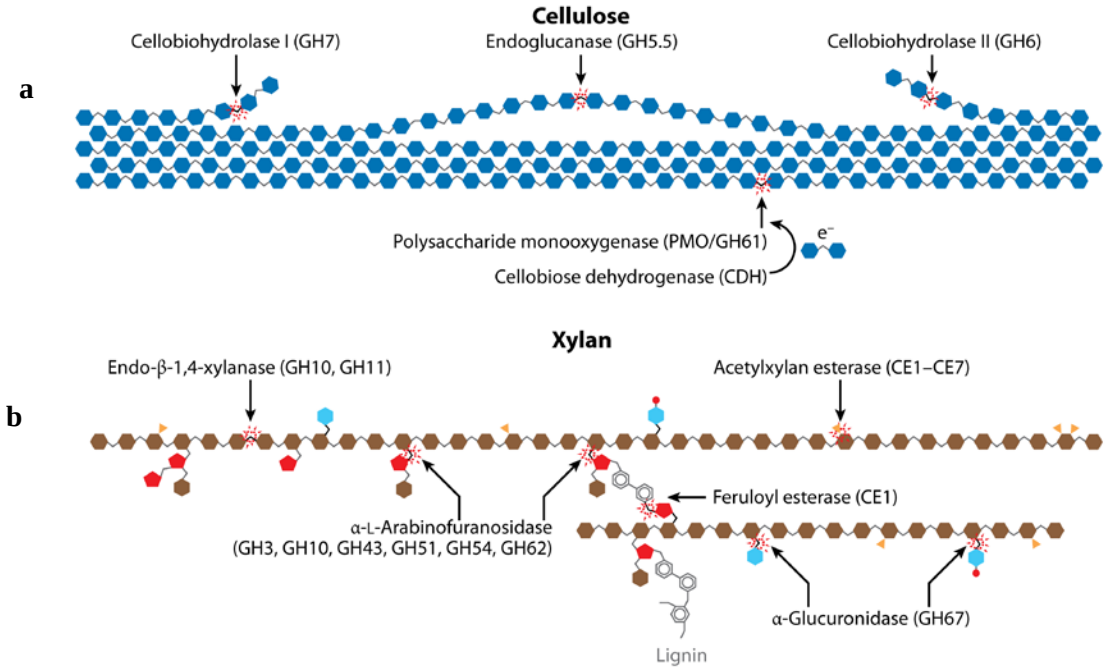


Figure 1.5 Schematic representations of main enzymatic activities involved in the degradation of (a) cellulose and (b) xylan; GH = glycoside hydrolase; CE = carbohydrate esterase; PMO = polysaccharide monooxygenase. Modified from Glass et al. (2013).



The microbial fermentation of carbohydrates generates VFA, CH_4, and CO_2 (Russell and Hespell, 1981). Rumen fermentation and VFA (mainly acetate, propionate, and butyrate) production is diet dependent; with the form of carbohydrate present being the most important factor influencing the concentration and profiles produced (Van Soest, 1994). Ruminal fermentation of structural, as compared to non-structural, carbohydrates typically results in lower total VFA and propionate concentrations but greater acetate concentration (Van Soest, 1994), causing a greater acetate:propionate ratio in the rumen fluid of animals fed forage diets. The VFA formed in the rumen are absorbed across the ruminal epithelium, from which they are carried by ruminal veins to the portal vein and hence through the liver. However, small proportions (10-20% in sheep and up to 35% in dairy cattle) reach the omasum and abomasum and are thus absorbed from these organs (France and Dijkstra, 2005). Continuous removal of VFA from the rumen is important not only for distribution, but to prevent excessive and damaging decline in the pH of rumen fluid. According to NRC (2001), absorbed VFA in the rumen form a major metabolic fuel for the mucosal tissue and may account for up to 75–80% of the digestible energy requirement of the animal. In sheep and

cows at maintenance levels, the VFA provide 50 to 70% of the digestible energy intake and 40 to 65% of the digestible energy intake in lactating cows (France and Dijkstra, 2005).

The rate of gas production in the rumen is most rapid immediately after diet consumption and may exceed 30 l/h in the cow (McDonald et al., 2011). The typical composition of rumen gas is 40% CO_2, 30–40 % CH_4, 5% H_2, with small and varying proportions of oxygen and nitrogen (N) from ingested air (McDonald et al, 2011). Carbon dioxide is produced partly as a by-product of fermentation and partly by the reaction of organic acids with the bicarbonate present in the saliva; and H_2 is produced as a result of carbohydrate fermentation and, if not efficiently removed from the rumen, can inhibit the metabolism of rumen microorganisms (Janssen, 2010). The basic reaction by which CH_4 is formed is the reduction of CO_2 by H_2, some of which may be derived from formate. Methanogenesis, however, is a complicated process that involves folic acid and vitamin B12 (McDonald et al, 2011). About 4.5 g of methane is formed for every 100 g of carbohydrate digested, and the ruminant loses about 7% of its feed energy as CH_4 energy generated from fermented carbohydrates (McDonald et al., 2011). Ruminant livestock produces approximately 80 million tonnes of CH_4 annually and this value accounts for nearly one-third of anthropogenic CH_4 emissions (Beauchemin et al., 2008). Feeding diets that contain high concentrations of structural carbohydrates such as low quality forage results in greater energy loss as CH_4 into the environment than feeding grain to ruminants. This is because more H_2 is generated during fermentation of structural carbohydrates where acetate and butyrate are the major VFA produced (Van Soest, 1994). In contrast, fermentation of starch and other non-structural carbohydrates (NSC) favours propionate production, which acts as a hydrogen sink and thereby reduces the amount of H_2 available for the reduction of CO_2 to CH_4 (Janssen, 2010). However, improving forage quality by harvesting less mature forages and ensuring proper storage will result in a greater proportion of NSC to NDF or the NDF will be less lignified, which can decrease CH_4 emissions per unit of product (meat or milk) (Knapp et al., 2014).

Ruminal fermentation of carbohydrates with end products from protein hydrolysis is also utilized for the maintenance and growth of the microbial community. Amino acids (AA) from microbial fermentation of proteins can be deaminated in the rumen to yield NH_3, carbon skeletons, and also VFA (Kingston-Smith et al., 2008). Ammonia cannot be taken up by the

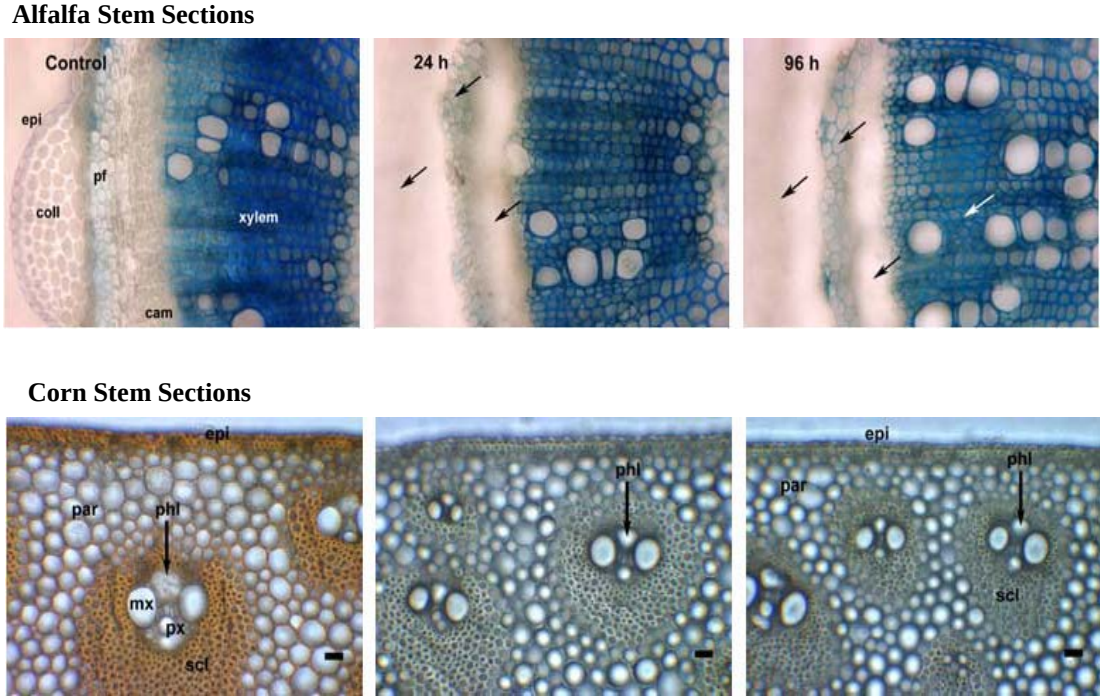
animal for growth unless first assimilated by rumen microbes. Thus, rumen microbes use the NH_3 and energy to synthesize their own AA or proteins (MP; microbial protein). However, in situations where the rate of proteolysis exceeds the relative rate of carbohydrate degradation, NH_3 production can exceed the capacity for it to be assimilated by the microbial community. The excess is then absorbed across the rumen wall and metabolized to form urea in the liver, which is excreted to the environment by the animal as nitrogenous waste (urine) (MacRae and Theodorou, 2003; Kingston-Smith et al., 2008) or recycled back to the rumen (Bach et al., 2005). Urea that is recycled back to the rumen can be turned into NH_3 by microbial urease and used by some members of the microbial community to synthesize AA. Metabolizable protein reaching the small intestine is the net result of the production of MP, bypass protein from the rumen, and endogenous protein (Van Soest, 1994). The MP leaving the rumen may represent about 64% of metabolizable protein absorbed in the lower digestive tract of the animal (NRC, 2001). Also, MP contains both essential and non-essential AA, in proportions that similarly match the overall AA spectrum of proteins being deposited in the tissues of animals (Nolan and Dobos, 2005). However, the total amount of MP flowing to the small intestine depends on the availability of nutrients and their efficiency of utilization by ruminal microbes (Bach et al., 2005).

Limitations to forage digestion

Ruminants have the ability to convert low quality feeds into high quality protein (milk or meat) for human consumption. However, the conversion of fibrous forages to meat and milk is relatively inefficient as plant cell walls recovered from feces are still fermentable (Krause et al., 2003). The extent of cell wall polysaccharide digestion by ruminants is largely a function of the rate of digestion and retention time in the rumen (Mertens, 1993). Cell wall matrix structure reduces rate and potential extent of digestion below the intrinsic rates of digestion of the polysaccharides due to the impact of ferulates and lignin (Paulson et al., 2008). Beyond the chemical structure, tissue organization and cuticular layers of grasses, legumes, and cereal grains also act as a barrier for microbial penetration to plant cell walls in the rumen (Selinger et al., 1996). Therefore, rumen bacteria and fungi cannot move from the interior of one plant cell to the next plant cell by digesting the intervening cell wall if that wall is lignified (Engels, 1989). This restrict cell wall digestion of lignified tissues to the

interior edge of the wall for individual plant cells, which is the least lignified layer, and the plant cell must be ruptured by mechanical grinding, chopping, or mastication to allow access into the cell by microbes (Wilson and Mertens, 1995). One of the major differences in fiber degradation among plant species is between grasses and legumes. Legume leaves have faster rates of cell wall digestion because they contain larger proportions of non-lignified tissues than grass leaves (Paulson et al., 2008). Legume stems also exhibit faster rates of cell wall digestion than do grass stems because of the presence of more non-lignified tissues in legume stems, but potential extent of digestion is greater for grass stems (Paulson et al., 2008). The difference in the degree of stem digestibility is because the thick, lignified grass sclerenchyma cell walls are extensively digested with long rumen residence time whereas similar thick, lignified legume xylem walls show only minimal digestion (Figure 1.6, Jung, 2012). Clearly, the complexity of legume cell wall lignification and how it affects digestion is still not identified.

Figure 1.6 Schematic representations of *in vitro* rumen digestion of alfalfa and corn stem tissues after 24- and 96-h incubations. Non-lignified tissues in alfalfa and corn stems are quickly and completely degraded: epidermis (epi), collenchyma (coll), cambium (cam) and phloem (phl). Lignified phloem fiber (pf) and xylem tissues in alfalfa are only partially digested in 24 h with little further digestion later whereas lignified corn epidermis, parenchyma (par), and sclerenchyma (scl) tissues are substantially thinned over a longer digestion time. Sourced from Paulson et al. (2008).



Retention time of feed particles in the rumen decreases as feed intake increases and particle size of the feed is reduced (Paulson et al., 2008). Controls of feed intake and particle size reduction are complex processes, and are strongly related (Wilson and Kennedy, 1996). Alfalfa disappeared from the rumen more quickly than ryegrass in dairy cows (Waghorn et al., 1989) and sheep (Grenet, 1989) at two 2-h and 4-h meal periods, respectively. The differences in rumen retention time between the two forage types may be partly due to the faster rate of degradation of the alfalfa, but particle size reduction was also faster for alfalfa (Paulson et al., 2008). The reduction in particle size may be partly due to the fragility or the brittleness of the stems, which may explain variation in chewing response (Mertens, 1997); thus, susceptibility of cell walls to mechanical rupture by mastication or chewing must also have been greater for the alfalfa than the ryegrass. The greater susceptibility to particle breakdown of legumes compared to grasses is also readily apparent when forages are mechanically ground; grasses tend to shred and produce long, thin pieces whereas legumes typically grind to finer and more cubical shapes (Paulson et al., 2008). This increased susceptibility to particle size reduction of legumes is likely due to a combination of stem brittleness, cell size of plant tissues, tissue organization, and cell wall thickening.

Apart from the intrinsic characteristics of the matrix of plant cell walls, other factors that modify the microbial and enzymic activity in the rumen also limit the rate and extent of forage digestion (Allen and Mertens, 1988). These include the chemical composition of the fiber and concentration of limiting substrates (non-fiber carbohydrates and N), the surface area available for enzymic attachment, rumen dilution rate due to passage rate, predation of bacteria by protozoa and other biological factors (substrate affinity, catabolic regulatory mechanisms, maximum growth rates, and maintenance requirements), as well as physio-chemical factors (pH, oxidation-reduction potential, temperature, osmotic pressure, hydrostatic pressure, surface tension, and viscosity) (Allen and Mertens 1988). Allen and Mertens (1988) explained that these factors determine the number of available attachment sites for microbes, the mass of fiber digesting microbes in the rumen, the species composition of the microbial population, and the ability of the different species to attach to and colonize the fiber, which together form one step in the process of fermentation lag. In summary, fermentation lag is determined by particle hydration and saturation of available attachment sites by microbes, which are in turn dependent on plant anatomy, animal factors such as

chewing and salivation, and microbial mass in the rumen (Jung and Allen, 1995). However, fermentation lag is hard to account for in various mathematical models of forage digestibility due to the difficulty in getting an accurate measurement *in vivo* (Allen and Mertens, 1988).

Many models, from simple to complex, have been developed to represent the process of digestion in the rumen. Most of these models recognize that the fiber fraction of feeds is not completely digestible and that the fermentability of the potentially digestible fiber is determined by the competition between rate of digestion and rate of passage from the rumen, as proposed by Waldo et al. (1972) for cellulose digestion (Figure 1.7). The rate of digestion and passage are regarded as kinetic constraints to ruminal digestion of plant cell walls. At the risk of representing the process of fiber digestion and passage in the reticulorumen in a simplistic manner, this model (Figure 1.8) is useful to demonstrate the relative importance of factors affecting ruminal fill and fiber digestibility because the data required to adequately parameterize this model are enormous (Jung and Allen, 1995). Allen and Mertens (1988) developed mathematical equations using this model to determine rumen fill and fiber digestibility. For rumen fill, the following equations were derived:

$$D = (f_d\,(d\text{FINTAKE}/dt)) / (k_d+k_p) \qquad \text{equation [1.1]}$$

$$I = (f_i\,(d\text{FINTAKE}/dt)) / (k_p) \qquad \text{equation [1.2]}$$

Fill can be calculated as the sum of the digestible (D) and indigestible (I) fiber pools in the rumen.

$$\text{Fill} = D + I = (d\text{FINTAKE}/dt)\,[f_d/(k_d+k_p) + f_i/k_p] \qquad \text{equation [1.3]}$$

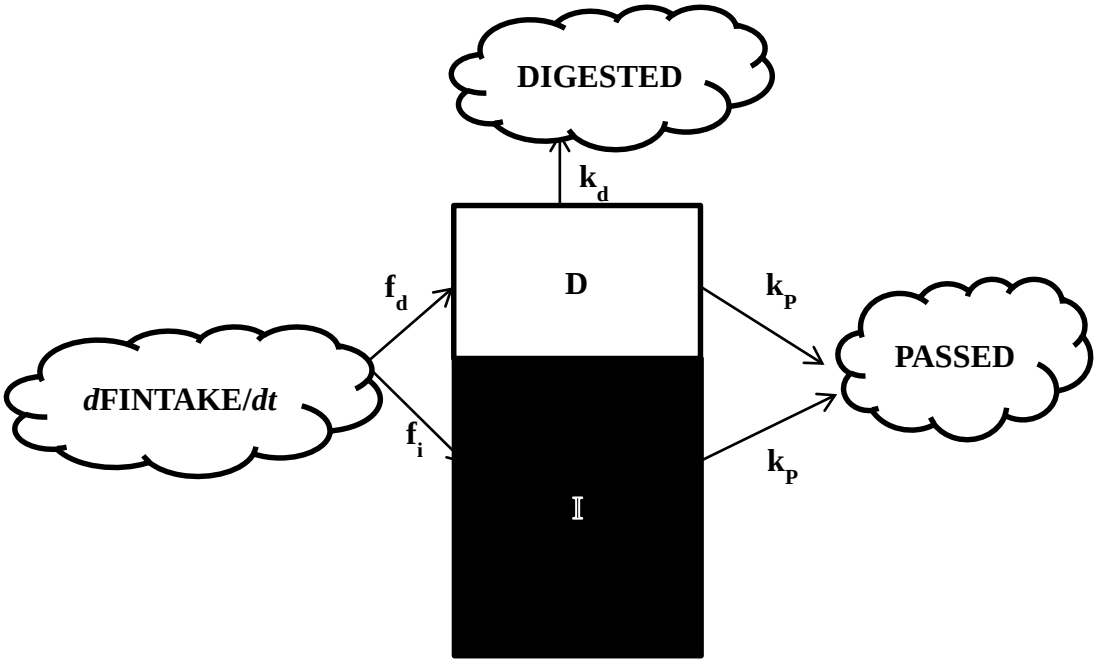
The weight of fiber in the rumen is dependent on the amount of fiber consumed per unit of time (dFINTAKE/dt), the fractions that are digestible (f_d) and indigestible (f_i), as well as rates of digestion (k_d) and passage (k_p). The fraction of fiber in the diet along with dry matter intake (DMI) determines dFINTAKE/dt and is an important determinant of fill. However, ruminal fill is most sensitive to changes in fiber content, followed (in order of decreasing sensitivity) by rate of passage, the fraction that is indigestible, and rate of digestion (Jung and Allen, 1995).

Digestibility is the amount digested per unit time (dDIGESTED/dt) as a fraction of the amount consumed per unit time (dFINTAKE/dt) and has been derived mathematically (as follows:

Digestibility = $f_d[k_d/(k_d+k_p)]$ equation [1.4]

This equation shows that digestibility is directly proportional to the fraction of fiber that is potentially digestible (f_d) and the rate of fiber digestion (k_d), and inversely related to the rate of total fiber disappearance (k_d+k_p). Thus extent of ruminal digestibility increases as ruminal retention time ($1/k_p$) increases.

Figure 1.7 Schematic representation of model of fiber disappearance from the reticulorumen. The amount of cell wall entering the rumen per unit time is *d*FINTAKE/*dt*. Cell wall is either potentially digestible (D) or indigestible (I). Potentially digestible cell wall disappears from the rumen by digestion and passage, whereas indigestible cell wall disappears by passage only. Fiber fractions and rates are represented as follows: digestible fiber as a fraction of intake (f_d), indigestible fiber as a fraction of intake (f_i), fractional rate of digestion (k_d), fractional rate of passage (k_p). Modified from Jung and Allen (1995).



Forage conservation

In most ruminant production systems, livestock derive between 40 and 90% of their feed requirements from forages (Charmley, 2001). In Canada, where the winter feeding period can last for five months or longer, successful forage conservation is crucial to production. Conserved forages are often the only options available to farmers wanting to preserve forage on a large scale, during those periods (winter or drought) when fresh forages are not available. Forage conservation also provides farmers with a means of preserving forage when production is faster than can be adequately utilized by grazing animals. This prevents lush growth from becoming too mature (Muck and Shinners, 2001). The goal of haymaking is to harvest a standing crop such as an alfalfa and preserve its high nutrient content (protein, vitamins, and minerals), digestibility, palatability, and green colour. Therefore, preserved forage of good quality is very important for the nutrition of ruminants, as well as for the quality and safety of meat or dairy products.

Forage is preserved as hay, haylage, or silage. Poor hay, haylage, and silage results in high conservation losses, unpalatability, and reduced intake, which in turn, cause decreased animal performance (Lindgren, 1991). Microbial activity in preserved forages can decrease the nutritional value and can lead to health problems for both animals and humans (Lindgren, 1991). In hay production, the crop is dried so that it is essentially biologically inactive both with respect to plant enzymic activity and microbial spoilage (Muck and Shinners, 2001). In silage making, the forage crop is fermented anaerobically by lactic acid bacteria present on the crop. Silage preservation depends on many factors; however, during storage the major determinants to producing good quality silage are 1) low pH to inhibit clostridia and other detrimental anaerobic microorganisms, and 2) anaerobic conditions to prevent the growth of aerobic spoilage microorganisms such as yeasts and molds (Muck and Shinners, 2001). Haylage is also a form of low moisture silage and, according to Finner (1966), a typical haylage would contain 400 to 600 g/kg DM.

There has been a trend over the last three decades or so for the proportion of forage conserved as silage to increase, while the proportion dedicated to hay has declined (Wilkinson et al., 1996). The reasons for this increase in silage production as a means of forage conservation may be due to the large quantities of forage that can be conserved in a

short time, its conservation is less weather dependent and thirdly, silage is well suited to mechanization (Charmley, 2001). However, preservation of forage as hay is recognized to be superior to preservation as silage with respect to protein quality for high producing ruminant animals (Petit and Tremblay, 1992). Also, in parts of the world where good drying conditions prevail, and similarly in humid climates where ensiling has been considered too difficult because of forage characteristics, high temperatures, or tradition, the production of hay still dominates (Muck and Shinners, 2001).

Hay production and factors affecting hay conservation

Hay production and storage as a means of conserving forages can be traced back to over 2000 years ago (Robertson 1983; McCartney, 2005). Through the modern technology of farming, haymaking has evolved from traditional hand production to more mechanized systems of harvesting, storage, and feeding. Hay is usually produced and stored as dry bales; however, due to its bulkiness, difficulty in handling and shipping, hay bales are often re-compressed after baling or processed into dehydrated and sun-cured pellets or cubes (Muck and Shinners, 2001). The rectangular baler remained the dominant hay harvesting system until the early 1970's when the large round balers and various types of loose hay mechanical stacking machines were developed. During these years, there was a lot of development and evaluation research occurring in North America and Europe on hay harvesting systems (McCartney, 2005). However, with all this advancement, research has shown that losses in quantity and quality still occur before, during, and after storage. Field loss is dependent on weather conditions during the drying period, on type of mechanization, and on the moisture content of the forage at time of baling (Thorlacius 1984; McCartney, 2005). Leaf loss from the field is estimated to be 62% and DM loss can be as high as 37% in rain-damaged, cured alfalfa hay (McCartney, 2005). Storage losses caused from spoilage and heating increase at moisture levels above 20%. This DM loss from poorly stored hay also translates to significant financial losses when lost nutrients have to be replaced by supplemental protein or energy products.

Producing high quality hay begins with mowing. Forage crops should be mown at the right maturity to optimize yield and quality. Quality in most forage crops declines rather rapidly as the crop enters a reproductive stage of development and growth begins to slow

(Rotz, 2003). The optimum maturity level varies among forage species, but normally this level occurs in the late vegetative to early reproductive stages. Mowing at this time provides a good yield, relatively low fiber content, and adequate energy and protein contents (Rotz, 2003). However, the challenge to mowing the forage at its optimum maturity is finding the right time of day to mow (McCartney, 2005), or the time when weather conditions are suitable for drying (Rotz, 2003).

On warm, sunny days when the crop is actively growing, the plant stores readily digestible (non-fiber) carbohydrates through the process of photosynthesis in the plant tissue and uses them for respiration at night (McCartney, 2005). Orr et al. (1997) observed that grazing animals consumed more grass and clover in the afternoon than in the morning. In the Western United States, Fisher et al. (2002) also observed that shifting hay mowing from early in the day to late in the day was effective in increasing forage preference by sheep, goats, and cattle. By cutting forages during the late afternoon, extra sugar is captured thus increasing the feeding value of the forage (McCartney, 2005). A crop mown in late afternoon will have reduced fiber and higher energy contents at mowing. However, this crop lays in the field for an extra night period, where at least portions of these carbohydrates are used in plant respiration. In temperate climates where weather conditions are variable, these carbohydrates are often used in plant respiration or lost due to rain damage (Rotz, 2003). With good drying conditions, some of the WSC can be preserved through the harvest and storage processes. For hay making in dry climates, Rotz (2003) suggested afternoon mowing as the best practice. However, for some areas, hay producers must weigh the need for extra drying time from late morning to late afternoon against the need for higher quality forage.

If the forecast is heavy rain and poor drying conditions, it is normally best to delay haymaking. However, one can mow when there is light rain when adequate to good drying conditions are expected, but this decision is dependent on the portion of total forage required at the highest level of quality by management or a farmer (Rotz, 2003). When rain occurs during field curing, yield or DM losses of up to 30% are commonly reported, with losses of over 50% reported with heavy rain damage (Collins, 1983; Rotz and Muck, 1994). Most of the DM lost consists of highly soluble and digestible plant nutrients that invisibly leach into the soil so that forage quality is excessively reduced (Rotz and Muck, 1994). Heavy rain can also cause leaves to sever from the stem of plant into the soil. This normally affects legumes

more than grass. This could be due to the lighter nature of the leaves of leguminous crops than grass. In a field study, Shepherd et al. (1954) measured increased leaf loss of 8.8% in alfalfa subjected to two rain showers and 36% with three rain showers.

There are many forage harvesting equipment manufacturers around the world, and as a result, computer forage programs have been developed to assist the forage producer in their choice of equipment (Rotz, 2001). Many different systems or combinations of machines and processes can be used to produce dry hay. The Prairie Agriculture Machinery Institute in Western Canada has tested the efficiency and performance of most North American and European hay harvesting equipment (McCartney, 2005). The primary mower designs available for cutting forage crops are either a sickle bar mower, or a rotary disk mower (Figure 1.8; Rotz, 2003). The sickle bar mower uses reciprocating knives, while the rotary disk mower has knives rotating at high speed. The sickle bar mower has been used worldwide for many years. It is a very reliable, low cost method of mowing. Animal power or a low horsepower tractor can pull it. The major disadvantage is limited mowing capacity, as field speed is limited by the cutting capacity of the reciprocating knives (McCartney, 2005). These knives are more likely to plug with heavy, wet or lodged forage. This limited capacity has led to the development and wide spread use of rotary mowers (Rotz, 2001; McCartney, 2005). Rotary mowers tend to have a higher power requirement and thus require a larger tractor and more fuel to operate per hour of use. However, the faster field speed reduces time, offsetting some of the increased fuel cost and reducing labor required for mowing (Rotz, 2003). Rotary mowers exert a certain amount of suction because of their high rotational speed and consequently small amounts of dust or soil move into the forage (McCartney, 2005). This can increase the ash content of the forage. The purchase price of rotary mowers is a little higher for a given width of cut (Rotz, 2003), and repair costs may be greater, particularly as the machine ages (McCartney, 2005); however, the overall cost of mowing is similar between these major mower types (Rotz, 2003). The type of mower used has little effect on mowing losses and the resulting forage quality (Rotz, 2003).

Figure 1.8 Pictures of sickle bar mower, which provides an economical cutting option for tractors with a lower horsepower (top) and rotary disc mower (bottom). Source http://www.deere.ca/en_CA/industry/agriculture/learn_more/index_brochures/hay_forage.page.

The need for rapid wilting or drying of mowed forage in the field to a moisture content of approximately 15–20% (McDonald, 2011) is well recognized, but accomplishing this task remains a challenge. Many factors affect the drying rate of mowed forage in the field (Rotz, 1995). Drying is restricted by: soil moisture, weather conditions (temperature, humidity, solar radiation and wind speed), forage (species, structure, stage of growth and

leaf:stem ratio of the plant), and structure and volume of the swath or windrow (Rotz, 2003; McCartney, 2005). In temperate climates, weather is often the most restrictive factor in drying, and of all the influences on quality imposed by weather, solar radiation level is the most important (Rotz, 1995). The energy from the sun is required to evaporate and move moisture out of the plant. This energy required is about 7 billion J, which is equivalent to the energy derived from combusting 270 l of fuel oil. The drying of hay by this level of energy removes about 3000 kg of moisture/1000 kg of hay produced (Rotz, 2003). Even though the sun is the main driving force, warm air temperature and low humidity also aid in drying mowed forage and enabling economic hay production. Moist soil under the swath can also slow drying by allowing moisture to move up into the swath (Rotz, 2003).

Dry matter losses and quality changes occur while the crop is wilting or drying in the field. These include plant respiration, rain, and machine induced losses (Rotz and Muck, 1994). The rate of moisture loss follows an exponential pattern so that a drop in moisture from 80% at cutting to 30%, may take as much time as dropping from 30–20% (Wilkinson, 1981). This is because plant respiration, which is a natural process of converting carbohydrates stored in the plant tissue to CO_2, heat, and moisture by the action of hydrolytic enzymes and epiphytic microbes present in living cells, continues after the forage is mowed until some lethal condition intervenes (Rotz and Muck, 1994). Plant respiration continues after cutting until moisture levels of 30–40% are reached and can result in dry matter loss of predominantly NSC, ranging from 2–16% (McCartney, 2005). Since this loss is primarily readily digestible carbohydrates, the loss increases the fiber content proportionally and reduces the energy content of the forage (Rotz, 2003). Also, plant respiration can result in loss of N to approximately 2.5% of the initial level (McCartney, 2005). Proteolysis proceeds rapidly from the time of cutting until terminated by either the attainment of a high DM content or a low pH value (Robertson, 1983). Rainfall, when it occurs can have the greatest effect on loss and quality of hay. Heavy rainfall can knock leaves and invisibly wash soluble carbohydrates, protein, and minerals from the plant material leaving greater fiber and reduced energy concentrations. It can also cause respiration to reoccur in drier forage and this results in a significant loss in DM and nutrients. Some studies have found field losses due to poor drying conditions can range as high as 4% per day (McCartney, 2005).

Most mowing devices used in hay production today include mechanical conditioning to help speed field drying of the crop. Conditioning modifies the plant structure to improve drying rate without causing leaf loss. This process eliminates the need for raking to turn the hay windrow over for drying. These conditioners are usually categorized as either roll or flail conditioners (Rotz, 2003). Rolls smash and/or break the plant stems, and flails abrade the waxy surface of the plant and break stems. Both processes can improve drying, but for alfalfa, roll devices are more effective with less field loss (Rotz, 1995). Some roll designs are promoted for faster drying, but field and laboratory studies consistently show little or no difference in the drying of alfalfa or grass treated with a commonly used crushing roll design (Shinners et al., 1991; Rotz and Sprott 1984). Roll conditioning is most effective on crops with thick stems such as an early cutting of alfalfa. Flail-type conditioners are better suited to grass crops, and they provide a greater throughput capacity when harvesting high yielding or entangled crops (Rotz, 2003). Generally, the two main conditioners' effectiveness in reducing field losses may be forage specific. Again, DM losses and the associated nutrient changes caused or promoted by conditioning increase with severity of conditioning (Rotz, 2003). Although more severe mower conditioning provides faster field curing, harvest losses are generally greater. Mowing and conditioning loss is mostly leaves, so to reduce loss with little effect on forage quality, Rotz, (2003) recommended less severe conditioning to obtain adequate drying with relatively low loss (1-2% of yield). However, considerable research and development is still ongoing, devoted to producing the fastest forage drying time (Savoie, 2001). Drying rate has been increased by 25-150% with new machines known as super conditioning or maceration equipment under good drying weather (Savoie, 1990), but these machines require at least twice the power of the mower conditioner and field losses can be very high when it rains. Thus, with rain damage very little can be done to prevent dry or partly cured hay from field losses. Once the plant cell has died, rainwater can dissolve its contents and leach them from the hay with the most digestible contents leaving first. In the case of light rain, the loss of nutrients may be minor but heavy rain leaches nutrients heavily and also compacts the windrows (McCartney, 2005). Also, chemical treatment referred to as a conditioner or drying agent can be sprayed on the crop at mowing to help speed drying (Rotz, 1995). The chemical affects the waxy surface of the plant to allow easier moisture removal. The most effective treatment is potassium and sodium carbonate-based solution.

This treatment has only been reported to be effective on alfalfa, and it is most effective on cuttings harvested in the summer months (Rotz, 2003).

As forage dries in the field, the top of the swath dries more rapidly than the bottom. Turning the swath can speed the drying process by moving the wetter material to the upper surface where it dries more quickly (Rotz, 1995). Spreading can also expose more of the crop to the radiant solar energy and drying air. There are three methods used to move hay swaths: tedding, swath inversion, and raking. These treatments can help speed drying, but the machines used for each process create additional loss by dislodging leaves and other plant material.

Hay tedders use long rotating fingers to stir, spread, and fluff the swath (Figure 1.9a). The tedder increases the area over which solar radiation reaches the swath. It also improves aeration and drying time by fluffing the windrow (McCartney, 2005). Tedding can improve the drying rate by 28-58% on the day it is applied. However, there can be mechanical loss of the leaves due to the high speed fingers fluffing the crop. Savoie (1990) found field losses of 4-8% when he tedded alfalfa (*Medicago sativa L.*) and losses of less than 2% with timothy (*Phleum pratense L.*). Again, he found that in a maritime climate swaths cut with a mower conditioner required 36 h to dry to 20% moisture under favorable weather conditions. Tedding twice reduced the drying time by 11 h or a full day. If a producer only uses the tedder once, then Savoie (1990) recommends doing it on the first day right after mowing. This is because when tedding is done on a relatively wet crop (above 50% moisture) the resulting loss is less than 3%; however, applied late in the drying process, the loss can be more than 10% (Rotz, 2003). The decision to use tedding should be made by comparing the probable loss from more time lying in the field to the known loss and cost of tedding. The increased machinery, fuel, and labor costs may only justify routine use of tedding on grass crops in wet climates (Rotz, 2001).

Windrow mergers or inverters have been used that gently lift and invert the swath (Figure 1.9b). The dry layer is moved to the bottom and the original bottom wet layer is moved to the top. Swath inversion is not as effective for improving drying as tedding, but shatter loss is very low. With less drying benefit, there is less potential for reducing rain and respiration losses. The added labor, fuel, and machinery costs of the operation are generally greater than the benefit received (Rotz and Savoie, 1991).

Raking is another form of swath manipulation (Figure 1.9c). Hay rakes are used to turn or roll together the windrows for easier pickup by the baler and to speed drying. Following the initial improvement, the increase in swath density can reduce drying rate, so the crop moisture content at raking is important. Raking also causes loss, and this loss is related to crop moisture (2% when wet to 15% in very dry crop) (Rotz, 2003). The best moisture content to rake hay for low loss and good drying is between 30 and 40% (Rotz, 2003). The losses that occur from raking are primarily alfalfa leaves, which is light. Thus if a light crop spread over the field surface is raked, losses can be more than double when narrower swaths are used (McCartney, 2005). In dry climates, hay can be raked at night or early morning when leaves are moist and less prone to shatter. Raking at the proper time can reduce field-curing time by a few hours to allow an earlier start at baling.

Dry hay is compressed and packaged in one of four forms: bales, stacks, cubes, or pellets for easier handling and transportation (Muck and Shinners, 2001). Hay balers are capable of producing bales of many sizes and shapes. Small rectangular bales have been most popular over the past fifty years, but now large round and large rectangular bales are becoming the predominant hay packages (McCartney, 2005). This traditional small rectangular bale is a viable option, but handling bales of this size (h × w; 35 × 46 cm) and weight (15-35 kg) tends to require considerable manual labor (Muck and Shinners, 2001; Rotz, 2003). Balers producing these large packages also offer greater baling capacity, harvesting up to twice the hay per hour as the small package balers. A popular option on livestock farms is the large round bale system, which was introduced in the early 1970's (McCartney, 2005). Large round balers require more power than small rectangular balers and the recommended minimum tractor sizes vary with baler size from 35-55 kW (Rotz and Muhtar, 1992). The large round bale weighs between 200-900 kg and its diameter and length varies between 90-180 cm and 120-160 cm, respectively (McCartney, 2005). There are two types of large round balers depending on chamber design. The hard-core baler has a spring-loaded adjustable bale chamber that rolls the hay under continuous pressure from start to finish. The soft-core baler has a fixed bale chamber and the hay does not roll until it fills the chamber and exerts pressure on the rollers (Beacom, 1991). Typical DM losses during hay baling vary between 2 and 6% of the yield with the loss equally divided between pickup and chamber losses (Rotz, 2003). Chamber loss is influenced by baler design and crop moisture

content. These losses are about 1-3% in small rectangular balers and 0.5-2% in large rectangular balers. For large round balers the loss in DM varies with the two chamber designs; but the loss can be three times as much with a fixed chamber baler compared with a small rectangular baler. Chamber loss is mostly high quality leaf material; so excessive chamber loss reduces the nutritive content of the remaining forage. Excessive loss occurs at low feeding rates of hay into the baler and if the bale is rolled in the chamber too many times per unit of hay baled (Robertson 1983; Rotz 2001). These chamber losses can be minimized by conditioning the forage crop, maintaining the fastest ground speed possible while bailing, increasing the width of cut, and reducing the power takeoff speed in light crops (McCartney, 2005). By so doing, chamber loss would be below 3% and this will have relatively small effect on forage quality (Rotz, 2003). Completed bales are transported to the storage site with a tractor mounted loader or a wagon. Other machines have been developed to pick up the round bales in the field and transport them to the storage site.

Figure 1.9 Pictures of tedder (a), windrow merger (b), and hay rake (c). Source: New Holland hay and forage equipment (http://agriculture.newholland.com/us/en/Products/Hay-and-Forage-Equipment/Pages/products_selector.aspx).

(a)

(b)

(c)

The safe storage of hay requires that the moisture content of the harvested forage be below 20%. Respiration by epiphytic microorganisms (bacteria, fungi, and yeasts) on hay causes heating and further DM and nutrient loss during storage. Heating depends on the moisture of the hay, the bale size and density, the drying rate of the hay, and the microbial population in the hay. If baling is done at 20-30% moisture as a means of preventing leaf loss, microbial activity can still exist in the forage after baling. Hay does not become static until the bales reach a moisture content of about 12% and the humidity is below 65%. In these conditions, most epiphytic microbes such as fungi will not grow (Mahanna, 1994). Wittenberg (1997) observed that the bulk of moisture loss from hay stored at 24-35% moisture occurred between days 4-14 of storage, a period when stack temperatures were the highest. Enzyme activity and epiphytic microbes with the help of oxygen degrade NSC of the forage and these result in the production of heat, CO_2, and water. Thus, the temperature rise associated with aerobic respiration is an important indication of the initiation of epiphytic microorganism growth. Also, greater heating occurs as hay density increases, particularly in large bales (Rotz and Muck, 1994). Dry matter loss during the first month of storage varies from 1-8%, increasing with hay moisture content. In many parts of the world, it is extremely difficult to dry hay in the field to below 20% due to either high rainfall or high humidity. For

hay with more than 25% moisture, excessive loss and even spontaneous combustion can occur (Rotz, 2003). Although most loss occurs in the first month, a small loss of about 0.5% DM per month continues in hay stored in a shed. The loss increases for unprotected hay stored outside due to weathering on the exposed bale surface (outer 10-20 cm). Loss in large round bales stored outside varies widely, ranging from 3-40% (Rotz, 2003). This loss is mostly affected by weather, length and method of storage. Dry matter loss and heating of hay affects the concentration of most nutrients. The loss of NSC respired to CO_2 and water due to microbial activities causes acid detergent fiber (ADF) and NDF concentrations to increase proportionally (Wittenberg, 1997). Some crude protein (CP) is also lost.

Preservation of forage as hay is recognized to be superior to preservation as silage with respect to protein quality for the high producing ruminant animal (Petit and Tremblay 1992). The magnitude and duration of spontaneous heating, moisture content, and forage type all affect the amount of heat damage that may occur to forage proteins (Coblentz et al., 2004). Generally, the changes in concentration of CP are somewhat dependent on length of storage time. In the short term (< 60 days), concentrations of CP may actually increase (Coblentz et al., 2000) because of preferential oxidation of non-fiber carbohydrates. The long term effect of heating during bale storage is to decrease CP content, and this can be reduced by 0.25 percentage units per month due to volatilization of NH_3 and other nitrogenous compounds (Rotz and Muck, 1994); however, this loss is unlikely to continue indefinitely. Maillard or browning reactions occur in hays as a consequence of heating, and these reactions may impact the apparent digestibility of N more severely. Maillard reactions occur when carbohydrates are degraded in the presence of amines or AA to yield polymers that are largely indigestible in ruminants (Coblentz et al., 2004). Normally, heat-damaged CP is determined by quantifying the N or CP (6.25 × N) remaining in forage residues after digestion in acid detergent. This is normally referred to as acid detergent insoluble nitrogen (ADIN) or acid detergent insoluble crude protein (ADICP). Moisture also plays a critical role in the Maillard reaction; first as a catalytic effect, which is why silages are more susceptible to heat damage than forages conserved as hay, secondly, the moisture of the hay at baling stimulates spontaneous heating, which subsequently increases the probability of heat damage proteins. All forages have some indigestible protein that is inherently unavailable to livestock, but this fraction is generally small in most standing forages or unheated hays.

Concentrations of ADIN in unheated alfalfa can range between 3-6% of total N (Coblentz et al., 2004). Typically, the indigestible protein in unheated warm-season grasses represents a higher percentage of the total forage N, and it can exceed 20% of total N in dormant forages. Grass hays are typically more susceptible to heat damage than alfalfa or other legumes. For example, Coblentz et al. (2004) showed that small rectangular alfalfa bales exhibited reduced ADIN per unit of heating than small rectangular bermudagrass bales. This effect may partly be due to higher concentrations of hemicellulose, which is a reactive fiber component and a structural carbohydrate (Coblentz et al., 2004); or due to lower concentration of CP in grass than in legumes. The greater CP concentration in legumes may explain why ruminant nutritionists usually consider alfalfa to be seriously heat damaged when concentrations of ADIN exceed 10% of total N.

Forage protein value is assessed as the amount of AA available for absorption in the animal's small intestine. These AA may be derived directly from the dietary protein being offered or may be derived from MP synthesized in the rumen. Forage harvest and storage systems that increase the proportion of forage protein that is undegradable in the rumen without increasing the unavailable protein will result in greater concentrations of dietary protein available for absorption in the small intestine (Wittenberg, 1997). Theoretically, ADIN is unavailable to ruminants; however, Broderick et al. (1993) reported digestibility in lactating dairy cattle of -12.2% for ADIN on an unheated (ADIN = 4.4% of total N) alfalfa hay diet, but the digestibility increased to 35.8% when the diet contained steam-treated alfalfa hay with ADIN accounting for 16.3% of the total N in the forage. Also, McBeth et al. (2001) showed that digestibility coefficients for ADIN measured in lambs increased linearly with spontaneous heating in bermudagrass hays. While this may provide some benefit with respect to N retention and utilization, Coblentz et al. (2004) explained that it should not be viewed as a justification for allowing forages to heat intentionally in the bale. Protein bypassing the rumen has been assumed to be 80% digestible (NRC, 1996), but this clearly may vary with source and processing/handling conditions (NRC, 2001).

Hay preservatives

Preservatives allow forage to be baled at higher moisture content, thus reducing field drying time and the chances of rain damage. Baling hay at 25% moisture reduced field-curing time by about a day (Rotz and Muck, 1994). Hay baled at higher moisture may reduce mechanical losses, providing an increase in harvested yield (up to 7%) and harvested quality (Rotz, 2003). However, moist hay deteriorates rapidly in storage, thereby offsetting the benefit of reduced field loss. Thus, moist hay may benefit from treatment to enhance preservation. There have been a lot of effort directed towards the development of hay preservatives designed to inhibit microbial activity that may adversely affect the quality of hay during storage. Additives used for the preservation of high-moisture hay include propionic acid, organic acid mixtures, buffered acid mixtures, anhydrous ammonia, microbial inoculants and enzymes. Most preservatives are applied at the time of baling as either a granular or liquid product; however, some products such as anhydrous ammonia may be applied shortly after stacking (McCartney, 2005). The preservative usually needs to inhibit the production of fungal end products (spores and mycotoxins) that have direct adverse effects on hay handlers and livestock. The product also requires easy application and safe handling procedures, no adverse response by animals consuming treated hay, lack of residue in animal products, and sufficient cost recovery (Wittenberg, 1997).

Organic acids are effective in preventing fungal invasion of moist hay during storage when adequate levels are applied. Propionic, acetic, isobutyric and formic acids, at levels of 1-2.5% of wet forage weight significantly reduced DM losses; and on average increased crude protein. Most commercial acid-based preservatives contain propionic acid or a propionic-acetic acid mixture which has been shown to reduce mold growth, heating in high moisture baled hay, and short-term DM loss (Rotz, 2001). On the contrary, DM loss over a longer storage period (> 4 months) was not reduced in either small or large bales (McCartney, 2005); thus, it maintained almost the same level of microbial activity compared to untreated high moisture hay (Rotz, 2003). The reason for this effect is that moisture was retained in the high moisture propionic-treated hay due to the long storage period, and the moisture supported microbial growth, but at a lower rate, because of the presence of the preservative. Typical application rates of propionic-based preservatives are 1-2% of the weight of the wet hay (Rotz, 2003), although applications as high as 2.5% by weight have

been reported (Robertson, 1983). Economic benefit from applying organic acid preservatives at the typical rates is only justified when it is used to avoid damage from rain (Rotz, 2001).

The corrosive nature and the high vaporization losses of the original acid products can cause application problems (Wittenberg, 1997). A dilute acid product, which is less corrosive and requires a high application rate, was developed. A second approach was to neutralize the acids. Neutralized propionates include ammonium, calcium, or sodium salts of propionic acid or use a buffer, thus making the product less corrosive and less volatile (Wittenberg, 1997). Laboratory results indicate that neutralized acids are less effective in preventing fungal invasion of moist hay, however, because they do not volatilize during field application and the recommended application rates are similar to that of the acid that is not neutralized (Wittenberg, 1997).

Anhydrous ammonia is perhaps the most effective hay preservative (Robertson, 1983; Thorlacius and Robertson 1984; Mir, 1991). Application rates of ammonia depend on hay moisture content, and are recommended to be approximately 2% of forage weight for hay that is 25-30% moisture (Thorlacius and Robertson, 1984). Mir et al. (1991) found that ammoniation raised the CP content of large round baled brome-alfalfa and alfalfa hay harvested at less than 20% and at 30% moisture, compared to non-ammoniated, field-cured hay at less than 20% moisture. The ammoniated hay was free of mold even after 14 weeks of storage. It was concluded that ammoniation was effective in preserving the quality of the high moisture hay, which would have spoiled otherwise. In the past, anhydrous ammonia was an economical method of hay preservation. Presently however, anhydrous ammonia is not widely used as forage preservative because of its high cost and volatile and caustic nature in relation to animal and human safety issues (McCartney, 2005). A cost benefit analysis of adding anhydrous ammonia limits application. Unless the anhydrous ammonia is applied to low quality forages or crop residues in years of forage shortages, it is not economical (McCartney, 2005). Ammonia treatment of forage has caused toxicity to animals when applied at high application rates (greater than 3% of hay weight) on alfalfa hay (Rotz, 2001). Anhydrous ammonia can cause burns, blindness, and even death when humans are directly exposed.

Microbial inoculants for forage preservation are widely accepted tools that are alternatives to organic acids in forage ensiling systems because they meet all the criteria of a

desirable preservative agent (Wittenberg, 1997). Most microbial hay inoculants marketed today were initially developed to aid in the fermentation of silage or haylage. Many of the early studies focused on lactic acid anaerobes commonly used in silage preservation, including the genera *Lactobacilli, Pediococci*, and *Streptococci*. A wider range of organisms, including the genera *Bacilli*, are being investigated based on their ability to survive in the early stages of storage within the bale microenvironment and on their ability to inhibit or modify fungal activity (Wittenberg, 1997). In the late 1980's, aerobic bacterial hay inoculants specifically designed for alfalfa hay were introduced into the market place (Wittenberg, 1997). One of such commercial product contained *Bacillus pumilus* and this had been marketed as permitting hay to be baled in the range of 20-25% moisture and reducing mold and heating in storage (Mahanna, 1994). However, certain products containing *Bacillus* bacteria which are better suited to the aerobic hay environment are showing little scientific evidence that they can provide substantial improvement in preserving moist hay. Mir et al. (1995) did not find any advantage of using *Lactobacillus plantarum* on alfalfa round bale forage at 18% moisture content. Also, Baah et al. (2005) did not find any reduction in heating or in spoilage microbes on alfalfa baled at 19% moisture with *Lactobacillus buchneri* 40788 but reported improvement in timothy baled at 20% moisture. Baron (1988) found that live bacterial culture, *Bacillus subtilis* or 12% lactic acid extract, were not effective at any moisture level for use in hay harvesting and storage. Since the moisture content of baled hay is lower than silage or haylage, it may be that the moist hay may not support the growth of spoilage microbes as compared to silage or haylage, hence the ineffectiveness of these inoculants in hay production. Other factors such as variations in internal bale temperatures, chemical composition, pH, and interactions between different microbes in the forage may be reasons for inconsistencies in the use of microbial inoculants as hay preservatives.

Enzymes additives, like microbial inoculants, have been used widely in silage or haylage preservation. Enzymes normally used are primarily cell-wall degrading or fibrolytic enzymes, and the principles behind their usage are: (1) promote plant cell break down rendering the cellulose and starch found in the plant fiber (ADF and NDF) more accessible to desirable acid-producing bacteria; and (2) partially breaking down plant cell walls so that animal performance on the hay would be more similar to performance on hay harvested at a more immature stage (Muck and Shinners, 2001). While they do not directly prevent mold

growth, enzymes will make nutrients available to desirable lactic acid bacteria (LAB); thereby increasing the desirable LAB. These products have been successful, largely in grasses, in breaking down cell walls (Muck and Kung, 1997) but much less successful in terms of animal performance (Kung and Muck, 1997). It appears that the crude enzyme extracts are breaking cell wall linkages that are readily attacked by rumen microorganisms and leaving a cell wall that is less digestible (Muck and Shinners, 2001). These enzyme products would be promising if they acted on chemical linkages in plant cell walls that limit the activity of fiber-degrading rumen microorganisms.

Unlike cellulase and hemicellulase enzymes, certain ferulic acid esterase (FAE) enzymes can hydrolyze ester linkages that bind carbohydrates to lignin. Several studies have shown that esterase enzymes can complement cellulase and hemicellulase enzyme effects on plant cell walls, thereby increasing DM or fiber degradability (Yu et al., 2005; Krueger et al., 2008a; Lynch et al., 2013). Some strains of *L. buchneri* have been found not only to produce organic acids, but also FAE enzymes (Nsereko et al., 2006a). This organism has been used as a silage inoculant to reduce growth of spoilage organisms. Silage treated with this organism was reported to improve NDF degradability by 5 to 7 units (Nsereko et al. 2006b). This is because of the simultaneous catalytic and preservative effect of the bacterial inoculant on preserved forages. Thus, adding a ferulic acid esterase bacterial inoculant to cellulase-hemicellulase enzyme preparations could increase the potency of fibrolytic enzymes on hay. Thus, it may be possible that combining fibrolytic enzymes with ferulic acid esterase bacterial inoculants would improve (not just preserve) the quality of the baled hay.

Exogenous fibrolytic enzymes in ruminant production systems

The efficiency by which ruminants obtain energy from structural plant polysaccharides and, in turn, produce high quality meat and milk protein is increasingly important if the demands of an expanding human population are to be met (Meale et al., 2014). In addition, if the demands for reduction in methane production are to be met, then the efficiency by which ruminants utilize forage cell walls to produce milk and meat is even more important, as increases in enteric methane production are normally associated with ruminants fed forage diets. Various strategies have been attempted to improve forage quality for ruminant livestock including treatment with physical agents such as heat, steam, and

pressure; with chemicals such as acids, alkalis, and NH_3; with biological agents such as white rot fungi; via natural selection, breeding, or molecular engineering and enzyme technology (Adesogan et al., 2014). However, none of these methods is widely used for improving forage quality and ruminant animal performance. This is due to the capital and energy intensive nature of physical methods such as steam or pressure explosion, the potential of pelleting, chopping, or grinding to limit salivary buffering of ruminal acids and retention time in the rumen (decreasing digestibility), the cost and corrosive and/or hazardous nature of chemicals such as NH_3 and sodium hydroxide, the potential for extreme DM losses following hydrolysis by white rot fungi, and the prolonged nature of breeding approaches (Adesogan et al., 2014). Exogenous fibrolytic enzymes are increasingly considered as cost-effective means of improving feed efficiency (Krause et al., 2003), and their use in forage preservation may be desirable as they are not corrosive and/or hazardous, unlike chemical treatments. Furthermore, fibrolytic enzyme costs have been declining due in part to more efficient production systems.

Source of fibrolytic enzymes and enzymic activities

Research on effects of forage cell wall degrading or fibrolytic enzymes started as early as the 1960's as reviewed by Beauchemin et al. (2003) and Beauchemin and Holtshausen (2010). Most research has centered on the use of exogenous fibrolytic enzymes to increase fiber digestion and thus digestible energy intake, but responses have been variable. In some studies, the enzyme formulations increased *in vivo* fiber digestibility, average daily gain (ADG) and feed efficiency (ZoBell et al., 2000; Titi, 2004; Krueger et al., 2008b), and milk production (Gado et al., 2009; Klingerman et al., 2009) of ruminants. However, in many cases, the efficiency of growth or milk production in ruminants has not been improved (ZoBell et al., 2000; Rojo et al., 2005; Arriola et al., 2011a).

Enzymes are naturally occurring biocatalysts produced by living cells to bring about specific biochemical reactions. In the case of ruminants, they are produced by the animal itself or by microbes naturally present in the gut. In the context of feed additives for ruminants, enzymes are employed to perform the degradative reactions by which feedstuffs such as hay or silage are degraded into their chemical components and/or simplest or

absorbable form, such as simple sugars and amino acids (McAllister et al., 2001). These are in turn used for cell growth, either by ruminal microorganisms or by the host animal.

Commercial ruminant enzyme additives contain concentrated enzymic activities that are involved in degrading fiber. They are derived primarily from four bacterial *(Bacillus subtilis, Lactobacillus acidophilus, L. plantarum,* and *Streptococcus faecium*) and three fungal (*Aspergillus oryzae, Trichoderma reesei,* and *Saccharomyces cerevisiae*) species (McAllister et al., 2001). Enzyme preparations for ruminants are produced through microbial fermentation, beginning with seed culture and growth media. Once the fermentation is complete, the enzyme protein is separated from the fermentation residues and source organism. Although the microorganisms from which the enzymes are derived only constitute a very limited group, the types and activity of enzymes produced can be diverse depending on the strain selected, the substrate they are grown on, and the culture conditions used (Gashe, 1992; Lee et al., 1998; Meale et al., 2014).

Enzyme activity is assayed by measuring either the disappearance of a defined substrate or the generation of a product from the biochemical reaction catalyzed by the enzyme over time (McAllister et al., 2001). Activities of enzymes are most commonly measured using the latter approach by the feed industry, and are expressed as the amount of product produced per unit time. In the case of carbohydrases or enzymes that hydrolyze carbohydrates, the production of free sugars is the most common product measured. These measurements must be conducted under conditions closely defined with respect to temperature, pH, ionic strength, substrate concentration, and substrate type, as all of these factors can affect the activity of an enzyme (Headon, 1993; McAllister et al., 2001). Enzyme activities of commercial enzyme products are typically measured at the manufacturers' recommended optimal conditions (Beauchemin et al., 2003). A temperature of approximately 60°C and a pH between 4 and 5 are the optimal conditions for most commercial enzymes (Coughlan, 1985). However, the optimal temperature and pH for assessing enzyme activity are not representative of the conditions in the rumen, which is closer to a pH of 6.0 to 6.7 and 39°C (Van Soest, 1994). Synthetic substrates such as dyes or chromophores can also be used to examine enzyme activity by measuring the release of the dye or chromophore linked to molecules chemically similar to natural substrates (Biely et al., 1985). These synthetic substrates offer uniformity among assays; however, they do not represent the same substrates

found in feeds such as cereal grains or forages. Additionally, the conditions used to assess enzyme activity are not representative of that in the digestive tract, where ultimately the level and persistence of enzyme activity may be most important (McAllister et al., 2001). For these reasons, measurement of enzyme activity using traditional assay techniques may have limited relevance to the potential worth of an enzyme as a feed additive for ruminants.

Currently, biological assays using mixed ruminal microorganisms incubated with complex substrates has been one approach by researchers to identifying enzyme preparations that are more suitable for use in ruminants (Meale et al., 2014). After the addition of enzymes, these *in vitro* incubations measure the digestion of ingredients commonly included in ruminant diets (i.e., grains, hay, silage, or straw) by recording the production of gas that arises from the fermentation process and the digestibility of the feed DM and fiber at a given incubation time. Using this system, several enzyme preparations can be simultaneously screened for their effectiveness with different application methods and rates (Meale et al., 2014) before embarking on the more costly *in vivo* study (Beauchemin and Holtshausen, 2010). However, extrapolation of information from these procedures to whole animal situations is limited, because they are not very representative of *in vivo* conditions and do not account for the differences in mixed ruminal microorganisms sampled from different animals (Hristov et al., 2012; Meale et al., 2014). Furthermore, these systems do not account for the possible impact of exogenous enzymes on biological parameters such as feed intake, rate of passage, or postruminal digestion of nutrients (McAllister et al., 2001).

Presently, *in vitro* screening of exogenous enzymes looks promising, because it provides a cost effective, and less time consuming, means of screening large numbers of products with specific substrates that can be used to predict possible *in vivo* responses (Beauchemin and Holtshausen, 2010).

Ruminant production responses to exogenous fibrolytic enzymes

Final assessment of the true value of exogenous enzymes for ruminants in terms of improving feed utilization can only be assessed through the use of animal production trials. A varity of effects of using cell wall degrading enzymes in ruminant diets has been reported in past and recent studies. Different domestic ruminants at various stages of production have been used. Various types of forages have been fed, and the enzyme products in those studies

were given to the animals in diverse ways at the time of feeding; sprayed onto forage, added to concentrate, sprayed onto the total mixed ration (TMR), added as dry powder to feed, or ruminally infused (Beauchemin and Holtshausen, 2010).

Beef cattle

Evidence that exogenous enzymes could improve average daily gain and feed efficiency in beef cattle was first recorded in a series of ten feeding trials reported more than five decades ago (Burroughs et al., 1960). Since then, adoption of enzyme technology has been slow, as the cost of enzymes relatively outweighs that of other additives, such as ionophores, antibiotics, and implants (Beauchemin et al., 2003; Meale et al., 2014). A review by Meale et al. (2014) of several studies using beef cattle has been summarised (Table 1.1). The authors reviewed studies published in English referred journals and selected those with minimal experimental errors. The ultimate goal of using enzymes in beef cattle feed is to increase ADG and feed conversion efficiency. Although responses to exogenous enzymes are expected to be greater in beef cattle fed roughage-based diets as compared with high-grain diets, many exogenous enzyme formulations have shown promising effects in cattle fed barley-based finishing diets (Beauchemin and Holtshausen, 2010). However, practical responses may not always show the expected results. Beauchemin et al. (1995) applied a mixture of xylanase (Xylanase B; Biovance Technologies Inc., Omaha, NE) and cellulase products (Spezyme CP; Genencor, Rochester, NY) and increased ADG of steers fed alfalfa hay or timothy hay by 30 and 36%, respectively (Table 1.1), but had no effect when applied to barley silage. These positive responses were attributed to an increase in digestible DM intake; however, it was noted that forage type influenced the optimal dose required to elicit these responses demonstrating the importance of interactions between dosage, enzyme, and substrate. Increases in ADG for alfalfa hay was seen when low to moderate amount of enzymes were applied, but only high level increased body weight (BW) gain for timothy hay (0.25 to 1.0 l/t DM for alfalfa hay versus 4 l/t DM for timothy hay). Applying the same enzyme formulation to a barley grain diet improved feed efficiency by 11%, yet performance was unaffected when enzymes were added to corn (Beauchemin et al., 1997). The concentrates portion of this study was 95 % on a DM basis. Supplementing a similar exogenous enzyme mixture (FinnFeeds Int. Ltd., Marlborough, UK) increased ADG of steers by 10% when applied to both the grain and forage portions of the diet (McAllister et al.,

1999) and resulted in a 28% increase in ADF digestibility (Krause et al., 1998). These studies indicate the application of a xylanase and cellulase enzyme formulations is promising in terms of increasing ADG in cattle when applied to either barley grain or forage diets; however, the use of this enzyme formulation is not recommended in diets based on corn grain or barley silage due to its apparent lack of effectiveness with these feeds (Meale et al., 2014). In agreement with the latter statement, DiLorenzo et al. (2011) observed no effects of supplementing an amylase enzyme formulation (600 kilo novo units/kg of dietary DM; RumiStar; DSM Nutritional Products, Inc., Kaiseraugst, Switzerland) to either a dried-rolled corn or steam-flaked corn diet. On the contrary, a study by Tricarico et al. (2007) reported an *A. oryzae* extract containing α-amylase activity quadratically increased ADG when included in either a cracked corn or high-moisture corn and corn silage diet, but had no effect when included with alfalfa hay, cotton seed hulls, or steam-flaked corn. Grain processing affects digestibility, thus lack of response to amylases may reflect the fact that starch digestion is generally not limited in the rumen, provided that the grains are adequately processed (McAllister and Cheng, 1996).

ZoBell et al. (2000) observed no effects on ADG or feed efficiency when applying an experimental exogenous proteolytic enzyme (Danisco-Agtech, Waukesha, WI) to either a barley-based growing (65:35 forage to concentrate ratio; DM basis) or finishing diet (20:80 forage to concentrate ratio; DM basis) compared to McAllister et al. (1999), who observed an increase in DMI when this enzyme was applied (0.5 l/t DM) to barley silage as well as an increase in ADG when it was applied to a finishing TMR at 3.5 l/t of TMR. Recently, the same enzyme product during the growing phase increased DMI of steers by 14.8%, but an increase in ruminal passage rate reduced NDF digestibility (4.1%) and, as a result, this increase in DMI was not reflected in improvements in BW gain or feed efficiency nor were any effects observed when this same enzyme was added to a finishing diet (Vera et al., 2012). Comparatively, Balci et al. (2007) applied Promote N.E.T. (60 g/d; Agribands Int., St. Louis, MO) with cellulase and xylanase activities to a corn and barley diet and observed increases in ADG and feed conversion efficiency. Eun et al. (2009) supplemented both growing and finishing diets with a commercial enzyme product (Fibrozome; Alltech Inc.) and observed no effect on growth performance, despite minor improvements in carcass characteristics. Lewis et al. (1996) applied Grasszyme (FinnFeeds Int. Ltd., Marlborough,

UK) to a grass hay and barley diet (70:30) and measured the impact of application time before feeding and the portion of the diet to which the enzyme was applied. There were no effects on DMI; however, digestibility of DM, NDF, and ADF increased when the enzyme was added to the forage either 24 h before or at the time of feeding. Most of the studies on exogenous fibrolytic enzymes usage have focused on application at feeding and less at preservation (Adesogan et al., 2005). With this in mind, Krueger et al. (2008b) applied an enzyme mixture (Biocellulase A20; Loders Croklaan, Channahon, IL) to bermudagrass hay at three different times of application, immediately after cutting, at bailing, or at feeding; and although enzyme treatment at cutting increased DMI, no effect was observed on final live weight, ADG, or feed conversion efficiency, regardless of the time of application. However, more studies need to be done to ascertain the best time of enzyme application.

Table 1.1 Summary of exogenous polysaccharide-degrading enzyme effects on production traits and total tract apparent digestibility of nutrients in beef cattle. Source: Meale et al. (2014).

Source[1]	Experimental design[2] (number of cows)	Product/manufacturer	Declared primary activities	Application level	Forage level in basal diet	Effects[3]			
						DMI	ADG	FCR[4]	Total tract digestibility
Beauchemin et al., 1995	CRD (72)	Xylanase B[5] and Spezyme CP[6]	Xylanase and cellulase	40 to 316 FPU[7]/kg DM	91 to 96.7%	↑[8]	↑[9]	-	NR
Lewis et al., 1996	LSD (5)	Grasszyme, FinnFeeds Int.[10]	Xylanase and cellulase	1.65 ml/kg forage DM	70%	-	NR	NR	↑DM, NDF, and ADF
Beauchemin et al., 1997	CRBD (56)	Xylanase B[5] and Spezyme CP[6]	Xylanase and cellulase	4.0 l/t concentrate DM	4.90%	-	-	-	NR
Beauchemin et al., 1999	CRBD (1,200)	Pro-Mote[5]	Xylanase and cellulase	1.4 l/t DM	7.8%	-	↑	-	NR
McAllister et al., 1999	CRD (98 and 66)	FinnFeeds Int.[10]	Xylanase and cellulase	1.25 to 5.0 l/t DM	70 to 82.5%	↑[11]	↑[12]	-	-[13]
ZoBell et al., 2000	CRD (32)	FinnFeeds Int.[10]	Xylanase and endoglucanase	15,880 and 5,580 IU/kg TMR[14] DM	20 to 65%	-	-	-	NR
Balci et al., 2007	CRD (16)	Promote N.E.T.[15]	Xylanase and cellulase	60 g/d	*Ad libitum* wheat straw	NR	↑	↑	- (*in vitro)*
Tricarico et al., 2007	CRBD (120, 96, and 56)	Amaize[16]	Amylase	580 to 1,160 DU[17]/kg DM		-	↑[18]	-	NR
Krueger et al., 2008	CRD (50)	Biocellulase A20, Loders Croklaan, Channahon, IL	Xylanase and cellulase	16.5 g/t	Ad libitum access to hay	↑[19]	-	-	↑DM, NDF, and CP[19]
Eun et al., 2009	CRD (60)	Fibrozyme[16]	Endoglucanase, exoglucanase, xylanase, and amylase	1 to 2 g/kg TMR DM	20 to 58%	-	-	-	NR
DiLorenzo et al., 2011	CRBD (32)	RumiStar[20]	Amylase	600 kilo novo units/kg DM	5.1%	-	-	-	-
Vera et al., 2012	CRD (48)	Danisco-Agtech, Waukesha, WI	Protease	0.52 g/kg DM TMR	25 to 63.4%	↑[21]	-	-	↓NDF,[12] ↑DM, N, NDF, and ADF[12]

[1]In chronological order.
[2]CRD = complete randomized design; CRBD = complete randomized block design; LSD = Latin square design.

[3]↑ = increase; ↓ = decrease; - = no statistically significant effect; NR = not reported.
[4]FCR = feed conversion rate.
[5]Biovance Technologies Inc., Omaha, NE.
[6]Genencor, Rochester, NY.
[7]FPU = filter paper units of cellulase.
[8]Dependant on forage and application rate (increases seen at alfalfa level 3).
[9]Dependant on forage and application rate (increases seen at alfalfa level 1, 2, and 3 and at timothy hay level 5).
[10]Finnfeeds International, Marlborough, Wiltshire, UK.
[11]Only the greatest amount of enzyme application (5.0 l/t) in the backgrounding study.
[12]Only in the finishing stage.
[13]Digestion experiment with sheep.
[14]TMR = total mixed ration.
[15]Cargill Animal Nutrition, Minneapolis, MN.
[16]Alltech Inc., Nicholasville, KY.
[17]DU = dextrinizing unit.
[18]Quadratic increase observed in experiment 2 only.
[19]Dependent on time of enzyme application before feeding.
[20]DSM Nutritional Products, Inc., Kaiseraugst, Switzerland.
[21]Only in growing phase.

Dairy cattle

The effect of exogenous enzymes in dairy cattle was first examined in the mid-1990s as reviewed by McAllister et al. (2001) and recently there has been a surge of research activity in this area. With the same criterion described for enzyme research in beef cattle, Meale et al. (2014) reviewed the work of some researchers who supplemented the feed of dairy cattle with fibrolytic enzymes (Table 1.2). As the essence of adopting enzyme technology in beef cattle is to increase ADG and feed conversion efficiency, the essence of enzyme use in dairy cattle is to increase milk yield and milk components, such as milk fat percentage and milk protein percentage.

Holstein cows in early lactation were fed with a TMR treated with an esterase-xylanase enzyme product (Dyadic International Inc., Jupiter, FL) and increased milk yield, with no effects on milk components, recorded (Adesogan et al., 2007). Similarly, commercial α-amylase product Amaize (Alltech Inc., Nicholasville, KY) was examined in a study by Klingerman et al. (2009), where the enzyme was applied (4 g/kg TMR DM) to a diet containing mixed legume and maize silage; and milk yield increased, but no effects were observed on milk components. Also, in other related studies by DeFrain et al. (2005) and Tricarico et al. (2005), the same α-amylase product was applied on a TMR (0.1% and 240 to 720 dextrinizing units/kg TMR DM, respectively) containing alfalfa hay, alfalfa haylage, and maize silage diets, with no observed effects on milk yield or composition of milk components.

Studies that have reported positive effects of exogenous enzymes on milk components are few; for instance, Beauchemin et al. (2000) reported a 2% increase in milk true protein with a β-glucanase/xylanase/endoglucanase product. Similarly, Bowman et al. (2002), Sutton et al. (2003), and Eun and Beauchemin (2005) reported increased milk fat or protein. Rare occurrences of increasing milk yield have also been observed. For example, Yang et al. (1999) found milk yield was increased by 1.9 kg/d when an exogenous enzyme (Pro-Mote; Biovance Technologies Inc., Omaha, NE) composed mainly of cellulase and xylanase activities was applied to hay at 2 g of enzyme mixture/kg. This effect was attributed to a 12% increase in nutrient digestibility. Even though positive results have been reported, the application of exogenous enzymes to dairy cow diets has shown extremely variable results, and largely failed to improve production efficiency (Meale et al., 2014). Most studies as

reviewed by Meale et al. (2014) have shown no effect on milk yield (DeFrain et al., 2005; Hristov et al., 2008; Bernard et al., 2010; Peters et al., 2010; Ferraretto et al., 2011) or the production of milk components (Yang et al., 2000; Holtshausen et al., 2009; Bernard et al., 2010; Arriola et al., 2011a).

A recent study by Holtshausen et al. (2011) screened five doses of a fibrolytic enzyme additive (AB Vista, Marlborough, UK), and further assessed its efficacy *in situ* before the enzyme additive was fed to lactating Holstein dairy cows. The enzyme product improved fat corrected milk production efficiency in a dose dependent manner up to 11.3%; however, DMI decreased. Similarly, Arriola et al. (2011a) screened varying amounts of a fibrolytic enzyme product *in situ* before conducting a feeding trial. Milk production efficiency was increased in cows fed this enzyme product with a low-concentrate diet as compared with those fed either an untreated low-concentrate diet or a high-concentrate diet (treated or untreated). Therefore, it is evident that careful attention needs to be paid to the type and dose of enzymes being applied to dairy cattle diets (Meale et al., 2014).

Table 1.2 Summary of exogenous polysaccharide-degrading enzyme effects on production traits and total tract apparent digestibility of nutrients in lactating dairy cows. Source: Meale et al. (2014).

Source[1]	Experimental[2] design (number of cows)	Product/manufacturer	Declared primary activities	Application level	Forage level in basal diet	Milk production, kg/d	Effects[3]		
							DMI	Milk components	Total tract digestibility
Chen et al., 1995	CRD (36)	Digest M, Loveland Industries Inc., Greeley, CO	Amylase & protease	209 g/t[4]	34%	34 to 37	-	-	↑CP[5]
Rode et al., 1999	CRD (20)	Pro-Mote[6]	Xylanase & cellulase	1.3 kg/t TMR[7] DM	39%	36 to 40	-	↓MFP	↑DM, OM, NDF, ADF, and CP
Beauchemin et al., 2000	LSD (6)	Natugrain 33-L[8]	β-glucanase, xylanase, & endocellulase	1.22 to 3.67 l/t TMR	45%	30 to 31	↑	↑MPP	↑DM[9], and ↓NDF[10]
Kung et al., 2000[11]	CRD (30)	FinnFeeds Int.[12]	Cellulase, hemicellulase & xylanase	2 to 10 l/t fresh forage	50%	33 to 35 and 36 to 39	-	- or ↓MFP and MPP	NR
Hristov et al., 2008	LSD (4)	Alltech, Inc.[13]	Amylase & xylanase	10 g/cow per d	40%	30	-	-	↑DM, OM, and CP
Miller et al., 2008	CRBD (72)	Roxazyme G2 Liquid[14]	Xylanase & endoglucanase	2.15 and 4.30 ml/kg concentrate	Pasture and 6.7 kg/d grain supplement	28 to 29	-	-	NR
Gado et al., 2009[15]	CRD (20)	ZADO[15]	Protease, amylase, & cellulase	40 g/cow per d	70%	13 to 16	↑	-	↑DM, OM, NDF, and ADF
Klingerman et al., 2009	LSD (28)	Amaize[13] and an experimental preparation[14]	Amylase	0.4 g/kg TMR DM and 0.88 to 4.4 ml/kg	50%	44 to 47	↑	-	↑DM, OM, CP, and NDF[16]
Holtshausen et al., 2011	CRD (60)	AB Vista, Marlborough, UK	Xylanase & endoglucanase	0.5 to 1.0 ml/kg DM	52%	38	↓	-	NR
Arriola et al., 2011a	CRBD (66)	Dyadic International Inc., Jupiter, FL	Xylanase, exoglucanase, & endoglucanase	3.4 mg/g TMR DM	52 to 67%	32 to 36	-	-	↑All

[1]In chronological order.
[2]CRD = complete randomized design; CRBD = complete randomized block design; LSD = Latin square design.

[3]↑ = increase; ↓ = decrease; - = no statistically significant effect; NR = not reported; MFP = milk fat percentage; MPP = milk protein percentage; All = all nutrients studied.
[4]Applied to the grain portion of the diet.
[5]Interaction with grain processing.
[6]Biovance Technologies Inc., Omaha, NE.
[7]TMR = total mixed ration.
[8] BASF Corporation, Ludwigshafen, Germany.
[9]Only the low enzyme application level.
[10]Only the high enzyme application level.
[11]Two experiments.
[12] FinnFeeds International, Marlborough, Wiltshire, UK.
[13]Alltech Inc., Nicholasville, KY.
[14]DSM Nutritional Products Ltd., Basel, Switzerland.
[15]Molecular Biology Laboratory of the Ain Shams University, Cairo, Egypt. Questionable data; see discussion.
[16]Milk yield and digestibilities increased only by low level of an experimental amylase enzyme.

Small Ruminants

It was shown in the 1960s that feeding a mixture of amylolytic, cellulolytic and proteolytic enzymes (Agrozyme®; 1.5, 3, and 6 g/d), as well as a potent proteolytic enzyme (Ficin®, Merck and Company; 5, 10, and 20 mg/d) did not alter feed conversion or the ADG of fattening lambs fed ground corn or alfalfa hay (Theurer et al., 1963). McAllister et al. (1998) also found that fibrolytic enzymes (Finnfeeds International Inc.) did not increase feed intake or ADG by lambs fed alfalfa hay- or barley-based diets (McAllister et al., 1998). Similarly, Miller et al. (2008) fed a barley-based diet treated with a commercial exogenous enzyme (Roxazyme G2 Liquid; DSM Nutritional Products Pty Ltd, Basel, Switzerland) to Dorset-cross ewe lambs and observed no effects on DMI, ADG, feed conversion, or wool growth. Additionally, Rojo et al. (2005) fed exogenous amylases from *B. licheniformis* and *A. niger* (up to 2.90 g enzyme/kg DM sorghum; ENMAX, Mexico City, Mexico) and observed no effects on production performance in Suffolk lambs. On the contrary, Titi and Lubbadeh, (2004) reported increased milk production when a commercial cellulase enzyme (Maxicel 200L®, George A. Jeffreys Company Inc., Salem, VA, USA) was supplemented to a TMR diet for Awassi ewes and Shami goats at a level of 150g/t of alfalfa hay per portion of the TMR; while milk components increased for ewes, no effects were observed for goats when compared to the untreated control diets. Generally, the application of exogenous enzymes to the diets of small ruminants has had little impact on production performance; therefore, most research work in this area has focused on digestibility studies (Meale et al., 2014).

Some studies have reported no effect on nutrient digestibilities with the use of exogenous fibrolytic enzymes (Avellaneda et al. 2009; Awawdeh and Obeidat, 2011). Similarly, Giraldo et al. (2008) inoculated exogenous fibrolytic enzymes (12 g/lamb daily; Fibrozyme; Alltech Inc.) directly into the rumen of fistulated Merino sheep before feeding a grass–hay concentrate diet (70:30; DM basis) without affecting diet digestibility. On the contrary, Titi (2004) reported an increased DM, organic matter (OM), CP, and NDF digestibilites when TMR diets of Awassi lambs were supplemented (150g/t of alfalfa hay portion of the TMR) with cellulase commercial enzyme from *Trichoderma sp*. (Maxicel 200L®, George A. Jeffreys Company Inc., Salem, VA, USA).

Allowing for the myriad of exogenous fibrolytic enzyme preparations available, possible methods of application and types of diet to which they may be applied, different genetic potential and physiological status of animals, coupled with other factors, is not surprising that production responses to these additives have been highly variable.

Reasons for variable responses to exogenous fibrolytic enzymes

Inconsistencies in animal responses with added enzymes are multifactorial, and can possibly be attributed to four main factors: enzyme characteristics (e.g., differences in enzyme preparations, enzymic activities, units of activity added, pH, and temperature effects on activity), forage (e.g., type, maturity), animal (e.g., species, age) and management (e.g., diet, mode of enzyme application, application rate, interaction time of enzymes applied to feed) (Beauchemin et al., 2003).

Some exogenous fibrolytic enzyme (EFE) products were developed for other applications such as textiles, food, nonruminant diets, or paper. Therefore, such enzyme products often lack sufficient potency and specificity for improving the use of fibrous ruminant feeds (Adesogan et al., 2014). In particular, the optimal pH and temperature for fiber degradation by EFE products often differ considerably from those of the rumen.

Recently, Arriola et al. (2011b) compared the endoglucanase and xylanase activities of 18 EFE products from five companies at pH 3, 4, 5, 6, and 7 under a constant temperature of 39°C or at 20, 30, 40, and 50°C under a constant pH of 6. Exactly 78 and 83% of the 18 EFE products exhibited optimal endoglucanase and xylanase activities at 50°C, and 77 and 61% had optimal activity at pH 4 and 5, respectively. This shows that most EFE products would exhibit suboptimal activity under ruminal conditions, and this could explain the variable responses often obtained in ruminants. *Trichoderma reesei* is the main microbe used commercially to produce large quantities of cellulases and hemicellulases (Paloheimo et al., 2010); however, it exhibits maximum cellulose degradation efficiency at pH 5 (Adav et al., 2011; Glass et al., 2013). Therefore, alternative microorganisms that secrete copious quantities of cellulase with high degradation efficiency under ruminal conditions are needed (Adesogan et al., 2014). Stability of the EFE products at the optimal pH is also very important, as noted by Colombatto et al. (2004). About 90% of losses in endoglucanase activity where recorded by Arriola et al. (2011b) after 24 h of incubation in the 18 enzyme

products assayed. Thus, stability of enzyme products may prevent the loss of enzymic activity at ruminal conditions. The stability of enzymic activities in the ruminal digestive tract may be affected by the enzyme glycosylation, inhibition or inactivation of cofactors or coenzymes, or complementary enzymic activities in the EFE (Adesogan 2005; Adesogan et al., 2014). For instance, recent research has shown that polysaccharide monooxygenases can enhance the activity of cellulases (Glass et al., 2013). However, polysaccharide monooxygenases are metalloenzymes, which require copper to enhance the activity of cellulase (Quinlan et al., 2011).

Some reviews (Beauchemin et al., 2003; Adesogan, 2005) have described how enzyme activity is affected by enzyme factors such as the type, source (pre-discussed), and activities, feed factors such as the specificity to EFE, and form (powder or liquid). In addition, management factors such as the EFE application rate, the timing of EFE application relative to feeding, the targeted dietary component and proportion of the diet to which the EFE is applied, and animal factors such as the performance level and lactation stage.

Descriptions of EFE products in most research papers are still broad at best (Beauchemin et al., 2003). Enzyme preparations for ruminants are marketed chiefly on the basis of their capacity to degrade plant cell walls and as such, are often referred to as cellulases or xylanases. Therefore, most products tested in ruminants are described as cellulases and/or xylanases, with proteases, amylases, and esterases being investigated in fewer instances. However, in these commercial products, preparations seldom consist of single enzymes; secondary enzyme activities such as amylases, proteases, or esterases are invariably present (McAllister et al., 2001). Enzyme-feed specificity presents a major dilemma for formulating new ruminant feed enzyme products because most commercial ruminant diets contain several types of forages and concentrates (Beauchemin et al., 2003). For instance, degradation of cellulose and hemicellulose alone requires a number of fibrolytic enzymes (as pre-discussed) and differences in the relative proportions and activity of individual enzymes affects the overall efficacy of cell wall degradation (McAllister et al., 2001; Meale et al., 2014). A common experimental approach has often been to use enzymes that may not be suited to a specific feed, but rather to formulate enzyme mixtures that are suitable for a range of feed types (Beauchemin et al., 2003). However, this approach of adding enzymes to diets without consideration for specific substrates has contributed to the

highly variable results often observed when enzymes are used in ruminants, an outcome that has undoubtedly discouraged and delayed the adoption of the technology (Beauchemin et al., 2003; Meale et al., 2014).

It is practically impossible to compare EFE preparations on an equal activity basis, as there is a distinct lack of standardization in the methodology used to assess enzyme activities among labs; even with the same method employed for different enzyme products with the same level of endoglucanase activity, they may contain different levels of xylanase activity (Meale et al., 2014). Also, the same enzyme products with the same assay employed may contain different levels of endoglucanase or xylanase activities due to different batches of production. Therefore, one cannot select a promising fibrolytic enzyme product based on its enzymic activities; rather, this must be followed by an *in vitro* study to determine the efficacy of the enzyme on dry matter degradability (DMD) or fiber digestibility.

Some of the variations associated with the use of EFE products in ruminant diets are due to different doses and diets to which the enzymes are applied. Nonlinear responses have been reported for growing beef cattle and have been attributed to high doses of enzyme supplementation (Beauchemin et al., 1995). In that study, ADG of cattle fed alfalfa hay increased by 24 to 30%, with lower levels of added enzyme (0.25 to 1 ml/kg of DM) as a result of increased intake of digestible DM; but higher levels of enzyme (2 and 4 ml/kg of DM) were not effective. With timothy hay, a high level (4 ml/kg of DM) of exogenous enzymes increased ADG of cattle by 36% as a result of a 17% increase in ADF digestibility and a 14% increase in digestible DM intake. These studies demonstrate that high levels of enzyme addition can be less effective than low levels, and the optimal level of enzyme supplementation may depend on the diet. Interestingly, the commercial α-amylase product Amaize (Alltech Inc., Nicholasville, KY), examined in the three studies as discussed above in the dairy animal section, all fed a diet containing either alfalfa hay, alfalfa haylage and maize silage, or a mixed legume hay and maize silage diet and observed an increased (Klingerman et al., 2009) or no effect on milk yield (DeFrain et al., 2005; Tricarico et al., 2005) or change in composition of milk components (DeFrain et al., 2005; Tricarico et al., 2005; Klingerman et al., 2009). The dose of enzyme and the portion of the diet to which it was applied varied across studies, raising questions about the enzymes applicability for use in lactating dairy cows fed current diets (Meale et al., 2014). Colombatto et al. (2003) showed

that dose response of some EFE products is not linear, and that higher doses of certain enzyme products may actually limit *in vitro* OM degradability due to different biochemical properties of these enzymes (Vahjen and Simon, 1999). This shows that an *in vitro* study may be an important component in a systematic identification of a promising enzyme for ruminant studies.

Enzymes have also been applied in multiple ways, including application in powdered form or sprayed as liquid onto feed (hay, silage, whole or portion of TMR), or infused directly into the rumen. Applying enzymes in liquid form prior to consumption showed improvement (Rode et al., 1999; Kung et al., 2000; Yang et al., 2000) while infusion directly into the rumen of animals had no effect on the animals performance (Lewis et al., 1996; Giraldo et al., 2008). The close association of enzymes with feed may enable some form of preingestive attack of the enzymes upon the plant fiber and/or enhance binding of the enzymes to the feed, thereby increasing the resistance of the enzymes to proteolysis in the rumen. Exogenous enzymes may be expected to be more effective when applied to high moisture feeds (such as silages) compared to dry feeds (such as hay) because of the higher moisture content (Beauchemin et al., 2003). This is because water is a fundamental requirement for the hydrolysis of soluble sugars from complex polymers. However, in practice this may not always be the case. This is because applying exogenous enzymes in liquid form to dry feed may contain the water activity required by enzymes to initiate hydrolysis of the easily soluble carbohydrates.

Furthermore, some of the variations could be related to the different physiological state of animals selected for the study (Adesogan et al., 2014). For example, some of the studies with dairy cows have involved cows at different stage of lactation (early, mid, or late lactation). Schingoethe et al. (1999) compared the milk production response to dietary EFE application by cows less (38 to 94) than or greater (101 to 204) than 100 days in milk (DIM). The EFE increased milk production by 10.8% in cows less than 100 DIM, but had no effect on those greater than 100 DIM. Similarly, Nussio et al. (1997) also observed greater production responses during earlier rather than later lactation with EFE treatments. Early lactation and growing animals require greater levels of energy to meet the demands for milk and meat production. Thus, the use of exogenous fibrolytic enzymes has greater potential to

improve the productivity of high producing animals than animals at maintenance (Beauchemin et al., 2003).

Mode of action

Beauchemin and Holtshausen, (2010) argued that the mode of action of EFE is still relatively unknown, due to the complex nature of the ruminal microbial ecosystem and the process of fiber digestion. However, lack of understanding of the mode of action of EFE could be categorised under pre-ruminal or pre-consumption, ruminal, and post-ruminal effects (Meale et al., 2014).

Application of EFE onto forages or to the diets of ruminants before consumption can initiate fiber hydrolysis through DM loss (Krueger et al., 2008). Also, it can also cause the release of sugars (Nsereko et al., 2000), at least from partial solubilization of NDF and ADF (Morrison and Miron, 2000; Devillard et al., 2004; Arriola and Adesogan, 2013) and hydrolytic cleavage of ester linkages that attach phenolic acids in the cell walls to sugars (Anderson et al., 2005). The EFE dose, composition, substrate crystallinity and composition, environmental conditions, and the time that elapses between the enzyme application and feeding determine the degree of sugar released. Meale et al. (2014) argued that sugars released represent only a minute portion of the total carbohydrate present in the diet; thus it is difficult to attribute production responses solely to the generation of soluble carbohydrates before consumption.

Exogenous fibrolytic enzymes applied to feed before consumption bind to the feed and this makes them more active in the rumen, possibly because of their increased resistance to proteolysis and outflow of ruminal content, thus prolonging their residence time in the rumen. Hirstov et al. (1996) showed that enzymic activities declined when two different EFE products where administered directly into the rumen due to enzyme inactivation and passage of fluid from the rumen. Similarly, maximizing the proportion of the diet to which the enzyme is added is considered to increase the chances that the enzymes will remain active in the rumen (Beauchemin et al., 2003).

Many studies on pre-consumption effects of EFE have focused on application at feeding and less at preservation (Adesogan et al., 2005). Since most EFE exhibit optimal activities at pH 4 to 5 and 50°C, which differ from those in the rumen (Adesogan et al.,

2014), we can only optimize their effects, should they be added to feeds during storage at low pH (i.e., 4 to 5) and in relatively hot conditions (i.e., approximately 50°C). However, the application of EFE to preserve forages at storage can accelerate their aerobic deterioration. This is because growth of epiphytic microbes is stimulated by soluble sugars released by enzyme treatment, which could lead to a decrease of the preserve forage feed value if the time elapsed between enzyme application and consumption is sufficiently long (Wang et al., 2002). Therefore, to improve feed value of preserved forages, EFE can be applied with preservatives such as bacterial inoculants, which can minimise the spoilage organisms on preserved forage, and consequently conserve the soluble sugars for ruminants.

Most of the improvements in forage quality resulting from EFE application were previously attributed to ruminal effects (Beauchemin et al., 2003); though recent *in vitro* work comparing preingestive versus ruminal application indicates that this is not always true, especially for alfalfa hay (Arriola and Adesogan, 2013). Generally, exogenous enzymes are more stable in the rumen than previously thought and have been shown to be resistant to ruminal proteases (Hristov et al., 1998b; Morgavi et al., 2000b). For instance, Morgavi et al. (2001) found four commercial enzymes remained stable when incubated in ruminal fluid, pepsin, or pancreatin. Enzyme stability in the rumen is considered to be a result of glycosylation and is usually enhanced by adding exogenous enzymes to the feed before consumption (Fontes et al., 1995). However, nonglycosylated enzymes may also resist ruminal proteolysis, but their persistence in the rumen may depend on the microbial source from which they were derived (Fontes et al., 1995).

Supplementing ruminant diets with EFE increases the rate but seldom the extent of feed digestion (Krueger et al., 2008). Therefore, Meale et al. (2014) suggested that the positive response from present EFE are not a result of these preparations solubilizing substrates that would not be normally digested if retained in the rumen for a sufficient period of time; rather, an increase in total enzymic activity in the rumen can increase ruminal hydrolytic capacity, which can enhance the digestibility of the complete diet. Beauchemin and Holtshausen (2010) cited an unpublished study (Eun and Beauchemin), which showed that ruminal fluid from cows fed an enzyme-treated diet increased the *in vitro* digestibility of an enzyme-treated and untreated substrate. This indicates that exogenous fibrolytic enzymes can increase the hydrolytic capacity of ruminal fluid. As such, digestibility of both non-

fibrous and fibrous fractions can increase; explaining why EFE can also be effective in increasing the digestibility of non-fiber fractions in high concentrate diets (Arriola and Adesogan, 2013). Though EFE have the potential to increase the hydrolytic capacity of the rumen, this effect is often not optimized because many of the enzyme activity tests are done under conditions that overestimate their activity relative to those in the rumen.

Given that exogenous enzymes represent only a fraction of enzyme activity in the rumen combined with the inherent capacity of the ruminal microbiota to digest fiber, it is difficult to attribute an increase in fiber degradation by exogenous enzymes to direct hydrolysis alone (McAllister et al., 2001). A synergistic relationship between exogenous enzymes and rumen microbiota, and an increase in bacterial attachment are other likely modes of action of exogenous enzymes in the rumen (Meale et al., 2014). Giraldo et al. (2008) and Gado et al. (2009) showed that exogenous enzymes increased microbial growth and production of MP. Furthermore, Morgavi et al. (2000a) showed that synergism acts to increase the effects of both indigenous ruminal microbes and EFE so that the combined response exceeds the additive effects of each individual component. However, increased amounts of EFE can also compete with the ruminal microbial populations for cellulose binding sites on feed (Morgavi et al., 2000b), potentially explaining the lack of or even negative responses observed with the increased amounts of exogenous enzyme supplementation *in vivo*. For exogenous enzymes to be effective, it is important that they complement and not replace the existing natural enzyme activities produced by ruminal microbes (Meale et al., 2014).

Adding exogenous fibrolytic enzymes to ruminant diets can also impact nutrient digestion in the hindgut. Hristov et al. (1998a) reported that approximately 30% and 5% of xylanases and endoglucanase, respectively, can escape ruminal fermentation and are active in intestinal digesta of ruminants. Depending on application level, other EFE may also bypass the rumen and increase polysaccharide-degrading activities in intestinal digesta (Chesson, 1994). This along with other reports (Fontes et al., 1995; Morgavi et al. 2001) seems to indicate that xylanases are more resistant to ruminal and abomasal conditions than endoglucanases. Presently, although post-ruminal effects can be documented, they are thought to account for a minor component of any positive responses observed with existing enzyme preparations, with improvements primarily arising from positive alterations in rumen

function (Meale et al., 2014). However, to date, no studies have directly quantified the relative importance of EFE by pre-consumption, ruminal or post-ruminal action either on animal performance or feed digestibility.

Methods used to evaluate forage feeds for ruminants

Feeding can account for up to 60% of the costs of livestock production, and even under intensive concentrate feeding used in western ruminant animal production, forages continue to represent the single most important feed resource (Jung and Allen, 1995). However, depending on species, variety, physiological maturity, regrowth, season, time of harvest, cutting height, fertilization, and other factors, forages are inherently variable in nutritive value (Adesogan, 2002). Feed evaluation methods are required to measure the capacity of the forage in question to sustain animal production and to supply the nutritional demands of the specific animal species (Beever and Mould, 2000). Feed analysis is also valuable for quality assurance in feed manufacturing and for identifying the presence and concentrations of undesirable substances in feeds, which adversely affect animal health and productivity (Adesogan, 2002). Feed analysis is therefore indispensable for efficient resource use and profitability in livestock production.

Proximate and Van Soest analysis

Originally, the most extensive information about the composition of foods was based on a system of analysis described as proximate analysis, wet chemistry, or Weende system, devised over 100 years ago by two German scientists, Henneberg and Stohmann (Burns, 2011; McDonald et al., 2011).

The proximate or Weende system of analysis (gravimetric method) was initially devised to separate the carbohydrate fraction of animal feedstuff, especially forage, into two general categories (Figure 1.12; Burns, 2011). These were crude fiber (CF) and nitrogen-free extract (NFE). This traditional laboratory method involves various chemical, drying and burning procedures to determine the major chemical components within the forage. The complete analysis divides feed into six fractions:

Moisture (water) – this is weight loss when a known weight of feed is dried to a constant weight at 105ºC (Burns, 2011). This method is satisfactory for most feeds, but with

a few, such as silage, significant losses of volatile material (short-chain fatty acids and alcohols) may take place (McDonald et al., 2011). Therefore, for silages, dry matter (DM), which is the percentage of the forage that is not water, may be underestimated.

Ash (mineral) – this is determined by ignition of a known weight of the feed at 550 or 600 °C until all carbon has been removed (Burns, 2011; McDonald et al., 2011).

Crude Protein (CP) – calculated from the N content of the feed, determined by a modification of a technique originally devised by Kjeldahl over 100 years ago or by the current Dumas technique (McDonald et al., 2011). It is assumed that the N is derived from protein containing 16% N, and by multiplying the N amount by 6.25 (i.e. 100/16), an approximate protein value is obtained. This is not 'true protein' since the method determines nitrogen from sources other than protein, such as free amino acids, amines, and nucleic acids, and the fraction is therefore designated CP. When excessive heating has occurred in the forage, such as in poorly managed hay or silage, a portion of the crude protein may be unavailable. The crude protein analysis gives no indication that excessive heating may have rendered a portion of the protein unavailable. If heat damage is suspected, an analysis for bound protein or unavailable or insoluble protein is often requested. Laboratories typically report the bound protein as ADICP, unavailable or insoluble crude protein.

Ether extracts (EE) – this fraction is determined by subjecting the feed to a continuous extraction with sulphuric acid and then petroleum ether for a defined period.

Crude fiber (CF) – this is obtained by subjecting the residual feed from ether extraction to successive treatments with boiling weak acid and alkali of defined concentration; the organic residue is the CF. The residue contains cellulose, lignin, and hemicelluloses, but not necessarily the whole amounts of these are present after treatment with the boiling acid and alkali, a variable proportion of the cell wall material, depending upon the species and stage of growth of the plant material, is dissolved during the CF extraction (McDonald et al., 2011).

Nitrogen-free extract (NFE) – calculated by difference, that is, 100 – (water + ash + CP + EE + CF). The NFE fraction is a heterogeneous mixture of all those components not determined in the other fractions.

The overall intent of the proximate analyses is to chemically separate the less digestible carbohydrate fraction (CF or cell wall components) of the DM from the more readily digestible fraction (NFE or cell content components). This separation, however, is generally not achieved by the chemical extractions used and thus, is contained in the NFE (Burns, 2011). This leads to an underestimation of the fiber and an overestimation of the NFE. Thus, the NFE fraction includes starch, sugars, soluble protein fructans, pectins, organic acids, and pigments, in addition to components of the CF mentioned above (Burns, 2011; McDonald et al., 2011). The proximate analyses therefore fails to properly distinguish between cell contents and cell walls.

While the proximate system has some limitations for the analysis of forages, portions of it are widely used today. Most typical forage analyses use the moisture and CP procedures from the proximate system to determine percent DM and CP. Ash (total mineral content) and EE are not commonly determined in a typical forage analysis. The original CF analysis has been replaced with the newer detergent analysis (Van Soest, 1967).

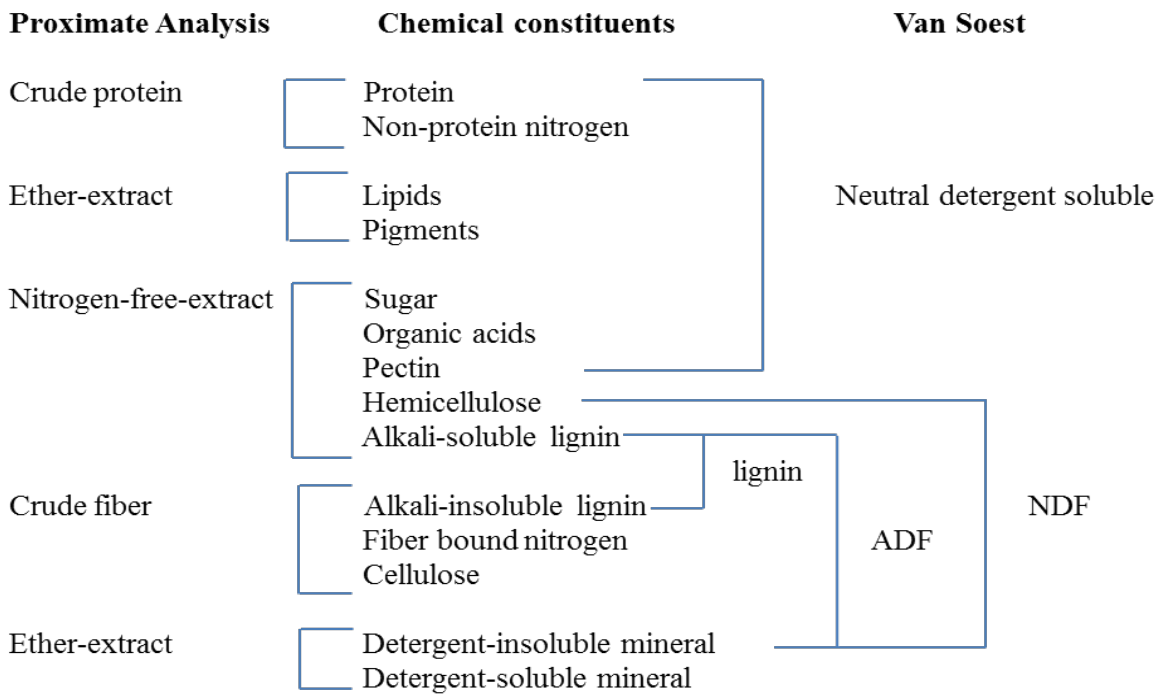
The limitations of the proximate analysis were recognized early on, generating the impetus for improved methodologies especially for fibrous diets. Subsequently in the 1960's Van Soest developed alternative procedures for determining fiber (Figure 1.10). The method determines fiber fractions according to their degradability as those insoluble in neutral solutions of sodium lauryl sulphate and ethylenediamine tetraacetic acid. As these fractions consist mainly of lignin, cellulose, and hemicellulose, they can be regarded as a measure of the plant cell wall material (NDF) (Burns, 2011; McDonald et al., 2011). The analytical method for determining NDF was originally devised for forages, but it can also be used for starch-containing feeds provided that an amylase treatment is included in the procedure (McDonald et al., 2011).

Subsequent treatment of the insoluble residue, or NDF, by acid detergent solution (sulphuric acid and cetyltrimethyl-ammonium bromide), results in further degradation of the cell wall leaving an insoluble residue of lignin and cellulose fractions of plant material (ADF) but also includes silica (Burns, 2011; McDonald et al., 2011). The solubilized fraction is termed hemicellulose, which is determined by difference (NDF – ADF). The ADF is further treated with 72% sulfuric acid or permanganate to generate a better estimate of lignin.

The difference between ADF and its lignin plus ash or silica concentration is termed cellulose (Burns, 2011).

The Van Soest method of analysing the fiber composition of forage or feed creates the possibility of predicting the intake and nutritive value of the test substrate (Mould, 2003). However, it has its own shortcomings. For instance, the NDF is considered to be the entire fiber fraction of the feed, underestimates cell wall concentration because most of the pectic substances in the wall are solubilized (Theander and Westerlund, 1993). As a result, NDF is a poor estimate of cell wall concentration for pectin rich forages such as legumes but not for grasses (Jung, 2012). Heat-damaged proteins, as discussed above in preserved forages, are also retained in NDF or ADF (Coblentz et al., 2004) which will overestimate fiber content. These shortcomings of NDF as a method to determine cell wall centration are certainly a problem if one is interested in the plant cell wall as a biological structure, but as pointed out by Van Soest (1994), these inconsistencies are not of concern if fiber is defined as the partly digestible fraction of feeds.

Figure 1.10 Schematic representation of the comparison of proximate and Van Soest analytical systems. ADF= acid detergent fiber; NDF= neutral detergent fiber. Modified from Mould (2003).

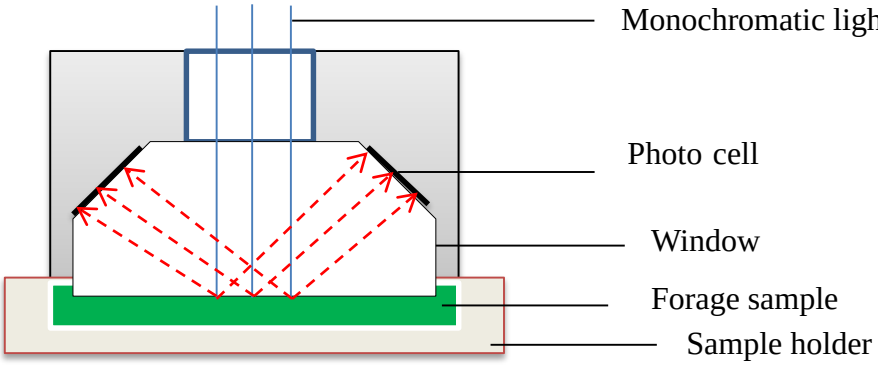


The industrial sector has provided significant advances in instrumentation for the improvement in efficiency and precision in estimating nutritive value of feed. Some of the major contributors are:

The Ankom incubator and fiber apparatus developed by Ankom Technology Corp. (Fairport, NY, USA) which was made available in the mid-1990s, permits 24 samples to be processed simultaneously for sequential or individual NDF and ADF determinations using Van Soest method of analysis (Burns, 2011).

Application of near-infrared spectroscopy (NIRS) to agriculture is the topic of a recent monograph with basic aspects and its specific application to forages and feedstuffs addressed (Roberts et al., 2004; Burns, 2011). This method of analysis involves the drying and grinding of samples which are then exposed to infrared light in a spectrophotometer (Figure 1.11; Schroeder, 2004). Each major organic component of forages (and grain) will absorb and reflect near-infrared light differently. The reflected infrared radiation is converted to electrical energy and fed to a computer for interpretation. The reflected energy from the sample provides information on its composition but, unlike normal spectroscopy, is not related directly to concentration since the sample is heterogeneous. Therefore, empirical relationships are derived by calibrating the reflected spectrum with samples of known composition programmed into the computer, which were determined by standard methods. In summary, when a similar feed sample is evaluated by NIRS, the computer compares the wavelength reflections caused by the sample, and matches them to the previously tested samples (Schroeder, 2004; McDonald et al., 2011). With forages, particularly grass and cereal silages, NIRS is now routinely used to determine not only chemical composition but also a range of feed characteristics, such as digestibility, metabolisable energy, nitrogen degradability in the rumen, and potential silage intake (McDonald et al., 2011). Near infrared reflectance spectroscopy is a rapid and low-cost computerized method to analyze forage and grain crops for their nutritive value; however, accuracy depends on a calibration set that must be developed from an adequate number of wet chemistry samples, similar to those being analyzed (Schroeder, 2004).

Figure 1.11 Schematic representation of how near infra-red reflectance spectroscopy reads a prepared plant sample. Modified from Schroeder (2004).



In vitro digestibility and gas production

The nutrient composition of feed is commonly estimated by chemical analysis (proximate analysis). This provides information about the concentrations of nutrients (DM, NDF, CP, ash) as well as the inhibitors and structures that may impact the availability of nutrients. This procedure is easy and fast. However, it does not provide sufficient information about the true nutritive value of the feed. It is the digestive efficiency, by which a ruminant animal utilizes feed nutrients, that has a significant impact on animal productivity performance and waste production (Cherney, 2000). Thus, animal experimentation is the ultimate index of feed quality; however it is often too costly, labor intensive, and protracted to be routinely practicable. Therefore, animal performance is generally estimated from animal-based simulation (*in vitro*) techniques that measure related parameters such as digestibility, degradation, fermentation, and passage. Various *in vitro* techniques have been used in the past as alternatives to animal experiments. These consist of the use of ruminal fluid, buffers, chemical solvents, or commercial enzymes. Another technique uses gas production (GP) as an indirect measure of the *in vitro* digestion.

All batch culture *in vitro* techniques currently in use to measure fiber degradability are based on the method developed by Tilley and Terry (1963) for determining DM degradability (DMD). This *in vitro* method involves a 48-h incubation of feed samples with ruminal fluid at 39ºC from fistulated ruminants. The ruminal fluid is diluted with a buffer

solution similar in characteristics to saliva and saturated with CO_2 to maintain anaerobic conditions. This is followed by digestion with an acid-pepsin solution for another 48 h to remove undegraded plant cell matter and microbial protein (Beever and Mould, 2000). Disappearance of sample DM after the two digestion steps is determined by weighing the residue collected by filtration, drying the residue, and calculating degradability. This protocol mimics the ruminant digestive tract where feeds are first subjected to microbial digestion in the rumen followed by protein digestion in the abomasum. The main shortcoming of this method is the disregard of the post-rumen digestion (Adesogan, 2002). The acid-pepsin solution used is not able to effectively remove most bacterial and residual feed proteins; therefore, to improve post-rumen digestibility Goering and Van Soest (1970) demonstrated that neutral detergent solution was more effective. In this method, the same protocol is used except that after the 48 h ruminal fluid digestibility, the feed sample residues are extracted with the neutral detergent solution. Digestibility of fiber is calculated as the difference in NDF present in the feed sample compared the residual NDF remaining after the incubation.

The Ankom incubator and fiber apparatus developed by Ankom Technology Corp. (Fairport, NY, USA) were introduced to improve the estimation of *in vitro* digestibility. The method consists of digesting forage samples in filter bags suspended in a mixture of buffered solution and rumen fluid for different periods of time, within rotating digestive jars in an insulated incubator ($Daisy^{II}$ incubator). The technique significantly reduces the labor input associated with Tilley and Terry (1963) *in vitro* digestibility estimation because it removes the need for filtration and allows batch inoculation of several samples with the ruminal fluid – buffer mixture. Several authors have shown that the technique gives relatively accurate predictions of *in vitro* apparent and true digestibility (Julier et al., 1999; Vogel et al., 1999; Wilman and Adesogan, 2000) and it has the potential to be used to estimate the rate and extent of degradation of feeds (Holden, 1999).

The *in vitro* method involving the GP system consists of the measurement of the volume of gas produced by fermenting feedstuffs using rumen fluid from fistulated ruminant and buffer solution (Menke et al., 1979) The *in vitro* GP technique also generates rate and extent of degradation data; but rather than measuring the disappearance of dietary components, it measures the appearance of fermentation gases notably CO_2, CH_4, and H_2. This technique, which collects and measures gas, can vary from the use of calibrated syringes

(Menke et al., 1979) and pressure transducers (Theodrorou et al., 1994) to computerized gas monitoring devices (Pell and Schofield, 1993). The computerized GP methods are expensive and may or may not handle large numbers of samples. While manual methods are cheap, they are labor intensive, restricted in capacity, and often generate inadequate kinetic data for precise descriptions of fermentation rates (Adesogan, 2002). Gas production is often assumed to be directly proportional to substrate digestion and hence, nutritive value. This could be misleading because GP is dependent on substrate composition, microbial population, and hexose utilization for microbial yield (Adesogan, 2002). Several authors have shown that less gas is produced from feeds high in propionate precursors relative to that in feeds high in acetate and butyrate precursors (Beuvink and Spoelstra, 1992; Beever and Mould, 2000; Williams, 2000). Others have shown that the NH_3 in high protein feeds can decrease GP by reaction with VFA (Schofield, 2000). All of these factors determine the quantity of gas produced during substrate fermentation; and so *in vitro* gas production values alone provide little direct information about rate and extent of degradation, apart from estimating fermentation rate (Beever and Mould 2000). Therefore, Schofield (2000) suggested that gas production data should be supplemented with measurements of substrate disappearance, VFA profiles, and microbial yield in order to give comprehensive nutritional information on the feed tested.

Animal digestibility techniques

It is the digestive efficiency by which a ruminant animal utilizes feed nutrients that has a significant impact on its performance and waste production (Cherney, 2000). Effects such as palatability, impact of diet composition on ruminal digestibility, and animal performance, or extent to which anti-nutritive factors influence feed intake cannot be determined with *in vitro* analysis methods. Thus, animal experimentation is the best index of feed quality.

During the course of batch culture incubation for *in vitro* analysis, no new feed nutrients enter the system, thus maintaining a constant pH. However, ruminants in commercial production consume numerous meals per day. This influx of nutrients with feeding can result in dramatic pH cycles in the rumen (Jung et al., 2004). For this reason, the *in situ* method was developed in order to expose feed samples to a more realistic microbial

growth environment that incorporates these pH dynamics. Feed samples are placed in small porous bags made of indigestible synthetic fabric and then suspended in the rumen of fistulated animals for different periods of time, followed by determination of DM, NDF and CP after washing the residue with running water (Jung et al., 2004; McDonald et al., 2011). The pores of the bag allow entry of rumen microbes into the bags and efflux of digestion products. However, the pores must be small enough to prevent loss of undigested sample feed particles from the bags.

The *in situ* rumen degradability of feeds has received widespread attention partly because it is one of the few techniques that describe the kinetics of feed degradation in the rumen (Jung et al., 2004). Also, it has significantly advanced animal nutrition knowledge in protein metabolism in the ruminant (Adesogan, 2002). However, attempts to characterize the degradability of starch and NDF with this technique have yielded variable and sometimes conflicting results (Beever and Mould, 2000). Also, compared to *in vivo* methods, the tested feed is not subjected to mastication and rumination, and anti-nutritive factors that influence intake cannot be measured.

The gold standard by which the nutritive value of feed is measured is *in vivo*, where the target livestock species, of the correct breed, sex, and production level are selected and fed diets of interest. However, *in vivo* trials require large amounts of feed, take significant time to complete, strenuous and are expensive.

Determining the proportion of forage fiber digested by animals requires three basic measurements: amount of feed consumed, amount of fecal material excreted, and fiber concentration of the feed and feces (Jung et al., 2004). Animal productivity can also be determined by measuring body weights (BW), average daily gain (ADG), feed conversion efficiency, and milk yield.

Thesis objective

The western Canadian beef industry is a forage-based industry, and feed costs are the major expense for beef farms. Improved forage utilization would greatly benefit the beef industry, as the cow-calf herd is fed primarily forages. Animal feeding studies, as discussed previously, have been conducted using numerous enzyme products applied at various dose

rates, but the experimental conditions of these studies have varied widely. Various types of forages have been fed and the enzyme products in those studies were provided to the animals in a variety of ways (sprayed onto forage, added to concentrate, sprayed onto the total mixed ration, added as a dry powder to feed, etc.). Information on the enzyme products and their activity units were often not provided, or when the activity units were provided, conditions of the enzyme assays were not specified. Together, these factors make the interpretation of results difficult. Some of the variability in response to enzymes by ruminant is due to the fact that these 'first generation' enzyme products (i.e., enzymes produced for the textile and detergent industries) were not designed for use in feed. In the last few years, there has been an effort by some groups to develop 'second generation' enzyme products for use specifically in ruminant diets, and some of these enzyme products, when added to silage or total mixed ration, were shown to increase milk production efficiency in dairy cows by 10-15% (Arriola et al., 2011a; Holtshausen et al., 2011), due to an increase in fiber digestibility. Beauchemin et al. (2003) also proposed using *in vitro* techniques to screen potential products for improvements in forage degradability, before embarking on the necessary, but more costly, *in vivo* evaluation.

Furthermore, a third-generation silage inoculant (organisms that produce ferulic acid esterase activity, to break the ester linkages between ferulic acid and more digestible carbohydrates) has been reported to improve the feed value of barley silage, fiber digestibility, and feed efficiency of feedlot cattle (Addah et al., 2012) as well as *in vitro* fiber digestibility of alfalfa hay when applied with an exogenous fibrolytic enzyme at baling (Lynch et al., 2013). However, to date no studies have been published on the effects of exogenous fibrolytic enzymes with or without a third–generation inoculant applied at baling and at feeding on feed value and fiber digestibility of alfalfa hay and growth performance of ruminants.

The main objective of this study was therefore to determine the efficacy of fibrolytic enzymes on the nutritive value of preserved forage for ruminants, using growing lambs as a model for beef cattle. The hypothesis for this study was that enzyme additives, applied at the time of baling would increase fiber digestibility of hay and therefore intake, growth, and feed efficiency would improve when lambs are fed the forage and enzyme combination diet. In

addition, the response would be further increased when these enzymes were used in combination with a ferulic acid esterase bacterial inoculant.

The specific objectives of this study were to:

1. Evaluate the effects of different application rates of two 'second generation' EFE products on *in vitro* DM digestibility and gas production of alfalfa hay.

2. Determine if the use of a selected EFE product applied at a particular dosage in combination with a ferulic acid esterase bacterial inoculant at baling would improve feed quality of alfalfa hay after storage.

3. Evaluate in growing lambs the nutritive value (feed intake, growth, feed efficiency, and digestibility) of the alfalfa hay with exogenous fibrolytic enzyme applied at bailing with or without a ferulic acid esterase bacterial inoculant and at feeding.

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CHAPTER 2- Digestibility and growth performance of sheep fed alfalfa hay treated with fibrolytic enzymes and a ferulic acid esterase producing bacterial additive

Introduction

Forage is fundamental to ruminant production so forage conservation with minimal loss of dry matter (DM) and nutrients is paramount. Alfalfa is a widely used forage crop that is grown and preserved as hay, haylage, or silage in North America, Europe, and elsewhere. Its digestible energy content is largely determined by the digestibility of fiber. In recent years, the use of exogenous fibrolytic enzymes to improve the digestibility of forages has been the focus of considerable research, as reviewed by Beauchemin et al. (2003) and Adesogan et al. (2014). Some experiments conducted in vitro have reported an increase in DM and fiber degradability when ground forage was supplemented with enzymes during incubation (Colombatto et al., 2007; Gallardo et al., 2010). Consistent with these in vitro studies, some in vivo studies with sheep (Titi, 2004) and cattle (Feng et al., 1996) reported that enzyme supplementation of diets at the time of feeding improved intake and digestibility, resulting in greater average daily gain (ADG) of cattle (Beauchemin et al., 1999). However, other studies in sheep (McAllister et al., 2000; Rojo et al., 2005) and in cattle (ZoBell et al., 2000; Krueger et al., 2008b) reported no effects of enzyme treatments on ADG when applied at feeding. Similarly, other experiments reported increases in intake and digestibility of DM in lambs (Giraldo et al., 2008) and steers (Krueger et al., 2008b) with the use of enzymes applied to the diet, while others reported no effect in lambs (Miller et al., 2008; Awawdeh and Obeidat, 2011).

Inconsistencies in animal responses to added enzymes are multifactorial, and can possibly be attributed to four main factors: enzyme characteristics (*e.g.*, differences in enzyme preparations, enzymic activities, units of activity added, pH, and temperature effects on activity), forage (*e.g.*, type, maturity), animal (*e.g.*, species, age) and management (*e.g.*, diet, mode of enzyme application, application rate, interaction time of enzymes applied to feed) (Beauchemin et al., 2003).

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In most feeding studies, enzymes were applied to forage or to the total mixed ration immediately prior to feeding to promote interaction between the enzyme and substrate before ruminal fermentation. Association of the enzyme to the feed can cause the release of soluble sugars for use by the rumen microbes and the initial hydrolysis can create sites for microbial attachment to feed particles (Adesogan, 2005).

Applying enzymes to forage at the time of preservation could increase the period in which enzymes interact with fiber and may prevent competitive effects between rumen microbial enzymes and exogenous enzymes (Adesogan, 2005). Recently, Lynch et al. (2013) reported increased *in vitro* fiber degradability when wilted forage was supplemented with fibrolytic enzymes at baling and stored for 90 days, compared to an untreated control. Furthermore, applying enzymes to forage at baling could improve uniformity of application (Adesogan, 2005) and eliminate the labor associated with daily application of enzymes at feeding. However, few studies have compared the relative merits of applying enzymes at feeding compared with application at baling.

Many commercial enzyme products used to improve feed digestion by ruminants contain cellulases and xylanases (Adesogan et al., 2014). However, the extent of cell wall digestion is largely controlled by ferulic acid, which is linked by ether and ester bonds with lignin and by ester bonds with polysaccharides (Jung and Deetz, 1993; Ralph and Helm, 1993; Yu et al., 2005). Thus, use of cellulases and xylanases in combination with ferulic acid esterase (FAE) may be an effective way of hydrolyzing the cross linkages of the cell wall. Applying these additives at the time of baling the forage may provide ample time for their interaction with the cell wall. A third-generation FAE producing bacterial inoculant that breaks the ester linkages between ferulic acid and more digestible carbohydrates has been reported to improve feed value of barley silage as well as feed efficiency of feedlot cattle (Addah et al., 2012). Also Lynch et al. (2013) reported that the application of this inoculant together with a cellulase/xylanase enzyme product at baling increased *in vitro* fiber digestibility when compared to an untreated control of alfalfa hay after 90 days of storage. Improvement in *in vitro* fiber digestibility, with the use of enzymes and FAE inoculant applied at baling could lead to improved animal performance.

The objectives of this study were to: 1) determine the effects of using exogenous fibrolytic enzymes applied to alfalfa hay at baling compared with at feeding on feed intake, digestibility, and growth of lambs; and 2) determine if the effects of exogenous enzymes applied at baling could be enhanced when used in combination with a FAE producing bacterial inoculant. The hypothesis of the study was that an enzyme additive, applied at the time of baling, would increase fiber digestibility of hay and therefore the intake, growth, and feed efficiency of lambs, and that the response would be further increased when enzymes were used in combination with FAE.

Materials and methods

Enzyme activities

Two commercial enzyme products were used in this study: ENZ1 (Econase RDE-L, AB Vista, Wiltshire, UK) and ENZ2 (Rovabio Excel LC, Adisseo, Alpharetta, GA, USA). Methods described by Wood and Bhat (1988) and Bailey et al. (1992) were used to quantify endoglucanase and xylanase activities, respectively, from the enzyme products used. Carboxymethylcellulose sodium salt, medium viscosity (CMC; Sigma Chemical Co., St. Louis, MO, USA, catalogue No. C4888) and oat spelts xylan (Sigma Chemical Co., St Louis, MO, USA, catalogue No. X0627) were used as substrates for the determination of endoglucanase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) activities, respectively. Substrates, 0.1 M citrate phosphate buffer (pH = 6.6), and individual enzyme solutions were incubated in triplicate in an agitating water bath at 39ºC for 15 (EC 3.2.1.4) and 5 (EC 3.2.1.8) min. Endoglucanase (EC 3.2.1.4) activity for ENZ1 and ENZ2 was 71 and 6 µmol glucose equivalent/min/ml of enzyme, respectively; and xylanase (EC 3.2.1.8) activity for ENZ1 and ENZ2 was 847 and 106 µmol xylose equivalent/min/ml of enzyme, respectively.

In vitro study

An *in vitro* experiment using methods described by Colombatto et al. (2003) and Eun et al. (2007) was conducted at the Agriculture and Agri-Food Canada's Research Centre in Lethbridge, Alberta, Canada. The study was performed to select a suitable dose and enzyme for the animal experiments, as recommended by Beauchemin et al. (2003) and Adesogan et al. (2014). The experiment consisted of 24- and 48-h *in vitro* batch culture fermentation in

two runs each. The treatments included alfalfa hay harvested at 5% bloom, dried at 55°C and ground through a 1-mm screen (Standard model 100, Retsch Inc., Newtown, PA), with or without one of two commercial enzymes: ENZ1 and ENZ2 applied at 3 doses (2, 4 and 6 µl/g of substrate DM). Per incubation time, approximately 0.6 ± 0.01 g of ground sample was weighed into four replicate filter bags (F57, ANKOM Technology, Macedon, NY, USA) that were previously washed with acetone, dried at 55ºC for 24 h, and weighed. Enzymes were diluted with distilled water and applied onto the substrates in the bags using a pipette to supply three doses: 2.0, 4.0, and 6.0 µl concentrated enzyme product/g of substrate DM. The amount of diluted enzyme solution (200 µl/0.6 g of sample) was the same for all doses. An equal amount of distilled water was added to a set of filter bags to serve as the control. The filter bags were heat-sealed and placed individually into 250 ml glass vials. Anaerobic buffer medium (pH 7.0) was prepared as described by Eun et al. (2007) and added to the vials and then sealed with rubber stoppers. Vials were flushed with CO_2 prior to the addition of 48 ml of the anaerobic buffer medium. The vials were placed on a rotary shaker platform at 120 rpm in an incubator and kept for 48 h at 20ºC before addition of 12 ml of ruminal fluid to each vial. This was done to ensure adequate interaction time between the forage substrates and the enzymes, similar to the animal study.

Ruminal fluid was obtained 2 h after feeding from two ruminally cannulated, nonlactating Angus cows fed a diet that consisted of barley silage (600 g/kg DM), barley grain (350 g/kg DM) and a vitamin-mineral supplement (50 g/kg DM). Ruminal fluid was obtained from dorsal, ventral, and causal sections of the rumen, composited and strained through two layers of cheesecloth into an insulated flask under CO_2, immediately transported to the laboratory, and kept at 39ºC in a water bath while continually flushed with CO_2. After adding the ruminal fluid to the pre-warmed, buffered medium, the vials were again sealed with rubber stoppers, crimp-sealed with aluminum caps, and placed on a rotary shaker platform at 120 rpm in the incubator at 39°C for either 24 or 48 h. Blanks containing buffer medium, ruminal fluid, an empty filter bag with or without the different doses of each enzyme product were also inoculated in three replications for each incubation duration. Headspace gas production was measured after 3, 6, 12, 18, 24 and 48 h of incubation. Measurements were made using a 23-gauge (0.6-mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC) connected to a

visual display unit (Data Track, Christchurch, UK). Following measurement, the needle was left in the seal to allow for gas release. Pressure values were corrected for values from the blanks and the amount of substrate DM incubated and subsequently used to generate volume estimates using the quadratic equation described by Mauricio et al. (1999). After each incubation period, the appropriate vials were removed, placed in a water bath at 4°C to stop fermentation, and the filter bags were removed. Filter bags were hand washed under cold water until excess water ran clear, dried at 55°C for 24 h, and weighed to determine DM degradability (DMD).

Preservation study

Hay preparation and treatment application

Forage for the animal experiments was obtained from a stand of mainly alfalfa (*Medicago sativa* L.) with minor amounts of orchardgrass and other grass species grown at Coaldale, Alberta, Canada. The second cut of forage from this site was mowed on August 15, 2013 and wilted to a target moisture level of 10%. Six samples of the fresh forage (~1500 g) were randomly obtained from the field for the determination of the proportion of the alfalfa stems in bloom (~400 g/kg) and the species composition (mean ± SD) (alfalfa, 938.3 ± 3.2 g/kg DM; orchardgrass and other species, 61.7 ± 10 g/kg DM). The DM content of the wilted forage was predicted at the field using a Dani Portable Hay Moisture Tester (Dani Farm Supply, Red Deer, AB, Canada) on August 22, 2013. Immediately thereafter, one of three treatments was applied to mown forage in the field. Eleven sets of bales were replicated from adjacent windrows for each treatment, with a discard bale produced between treatments to avoid cross-contamination of treatments. Treatments were applied using a single-nozzle sprayer mounted on the pick-up of the forage baler. Two temperature recording probes (Dallas Thermochron iButtons, Embedded Data Systems, Lawrenceburg, KY, USA) were inserted near the geometric center of five bales selected at random from each treatment to measure the internal temperature every 4 h. Round bales of ~ 500 kg were made using a John Deere model 567 baler (John Deere Agriculture, AB, Canada). There was no rainfall from harvesting through baling and the weather conditions were dry and hot (*i.e.*, 30.5°C daytime high).

The treatments applied prior to baling were: (1) an untreated control with water, (2) hay with enzyme (Eb; Econase RDE-L, AB Vista, Wiltshire, UK), and (3) hay with enzyme plus FAE producing bacterial inoculant (EIb). Application rates were calibrated using the procedure described by Lynch et al. (2013) at a desired rate of 2 ml/kg forage (DM basis). The application rate and the enzyme used were based on the initial *in vitro* study. The water used for control and to dilute the enzyme for Eb and enzyme plus inoculant for EIb was applied at 2 ml/kg (fresh basis). The inoculant (for EIb; 11 GFT, Pioneer Hi-Bred Ltd., Chathan, ON, Canada) was applied at the manufacturer's recommended rate of 1 g/t fresh forage to achieve 1.3×10^5 cfu/g fresh forage and contained a mixed bacterial culture of 1.0×10^{11} cfu/g of *Lactobacillus buchneri* LN4017 (ATCC No. PTA-6138) that produced FAE, 2.0×10^{10} cfu/g of *Lactobacillus plantarum* LP7109 (ATCC No. PTA-6139), and 1.0×10^{10} cfu/g of *Lactobacillus casei* LC3200 (ATCC No. PTA-6135).

Pre-storage and post-storage sampling

Bales were transported to the Lethbridge Research Center (20 km from the baling site), where they were stored under an open sided pole barn with concrete floor until feed out. Immediately after the bales were produced, two samples were taken from the central line of the curved surface of each bale using an electrically powered core sampler (54 cm length × 4.5 cm diameter). After 90 days of storage, two core samples (~ 500 g each) were taken from the bales fitted with the temperature recording probes (Figure 2.1). Samples were composited by bale and a 20 g sub-sample of composited samples was dried in a forced-air oven at 55ºC for 72 h to determine pre-storage and post-storage DM content. The remainder was stored at –20°C prior to chemical analysis. Ambient temperature data were obtained from a weather station located 0.5 km of the hay storage site. Aerobic deterioration of bales was expressed as the accumulated daily temperature rise above ambient during 50 days of storage (ACT = 50 days; *i.e.*, the accumulated difference in daily temperatures of bales and ambient temperatures within 50 days of storage).

Figure 2.1 Pictures showing hay making and core sampling at baling.

Animal studies

Treatments and feeding

The effect of the enzyme-forage treatments on digestibility and growth were evaluated in separate studies using Canadian Arcott lambs. Both studies were conducted concurrently at the Lethbridge Research Center with a protocol approved by the Institutional Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care. Four dietary treatments were evaluated: control, Eb, EIb, and control forage plus enzyme added 2 h before feeding (Ef). Bales from each treatment were chopped as needed and stored in a barn in separate piles. Four out of the five bales inserted with temperature probes were selected for Eb and EIb treatments, and nine bales including those with the inserted iButtons were selected for the control and Ef. For the Ef treatment, enzyme was applied at the same rate (2 ml/kg forage, as-fed basis) as used for the Eb and EIb treatments. To obtain a uniform distribution of the enzyme on the forage for Ef, control hay was loaded into a Data Ranger feed mixer/delivery unit (American Calan, Northwood, NH) and 40 ml/kg (as-fed basis) of enzyme solution (enzyme:water, 1:19) was added gradually as the hay was being mixed. Before the study began, a dye was used to verify thorough distribution of the enzyme when applied in this manner. All diets also included a supplement to provide the necessary nutrients required for growth (Table 2.1). Lambs were also vaccinated using TASVAX® 8 (Schering-Plough Animal Health Limited, Upper Hutt, New Zealand) before the study began and lambs that showed signs of illness during the study were treated with

Micotil (Elanco, Division Eli Lilly Canada Inc., 150 Research Lane, Suite 120, Guelph, Ontario, Canada).

Table 2.1 Ingredients and chemical composition of the supplement for lambs in both animal studies.

Item	Supplement
Ingredients[1]	
Barley	700
Alfalfa (ground)	160
Canola meal	84
Dried molasses	25
Sheep mineral[2]	10
Dicalcium phosphate	5
Calcium carbonate	5
Ammonium chloride	5
6% Deccox premix[3]	0.132
Vitamin ADE	0.250
Chemical composition[4]	
DM (g/kg)	932 (7.3)
OM	923 (8.4)
CP	171 (8.2)
NDF[5]	238 (13.7)
ADF[6]	108 (6.0)
Hemicellulose[7]	125 (8.0)
Cellulose[8]	82 (7.7)
Lignin (sa)[9]	27 (2.9)

[1] Ingredients (kg/t) of the supplement in a pellet form.
[2] Sheep mineral (kg/100 kg) contained 93.1 kg; Dynamate® (Mosaic Co., Plymouth, MN, USA) 5.0 kg; $ZnSO_4$ 0.925 kg; $MnSO_4$ 0.835 kg; ethylene diamine dihydriodide (80%) 0.014 kg; Se premix (1%) 0.144 kg; $CoCO_3$ 0.0036 kg and canola oil 0.40 kg.
[3] 6% Deccox premix (Alpharma Animal Health, Mississauga, ON, Canada) contained 7.92 g of decoquinate per tonne of concentrate.
[4]Chemical composition based on g/kg analytical dry matter, except where indicated and with standard deviation in parenthesis.
[5] Neutral detergent fiber inclusive of residual ash and assayed using sodium sulfite and amylase.
[6] Acid detergent fiber inclusive of residual ash.
[7] NDF-ADF.
[8] ADF- lignin (sa).
[9] Lignin determined by solubilization of cellulose with sulphuric acid.

Digestibility study

Sixteen intact male lambs with initial BW (mean ± SD; 24.0 ± 2.6 kg) were used in a replicated 4 × 4 Latin square experiment. Before the start of the experiment, lambs were adapted to the crates and control diet for three weeks. Lambs were assigned to two groups, blocked (square) by initial weight, and each lamb within the square received a unique sequence of the four treatments diets over time. The experiment consisted of four 21-day

periods, periods, where periods were staggered by 1 week between groups 1 and 2, as only eight metabolic crates were available at a time. At the beginning of each period, lambs were adapted to the treatment for 17 days while in their individual pens, and then transferred to elevated metabolic crates for the last 4 days (collection phase). During the collection phase, total feces were collected into bags using collection harnesses attached to the animal (Figure 2.2), with bags changed twice each day (0830 and 1600 h). Lambs had *ad libitum* access to the hay (115% of average intake from the previous week) and water. The amount of supplement provided daily was adjusted weekly, and was equivalent to 0.20 of each animal's mean daily forage intake determined the previous week. The supplement was provided to meet the nutrient requirements of lambs growing at approximately 200 g/d (NRC, 2007). The supplement and daily allotment of hay was provided once at approximately 0900 h, after the orts from the previous day's offering were weighed and sampled. The supplement was offered in a separate trough 15 min before feeding the forage treatments, and it was consumed immediately. Forages and ort subsamples were collected daily and dried, with forages composited by period and orts composited by animal within period. Concentrate was subsampled at the end of each period and dried to determine DM content. Total feces collected for each lamb daily were weighed, thoroughly mixed, a 150 g (fresh sample) was dried for DM content, and dried samples were composited by lamb for each period. Any feces that occasionally escaped the collection bag were also collected, weighed, dried for DM, and later discarded. All samples were dried in a forced-air oven at 55ºC for 72 h. Lambs were weighed at the beginning and end of each period and prior to entering the crates for total collection.

Figure 2.2 Pictures of a lamb harnessed with fecal collection bag and put in a metabolism crate.

Performance study

A total of 32 lambs with initial body weight (mean ± SD; 23.5 ± 3.5 kg) were selected three weeks after weaning. Twelve days before the initiation of the study, lambs were gradually introduced to the control diet, and adapted to their individual pens (1.5 m × 1 m) matted with wood shavings. Lambs were blocked by sex (20 females and 12 males) and randomly assigned to one of the four dietary treatments as used in the digestibility study. Lambs were assigned to treatments such that mean initial weights were similar for all treatments. Subsamples of each forage treatment were obtained weekly, dried, and DM content was used to calculate forage DMI. A biweekly composite from each forage treatment was dried and stored for chemical analysis. Forage refusals (orts) were weighed and sampled daily, composited weekly, subsampled, and dried for the forage DMI calculation. A biweekly composite ort subsample was dried and stored for chemical analysis. All samples were dried in a forced-air oven at 55 ºC for 72 h.

The study lasted 110 days and was comprised of a 12-day covariate and a 98-day measurement period. Lambs were weighed on the two consecutive days before the start of the study to calculate initial body weight (BW) and then at two-week intervals to determine average daily gain (ADG; g/d). Total DMI (g/d), forage DMI (g/d), supplement DMI (g/d), and feed efficiency (gain:feed) were also estimated biweekly.

Figure 2.3 Pictures of lambs in their individual pens and treatment sampling.

Chemical and particle size analyses

Each pre-storage hay sample from the five replicate bales was freeze-dried, then ground through a 1-mm screen (Standard model 100, Retsch Inc., Newtown, PA), and analyzed for chemical composition. Aqueous extracts were obtained from samples cored on day 90 by blending 15 g of hay with 135 ml of distilled water in a Waring Blender (Waring Commercial, Torrington, CT, USA) for 30 s at full speed and subsequently filtering through four layers of cheesecloth. The pH of the filtrate was determined using a pH meter (VWR, Mississauga, ON, Canada).

Ground sample from the pre-storage hays and feed samples from the animal studies were analyzed in duplicate for DM, ash, neutral detergent fiber (aNDF), acid detergent fiber (ADF), lignin (sa), crude protein (CP) and acid detergent insoluble CP (ADICP). Analytical DM and ash (method 930.15; AOAC 2005) were determined by drying samples at 135ºC for 2 h and combusting samples in a muffle furnace at 550°C for 5 h respectively, followed by hot weighing. Organic matter (OM) was calculated as DM minus ash content. Sequential aNDF, ADF, hemicellulose (NDF minus ADF), cellulose (ADF minus lignin) and lignin (sa; solubilization of cellulose with sulphuric acid) were determined as described by Van Soest et al. (1991) using the Ankom 200® system (Ankom Technology Corporation, Fairport, NY, USA). Heat-stable α-amylase and sodium sulfite were used in the aNDF assay while ADF was analyzed with α-amylase omitted from the procedure. To determine CP (nitrogen × 6.25) samples were ground to a fine powder using a ball grinder (Mixer Mill MM200; Retsch Inc., Newtown, PA). Nitrogen in CP and ADICP was determined by flash combustion with gas chromatography and thermal conductivity detection (Nitrogen Analyzer 1500 series; Carlo Erba Instruments, Milan, Italy).

Dried forage orts and concentrate from the animal studies and feces from the digestibility study were also ground through a 1-mm-screen Retsch mill (Standard model 100, Retsch Inc., Newtown, PA) and analytical DM, ash, aNDF, ADF, and CP were determined as described above.

The particle size of chopped hay was determined using Penn State Particle Separator as described by Kononoff et al. (2003). The mass of particles on each screen of the separator was determined and a percentage of the total mass was then calculated.

Statistical analyses

All data obtained were analyzed using the MIXED procedure of SAS (SAS Ins. Inc., Cary, NC) and means were separated at $P < 0.05$ while trends were indicated at $P \leq 0.10$. The LSD was used to determine significant differences among means.

A randomized complete block design was used to analyze effects of the enzymes and dose for the *in vitro* study, using a model that accounted for all treatments individually (n = 7) and replication (n = 4):

$$Y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$$

where Y_{ij} is an observation, μ is the overall mean, α_i is the effect of treatment, β_j is the replication, and e_{ij} is the residual error.

An additional analysis of variance was conducted using a model that omitted the untreated control, but included a factorial arrangement of enzyme products (n = 2) and dose (n = 3), their interactions and replication (n = 4):

$$Y_{ijk} = \mu + P_i + D_k + \beta_j + PD_{ik} + e_{ijk}$$

where Y_{ijk} is an observation, μ is the overall mean, P_i is the effect of enzyme product, D_k is the effect of dose, β_j is the replication, PD_{ik} is the enzyme product by dose interaction, and e_{ijk} is the error term.

A model for a randomized complete block design was used to analyze pre-storage and post-storage effects of the three forage treatments as follows:

$$Y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$$

where Y_{ij} is an observation, μ is the overall mean, α_i is the effect of treatment (control, Eb, EIb), β_j is the replication (n = 5) or bale, and e_{ij} is the residual error.

A model for a replicated 4 × 4 Latin square design was used to examine the effects of forage treatments for the digestibility study:

$$Y_{ijkl} = \mu + S_i + P_j(S_i) + L_k(S_i) + D_l + + e_{ijkl}$$

where Y_{ijkl} is an observation, μ is the overall mean, S_i is the square (1, 2, 3, or 4), $P_j(S_i)$ is the period (1, 2, 3 or 4) within square, $L_k(S_i)$ is lamb (1 through 16) within square, D_l is the effect of diet (control, Eb, EIb, or Ef), and e_{ijkl} is the error term.

The effect of the four forage treatments on the performance of lambs was analyzed using a model for a randomized complete block design. The data from biweekly measurements (periods 1- 7) of intake and gain were analyzed using a repeated measure statement and covariance structure (autoregressive) that yielded the smallest Akike's information criteria and Bayesian information criteria value. Period data for any lambs that were ill and required treatment (10 lambs) were removed, but as all lambs recovered within days of treatment, the remaining data for those lambs was not discarded. Where appropriate, a slice command in SAS was used to examine treatment differences at each period for each dependent variable.

$$Y_{ijk} = \mu + D_i + L_{j(i)} + P_k + DP_{ik} + e_{ijk}$$

where Y_{ijk} is an observation, μ is the overall mean, D_i is the effect of diet (control, Eb, EIb, or Ef), $L_{j(i)}$ is effect of jth lamb in treatment i, P_k is the effect of period, DP_{ik} is the effect of diet across period, and e_{ijk} is the error term.

Results

In vitro study

The *in vitro* DMD of hay tended (P=0.07) to be affected by treatment after 24 h but not (P>0.05) after 48 h of incubation (Table 2.2). When the control was omitted from the analysis, an enzyme × dose interaction (P=0.04) occurred for DMD after 24 h of incubation. The interaction was observed because the highest dose (6 µl) of ENZ1 greatly increased DMD, but there was no dose effect for ENZ2. There was no effect of enzyme type (P>0.05) on DMD after 24 and 48 h of incubation. No differences (P>0.05) in gas production were observed for treatments for any period of incubation.

Preservation study

Pre- and post-storage chemical composition and storage characteristics

The chemical composition of the three hay treatments was similar pre-storage, except for DM content (Table 2.3). The pH of hay samples did not differ (P>0.05) among treatments after 90 days of storage. Figure 2.4 illustrates the heating pattern of the bales during storage. For the first 10 days, there were no differences (P>0.05) in mean temperature among

treatments; however, there were differences ($P<0.001$) for the subsequent 10-day period with greatest temperature for Eb, followed by EIb, whilst the control recorded the lowest mean temperature. From days 11–25, there was a steep rise in mean temperature from 26–35ºC in Eb bales compared with lower temperature fluctuations between 23 and 26ºC in EIb bales and a maximum temperature of ~20ºC recorded in control bales. Mean bale temperatures fell sharply from day 28 to day 45, then stabilized for the last 5 days in all bales but the lowest temperature was again recorded in the control. After a 50-day storage period, the mean internal temperature of control bales was the lowest ($P<0.001$) whilst Eb recorded the greatest temperature, with intermediate temperature registered for EIb (Table 2.3). Mean accumulated temperature above ambient during 50 days of storage (ACT = 50 days) of the control bales was less ($P=0.02$) than Eb, but it did not differ from EIb.

Table 2.2 Effect of exogenous fibrolytic enzyme on *in vitro* degradability of DM (DMD) and cumulative gas production of alfalfa.

Item		DMD		Cumulative gas production (ml/g DM)					
Enzyme[1]	Dose	24 h	48 h	3 h	6 h	12 h	18 h	24 h	48 h
Control[2]	0	0.645xy	0.687	20	35	61	86	98	117
ENZ1	2	0.625y	0.689	22	35	61	81	95	113
	4	0.618y	0.688	22	37	63	83	97	120
	6	0.662x	0.696	24	41	69	89	103	123
ENZ2	2	0.618y	0.689	22	38	64	83	96	114
	4	0.645xy	0.694	21	39	67	83	102	119
	6	0.625y	0.692	22	38	63	83	97	117
SEM[4]		0.0195	0.0484	2.0	2.1	3.5	6.7	8.6	7.5
P-value		0.07	0.84	0.62	0.35	0.19	0.27	0.42	0.52
Enzyme (control omitted)									
ENZ1		0.635	0.691	22	38	64	84	98	119
ENZ2		0.629	0.692	22	38	65	85	99	116
Dose[3] (control omitted)									
2		0.621y	0.689	22	37	63	82	96	113
4		0.631xy	0.691	22	38	65	86	100	119
6		0.644x	0.694	23	40	66	87	100	120
SEM (control omitted)									
Enzyme		0.0212	0.0023	1.5	1.1	3.4	7.1	9.1	7.9
Dose		0.0220	0.0030	1.6	1.4	3.5	7.2	9.2	8.0
Enzyme × Dose		0.0230	0.0046	1.9	2.0	3.9	7.4	9.4	8.4
P-value (control omitted)									
Enzyme		0.49	0.85	0.66	0.94	0.83	0.82	0.90	0.45
Dose		0.10	0.58	0.43	0.35	0.31	0.29	0.28	0.15
Enzyme × Dose		0.04	0.55	0.80	0.28	0.09	0.11	0.19	0.59

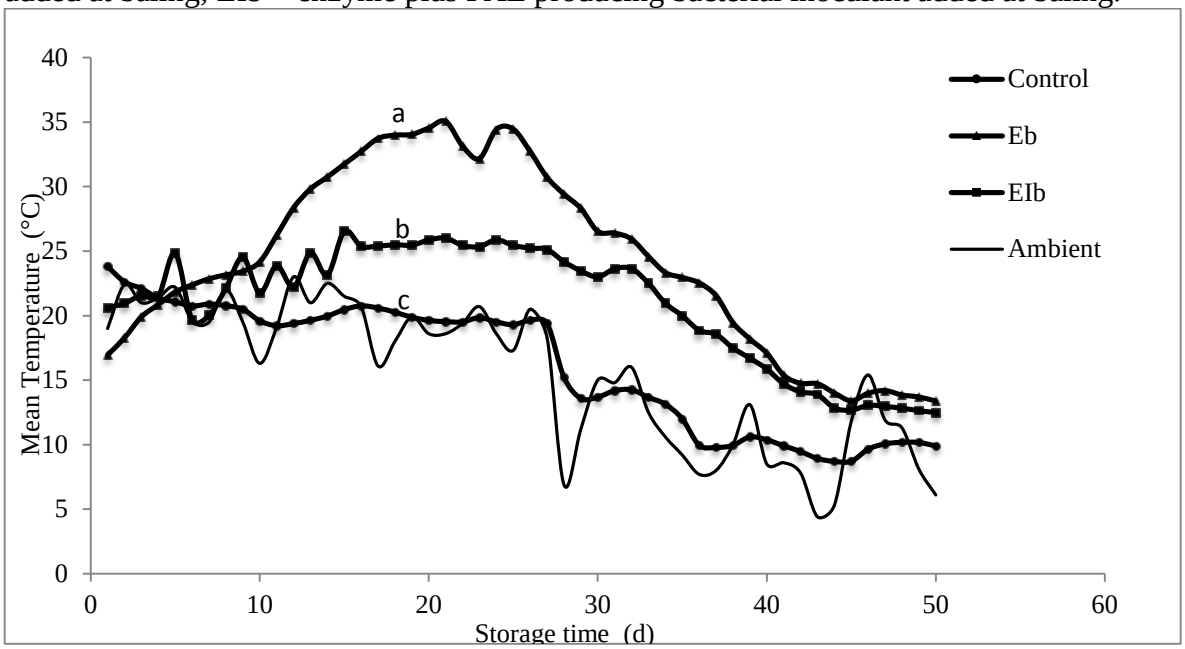
[1] ENZ1 = Econase RDE-L; ENZ2 = Rovabio Excel LC.
[2] Alfalfa treated with an equal amount of water (200µl) without any enzyme.
[3] ENZ1 and ENZ2 enzymes applied at 2, 4 and 6 µl/g DM.
[4] Standard error of means.
Different letters (x, y) following means within a category differ at $P \leq 0.10$.

Table 2.3 Chemical concentration just after baling, pH and heating characteristics after 90 days of storage of alfalfa hay with or without an enzyme and bacterial inoculant.

Item	Treatment[9]			SEM[10]	*P*-value
	Control	Eb	EIb		
Pre-storage[1]					
DM (g/kg)	891a	852b	870b	6.3	0.003
OM	909	909	908	7.9	0.60
CP	201	205	202	3.6	0.71
NDF[2]	462	464	456	6.9	0.71
ADF[3]	348	350	346	4.7	0.82
Hemicellulose[4]	114	114	109	3.7	0.69
Cellulose[5]	251	257	255	5.0	0.63
Lignin (sa)[6]	98	93	91	3.4	0.55
Post-storage					
pH	6.1	6.1	6.0	0.04	0.75
50-d average[7] (°C)	17.8c	26.8a	22.8b	0.69	<0.001
d[8] (°C)	68.4b	453.2a	275.5ab	82.82	0.02

[1] Chemical composition based on g/kg analytical dry matter, except where indicated.
[2] Neutral detergent fiber inclusive of residual ash and assayed using sodium sulfite and amylase.
[3] Acid detergent fiber inclusive of residual ash.
[4] NDF-ADF.
[5] ADF- lignin (sa).
[6] Lignin determined by solubilization of cellulose with sulphuric acid.
[7] Mean internal bale temperature during 50 days of storage.
[8] The accumulated difference in daily temperatures of bales and ambient temperatures after bales were stored for 50 days.
[9] Hay treatments; control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling.
[10] Standard error of means.
Different letters (a, b, c) following means within a row differ at $P < 0.05$.

Figure 2.4 Heating pattern of internal bale temperature of alfalfa hay treated at baling with or without an enzyme and bacterial inoculant after 50 d of storage. No enzyme treatment differed ($P = 0.88$) from control at d 0 to 10. Control differed ($P < 0.001$) from enzyme treatments and enzyme treatments differed ($P < 0.001$) at d 11 to 20, 21 to 30, 31 to 40 and 41 to 50. Different letters (a, b, c) show differences ($P < 0.001$) in treatments for the subsequent 10-day interval following d 0 to 10. Hay treatments: control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling.



The bales were stored for a minimum of 90 days and then fed to the lambs, and because both animal studies were conducted concurrently, the chemical composition of the treatments is shown in Table 2.4 and represents the feed used for the digestibility study. The mean DM content (g/kg) of control, Eb, and EIb hays (907, 903, and 908, respectively) was greater (P<0.001) than that of Ef (878), but OM, CP, ADICP, ADF, cellulose, and lignin (sa) contents expressed on a DM basis were not affected (P>0.05) by treatment. However, NDF concentration tended to be greater (P=0.08) for Eb compared with EIb and Ef, but not control; while hemicellulose concentration was greater (P=0.04) for Eb compared with all treatments. The proportion of fine particles on the bottom screen for each hay treatment was greater (P<0.001) for Ef compared to all other treatments, while it was greater for EIb compared to control but not Eb (Table 2.4).

Table 2.4 Effects of enzyme with or without a bacterial inoculant on chemical composition of feed for digestibility study.

Item	Treatment[9]				SEM[10]	*P*-value
	Control	Eb	EIb	Ef		
Chemical composition[1]						
DM (g/kg)	907a	903a	908a	878b	2.9	<0.001
OM	908	905	905	905	14.0	0.15
CP	194	201	201	198	4.0	0.36
ADICP (g/kg of CP)[2]	219	228	215	211	9.2	0.52
NDF[3]	479xy	496x	468y	467y	11.4	0.08
ADF[4]	364	369	354	353	7.3	0.23
Hemicellulose[5]	115b	127a	114b	114b	6.3	0.04
Cellulose[6]	281	284	273	270	6.1	0.25
Lignin (sa)[7]	83	85	81	83	1.9	0.36
Particle size[8] (%)						
Screen 1 (19 mm)	37a	31b	25c	25c	2.4	<0.001
Screen 2 (8 mm)	26a	24ab	26a	21b	2.4	<0.001
Screen 3 (1.18 mm)	29b	32ab	35a	36a	2.4	<0.001
Bottom screen	8c	12cb	14b	19a	2.4	<0.001

[1] Chemical composition based on g/kg analytical dry matter, except where indicated.
[2] Acid detergent insoluble crude protein on crude protein basis.
[3] Neutral detergent fiber inclusive of residual ash and assayed using sodium sulfite and amylase.
[4] Acid detergent fiber inclusive of residual ash.
[5] NDF-ADF.
[6] ADF- lignin (sa).
[7] Lignin determined by solubilization of cellulose with sulphuric acid.
[8] Proportion retained on various screens of the Penn State Particle Separator.
[9] Hay treatments; control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling; Ef = enzyme added to control at feeding.
[10] Standard error of means.
Different letters (a, b, c) and (x, y) following means within a row differ at $P < 0.05$ and $0.05 \geq P < 0.1$ respectively.

Animal studies

Digestibility study

There were no differences in total DM (P=0.11) and OM (P=0.12) intakes among treatments. Total CP (P =0.09) and hay CP (P =0.05) intakes were greater for lambs fed EIb compared with those fed control and Ef. Total hemicellulose intake was greater (P=0.005) for Eb compared with control and Ef, but not EIb, due to greater hemicellulose intake (P = 0.002) from hay for lambs fed Eb compared with the other treatments.

There was a trend for greater apparent total tract DM (P = 0.09) and OM (P=0.07) digestibility for Ef compared with control and Eb (Table 2.5). There was no effect (P=0.22)

of treatment on ADF digestibility; however, NDF (P=0.03) and hemicellulose (P=0.001) digestibilities were greater for Eb compared with the other treatments.

Performance study

Total and hay DM and OM intakes increased over the study, but there was no treatment by time interaction (P>0.05), thus only the mean intakes for the study are presented (Table 2.6). There was no effect (P>0.05) of treatment on digestible OM intake. There were also no treatment differences (P≥0.30) in fiber (NDF, ADF, and hemicellulose) intakes; however, total and hay CP intakes were greater (P<0.001) for lambs fed Eb and EIb than those fed control and Ef.

Initial body weight was similar (P>0.05) among treatments (Table 2.7). Feed efficiency tended to be affected (P=0.07) by treatment; gain:feed for EIb was 18% greater than control while Eb and Ef were similar to control. Tendencies towards increased gain:feed stemmed mainly from changes in ADG. There were differences (P=0.048) in ADG among treatments; ADG of lambs fed EIb was 21% greater than that of control lambs, whereas ADG of lambs fed Eb and Ef were similar to control lambs. There was no period × treatment interaction (P=0.18) for ADG.

Table 2.5. Effects of enzyme with or without a bacterial inoculant on intake of nutrients and coefficient of apparent total tract digestibility by male lambs (digestibility study).

	Treatments[2]				SEM[3]	*P*-value
Item	Control	Eb	EIb	Ef		
Total intake[1] (g/d)						
DM	1171	1168	1216	1151	28.7	0.11
OM	1055	1048	1093	1034	26.0	0.12
CP	241^{y}	249^{xy}	255^{x}	243^{y}	6.3	0.09
NDF	467^{xy}	483^{x}	480^{x}	438^{y}	15.3	0.05
ADF	335^{xy}	338^{x}	344^{x}	310^{y}	11.1	0.08
Hemicellulose	132^{b}	145^{a}	136^{ab}	128^{b}	4.9	0.005
Hay intake (g/d)						
DM	962^{xy}	961^{xy}	1008^{x}	938^{y}	25.3	0.06
OM	861^{xy}	858^{xy}	900^{x}	836^{y}	22.9	0.07
CP	205^{y}	213^{xy}	219^{x}	206^{y}	5.6	0.05
NDF	416^{ab}	432^{a}	429^{a}	386^{b}	14.6	0.04
ADF	311^{xy}	314^{x}	320^{x}	285^{y}	10.7	0.07
Hemicellulose	105^{b}	119^{a}	110^{b}	101^{b}	4.5	0.002
Digestibility						
DM	0.635^{y}	0.634^{y}	0.640^{xy}	0.648^{x}	0.0040	0.09
OM	0.645^{y}	0.643^{y}	0.649^{xy}	0.658^{x}	0.0040	0.07
CP	0.751^{ab}	0.742^{b}	0.755^{ab}	0.763^{a}	0.0049	0.03
NDF	0.437^{b}	0.480^{a}	0.430^{b}	0.430^{b}	0.0117	0.03
ADF	0.424	0.459	0.421	0.415	0.0140	0.22
Hemicellulose	0.460^{b}	0.524^{a}	0.446^{b}	0.458^{b}	0.0123	0.001

[1] Supplement intake + hay intake (DM basis).

[2] Hay treatments; control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling; Ef = enzyme added to control at feeding.

[3] Standard error of means.

Different letters (a, b) and (x, y) following means within a row differ at $P < 0.05$ and $0.05 \geq P < 0.1$ respectively

Table 2.6 Effects of enzyme with or without a bacterial inoculant on nutrient intakes for growing lambs (performance study).

	Treatment[3]				SEM[4]	*P*-value
Item	Control	Eb	EIb	Ef		
Total intake[1] (g/d)						
DM	1196	1209	1239	1201	54.1	0.54
OM	1081	1087	1198	1085	48.8	0.52
DOMI[2]	697	699	727	714	13.7	0.38
CP	240b	266a	268a	250b	4.6	<0.001
NDF	471	465	474	471	10.2	0.93
ADF	320	314	329	321	7.3	0.59
Hemicellulose	151	150	145	150	2.9	0.52
Hay intake (g/d)						
DM	982	997	1023	985	19.8	0.46
OM	882	891	919	884	17.9	0.45
CP	204b	230a	232a	212b	4.1	<0.001
NDF	421	416	424	421	9.5	0.94
ADF	297	291	305	298	7.2	0.60
Hemicellulose	124	124	118	123	2.5	0.30

[1]Supplement intake + hay intake (DM basis).
[2] Digestible organic matter intake, calculated using apparent OM digestibility from the digestibility study.
[3] Hay treatments; control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling; Ef = enzyme added to control at feeding.
[4]Standard error of means.
Different letters (a, b) following means within a row differ at $P < 0.001$.

Table 2.7 Effects of enzyme with or without a bacterial inoculant on body weight (BW), average daily gain (ADG) and gain:feed for lambs (performance study).

	Treatments[a]				SEM[b]	*P*-value
Item	Control	Eb	EIb	Ef		
Initial BW (kg)	23.9	23.7	24.4	24.2	1.11	0.97
ADG (g/d)	192b	206ab	233a	202b	10.2	0.048
Gain:feed	0.166y	0.181xy	0.198x	0.176y	0.0082	0.07

[a] Hay treatments: control hay; Eb = enzyme added at baling; EIb = enzyme plus FAE producing bacterial inoculant added at baling; Ef = enzyme added to control at feeding.
[b] Standard error of means.
Different letters (a, b) and (x, y) following means within a row differ at $P < 0.05$ and $P < 0.10$ respectively.

Discussion

In vitro study

The different enzyme products applied at various rates showed differences in DMD after 24 h but not after 48 h of incubation. This agrees with authors who reported that the use of exogenous fibrolytic enzymes applied to feed can affect degradation at early incubation times, but enzymes rarely affect extent of forage degradability (Colombatto et al., 2003; Krueger and Adesogan, 2008). Rate of enzyme application has been implicated in producing some of the variability in enzyme research results (Beauchemin et al., 2003). The enzyme product × dose interaction observed in the present study for DMD after 24 h of incubation occurred because ENZ1 responded positively to the high enzyme dose which was not the case for ENZ2. Colombatto et al. (2003) also showed that dose response of some enzyme products is not linear and that higher doses of certain enzyme products may actually limit OM degradability due to different biochemical properties of these enzymes (Vahjen and Simon, 1999). Based on the *in vitro* study, ENZ1 was selected and applied at 2 µl/g DM because at that dose DMD did not differ from ENZ2 applied at any of the three doses evaluated. Although the 6 µl/g DM dose of ENZ1 showed greater response than did the 2 µl/g DM dose of ENZ1, the higher dose was not used because it was deemed not economical, and not within the practical range that would be used commercially on farms.

Preservation study

Pre-and post-storage characteristics

Moisture content at baling is considered one of the major factors influencing DM recovery of hay (Rotz and Muck, 1994) and moisture content below 16% is considered desirable for large round bales because they generally remain stable after long periods of storage (Coblentz et al., 2004). The DM contents of treated and control bales in the present study were within the desirable range pre-storage, and no difference in DM between them post-storage was observed. Differences in DM content between treated bales and control bales pre-storage could be attributed to rapid drying of mown forage in the hot weather during baling resulting in the sequentially produced bales having a greater DM content. The treatments were applied in the field in the order of Eb, followed by EIb and then control to

minimize possible contamination between treatments. The concentrations of aNDF, ADF, and CP in hays pre-storage were as expected for alfalfa hay with minor concentrations of grass harvested in a mid-stage of maturity (NRC, 2001).

The rise in internal temperatures of bales during storage is affected by two phases, a short period of plant-associated respiration followed by plant microbial respiration (Coblentz et al., 2004). Thus, biological activity in hay does not necessarily terminate at baling because plants cells continue to be metabolically active for a few days after baling (Mahanna, 1994). Plants continue to undergo respiration after cutting; using sugars, O_2 and inherent enzymes to produce CO_2, H_2O, and heat. These by-products provide a favorable condition in the internal biome of the bale for spoilage microbes to obtain energy and grow by respiration. As a result, heat continues to build up in the bales resulting in a brownish reaction, which may reduce the nutritive value of the hay.

In the present study, elevated maximum temperature and greatest ACT=50 days recorded in Eb during the microbial respiration phase can be attributed to the combined effects of exogenous fibrolytic enzymes and indigenous organisms that could have contributed to utilization of soluble carbohydrates in the hay. This observation agrees with Lynch et al. (2014) who reported control alfalfa haylage as having the least ($P<0.001$) accumulated difference in daily temperatures of bales and ambient temperatures after 120 days of storage (ACT=120 days) compared with haylage treated with different enzyme products. In contrast, Lynch et al. (2013) applied the same enzyme product as was used in the present study at baling to alfalfa hay (also at 2 ml/kg), and reported that the control hay did not differ in ACT=50 days from the enzyme-treated hay. Although the same enzyme product was used in both studies, the enzyme lots were different and enzymic activities were greater in the study by Lynch et al. (2013) compared with the present study (endoglucanase; 345 *vs.* 71 µmol glucose equivalent/min/ml of enzyme; xylanase; 5608 *vs.* 847 µmol xylose equivalent/min/ml of enzyme). One might expect greater heating in hays treated with greater enzymic activities. However, the difference in internal bale temperatures of treatments between these two studies may partly be due to differences in the DM at baling and the storage sites for the bales. In the present study, internal temperature of Eb bales was greater than for control bales, but the internal bale temperature readings for all treatments were considerably lower than reported by Lynch et al. (2013), who did not find any difference

between control and enzyme treatments. Greater moisture content of hay at baling can cause spontaneous heating in hay (Coblentz et al., 2004). Generally, the DM content of bales at baling was less in the study by Lynch et al. (2013) than in the present study (812 *vs.* 871 g/kg). Thus, the greater DM content of control bales in the present study may have reduced microbial oxidation, and therefore internal bale temperature was less than in the study of Lynch et al. (2013). Also Lynch et al. (2013) stored bales in an open field subject to harsh weather (snow or rainfall), which may have increased the moisture in the bale and subsequently maximized the internal conditions for microbial oxidation; whereas, bales for the present study were kept in an open-sided barn with a concrete floor, which would have protected the bales from rainfall and snow.

Bacterial inoculants with FAE activity are used as alternatives to organic acids in silage preservation (Addah et al., 2012) because of their effectiveness in maintaining aerobic stability. The strain of *Lactobacillus buchneri* found in the inoculant used in the present study is known to produce acetic acid and extend the aerobic stability of alfalfa or grass silage by inhibiting growth of yeasts and molds (Muck, 2010). While we did not measure microbial load or fermentation characteristics of the bales, the reduction in mean temperature after 50 days of storage and the intermediate value of ACT=50 days in EIb compared with Eb, may indicate the preservative potential of the inoculant and suppression of activity of the inherent microbes and enzymic activity. Thus, the effect of the FAE producing bacterial inoculant applied to large round bales that do not exclude oxygen may be similar to that observed for silages during feedout and exposure to oxygen (Rotz, 2003; Bass et al., 2012). Hence, the FAE producing bacterial inoculant in EIb may have partly curtailed the interaction between the spoilage microbes and the exogenous fibrolytic enzymes. This result contradicts the study by Lynch et al. (2013) who reported the same FAE producing bacterial inoculant and enzyme combination as having the greatest ACT=50 days, when compared with control and enzyme alone treatments. The contradiction may be partly due to differences in DM at baling. Though FAE bacterial inoculant applied alone was not considered, *L. buchneri* strains applied by themselves during haymaking (Emanuele et al., 1992; Bass et al., 2012) or silage production (Nsereko et al., 2008) had little or no effect on preservation.

Generally, greater internal heating of Eb bales combined with greater fiber (aNDF and hemicellulose) content compared with the other treatments may indicate an enhanced

oxidative process by the enzyme product and inherent microbes of the hay. Enzyme treatment may have increased the solubilization of non-fiber carbohydrates and pectin, thereby providing substrate for growth of epiphytic microbial organisms. This process could have led to a proportional increase in the concentration of fiber components (Wang et al., 2002). The FAE producing bacterial inoculant activities may have partly negated the effect of the enzymes and epiphytic microbes such that there was no proportional rise in aNDF content for the EIb hay. On the contrary, greater aNDF content due to greater oxidation was reported when the same enzyme plus bacterial inoculant was used by Lynch et al. (2013). Oxidation usually causes a brownish coloration of hay. Visual appraisal of bales in the present study showed no discoloration in most of the EIb and control bales, but Eb bales showed classic signs of oxidation. The dissimilarities in fiber composition between the present study and Lynch et al. (2013) may partly be due to differences in cultivar, in addition to moisture content. Krueger et al. (2008a) applied an enzyme with esterase activity to two different bermudagrass hay cultivars and found differences in their fiber composition after a 16 h treatment period. Improvement in the nutritive value (fiber composition) of hay after an FAE bacterial inoculant with fibrolytic enzyme application may be cultivar specific.

Despite differences in internal heating of bales, there were no treatment differences in concentration of ADICP, a polymer formed as a result of heat-damaged protein in bales. Loss of ammonia and other nitrogenous compounds may reduce CP content due to greater oxidation (Rotz and Muck, 1994). However, CP contents of treated bales in the present study were numerically greater than the control, indicating virtually no loss in nitrogenous compounds during storage. Similarly, Lynch et al. (2013) recorded increased CP content in alfalfa hay treated with enzyme alone or with an FAE bacterial inoculant at baling. Even though the exact cross-linkage mechanism of xylan and lignin of dicotyledonous plants has not been identified, it has been suggested by Jung (1997) that tyrosine may be responsible for the cross-linkages of polysaccharides and lignin. Therefore, the high protein content normally associated with enzyme application at baling could be due to the proteolytic effect of the enzyme.

Animal studies

Digestibility and performance studies were conducted to provide true estimates of the nutritive value of the forages. Although DM, OM, and CP digestibilities were not affected by Eb treatment, the greater apparent total tract NDF and hemicellulose digestibilities could be due to the hydrolytic effects of enzyme addition. Long interaction time between the enzymes and inherent microbes may have weakened the fibrous cells during storage, thereby increasing total tract digestibility of the fiber. In a related *in vitro* study, the same enzyme treatment applied to alfalfa hay with a similar DM at baling (Lynch et al., 2013), or alfalfa haylage with greater DM at ensiling (Lynch et al., 2014), recorded no effect on *in vitro* DMD but increased NDF degradability, after 24 h of incubation. In the present study, greater NDF and hemicellulose digestibilities of the Eb hay did not reflect improvement in ADG of lambs likely because total digestible OM intake was not increased. Equally, Krueger et al. (2008b) applied an enzyme solution to grass at baling and enzymes increased NDF digestibility compared to the control for beef steers without any effect on ADG.

Applying enzyme and FAE producing bacterial inoculant together in EIb did not affect apparent total tract NDF digestibility. Lack of effect on NDF digestibility in lambs agrees with *in vitro* results obtained by Lynch et al. (2014) after 24 h of incubation and Kruger et al (2008a), who also applied an enzyme with an esterase activity to a bahiagrass and two bermudagrass hay cultivars after 48 h of incubation, but contradicts results obtained after 48-h incubation (Lynch et al., 2013). Because oxidation in bales caused by enzyme application was reduced by adding the inoculant, the effects of the exogenous enzymes may also have been curtailed to some degree, which would explain the lack of increase in NDF and hemicellulose content in EIb bales. Despite no effect on forage digestibility, lambs fed EIb had greater ADG and gain:feed compared with lambs fed control due to numerically greater digestible OM intake for lambs fed EIb compared with control.

The use of FAE producing bacterial inoculant applied to bales in this study is beyond the manufacturer's intended use of the product for ensilage of grasses. A similar inoculant applied alone on barley silage at ensiling increased gain:feed (P=0.03) of feedlot cattle, though there was a 7% decrease in DM intake for the inoculated silage (Addah et al., 2012). Xylanase–esterase enzyme applied to the total mixed ration of dairy cows increased the level and efficiency of milk production (Adesogan et al., 2007) but not when applied on alfalfa hay

and corn silage diet (Dhiman et al., 2002). Baah et al. (2005) also reported a 22% increase in DMI relative to a control in sheep for timothy hay (83% DM content) preserved with *L. buchneri* and partly attributed the difference in intake to be enhanced palatability of the *L. buchneri* treated hay. Conversely, a bacillus-based bacterial inoculant applied alone to alfalfa hay and baled at 72% DM did not improve DM intake, ADG, and gain:feed in lambs (Emanuele et al., 1994). It is possible that applying fibrolytic enzymes with FAE producing bacterial inoculant during baling of hay with greater DM caused slight increases in the palatability of the hay, which could have contributed to slight increases in digestible OM intake, and thus increased ADG. Because digestibility of OM and NDF of EIb hay was similar to that of control hay, the increase in ADG was not due to direct effects of the treatment on digestibility.

Using a mixer to obtain consistent application of the enzyme to hay for the Ef treatment did not affect DM intake, despite an increase in fine particles. Exogenous fibrolytic enzymes applied to feed just prior to feeding generally increases rate but not extent of digestion (Colombatto et al., 2003; Krueger and Adesogan, 2008) because enzymes tend to degrade the easily degradable fraction of feed. Thus, the increased apparent total tract DM and OM digestibilities for Ef over the control and Eb, despite no effects on NDF digestibility, shows that the addition of enzymes at feeding increased degradation of the easily digestible non-fiber carbohydrate fraction. This finding indicates that a long interaction time between the enzyme and forage approach is likely needed to cause measureable increases in NDF digestibility, as was the case for the Eb bales where interaction time was considerable. Similar degradation of the easily digestible non-fiber carbohydrate fraction may have occurred in Eb bales within hours of baling, but its proportional rise in NDF indicates that the easily digestible non-fiber carbohydrates fraction degraded by the enzymes was utilised by the epiphytic microbes in the hay. Nevertheless, the relatively small increase in DM and OM digestibilities for Ef did not reflect improvement in ADG or gain:feed. Likewise, Krueger et al. (2008b) applied an enzyme solution to dried grass at feeding and found that enzymes increased DM digestibility compared to the control for beef steers without any effect on DMI, ADG, or gain:feed.

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CHAPTER 3- General discussion and conclusion

Main findings and integration of results

Although some studies have shown that exogenous fibrolytic enzyme application to forages and diets improved forage digestibility and animal performance (Beauchemin et al., 2003; Adesogan et al., 2014), use of exogenous fibrolytic enzyme in commercial ruminant diets is very limited. This is because their use has produced equivocal animal performance results due to the wide array of conditions under which they have been tested and limited understanding of their mode of action (Beauchemin et al., 2003; Adesogan et al., 2014). Allowing for the myriad of exogenous enzyme preparations available, possible methods of application and types of diet to which they may be applied, it is not surprising that production responses to enzyme additives have been highly variable. So, with this in mind and with the problem of increasing cost of feed, a comprehensive understanding of the interrelationship among feed enzyme additives, preserved forages, and the ruminant's digestive system is required. Accordingly, additional study was required to secure consistency in ruminant performance using exogenous fibrolytic enzymes. This thesis project was therefore designed to determine the efficacy of fibrolytic enzymes on the nutritive value of preserved forage for ruminants, and it consisted of an *in vitro* study, preservation study, and two animal studies.

The *in vitro* study compared the effects of two enzyme products (ENZ1 or ENZ 2) applied at three different doses (2, 4 or 6 µl g/DM) on alfalfa hay DMD. This was done to select the best dose and enzyme product for the preservation and animal studies. The *in vitro* DMD of hay was greater for ENZ1 applied at 6 µl g/DM compared to the control but not for ENZ 2 after 24 h of incubation. This showed that dose response of some enzyme products is not linear and that higher doses of certain enzyme products may actually limit DMD due to different biochemical properties of these enzymes. Thus, ENZ1 was selected and applied at 2 µl/g DM, because at that dose DMD did not differ from ENZ2 applied at any of the three doses evaluated. Although the 6 µl/g DM dose of ENZ1 showed greater response than did the 2 µl/g DM dose of ENZ1, the higher dose was not used because it was deemed not economical, and not within the practical range that would be used commercially on farms.

The preservation study compared exogenous enzyme application with or without FAE bacterial inoculant at baling (EIb or Eb, respectively). After a 50-d storage period, the mean internal temperature of control bales was the lowest whilst Eb recorded the greatest temperature, with intermediate temperature registered for EIb. Mean accumulated temperature above ambient during 50 days of storage (ACT = 50 d) of the control bales was less than Eb, but it did not differ from EIb. This showed that applying exogenous fibrolytic enzyme product to alfalfa hay at baling contributed to a decrease in the aerobic stability of the hay. However, adding FAE producing bacterial additives with fibrolytic enzymes at baling improved aerobic stability compared with enzymes alone. As the microbial profile of the hay pre- and post-storage and fermentation profile of the hay after storage were not determined, it is difficult to deduce the extent of spoilage of the enzyme-treated hay. However, from classical sign of oxidation in bales through visual appraisal and with the relatively high heating recorded in the enzyme alone treated hay, there is a potential that enzyme alone application may have increased epiphytic spoilage organisms on hay.

The bales were stored for a minimum of 90 days and then fed to the lambs, through two animal studies (digestibility and performance studies) that were conducted concurrently. The treated diets consisted of control, Eb, EIb, and enzymes applied to the control at feeding (Ef). The chemical composition of Eb showed an increase NDF and hemicellulose contents, as well as their total tract digestibilities. However, improved fiber digestibility did not offset the decrease in non-fiber carbohydrate content, and thus ADG and gain:feed of lambs was not affected relative to control lambs. Adding FAE producing bacterial additives with fibrolytic enzymes negated most of the effects of enzymes on the fiber content of bales during storage and on improving fiber digestibility. However, ADG and gain:feed of the lambs was increased relative to control lambs due to a trend for increased digestible OM intake. Applying enzymes at feeding increased apparent DM and OM digestibilities but not fiber digestibilities, and had no effect on animal performance. Applying enzymes alone to the hay at baling increased the uniform distribution and attachment of the enzymes (Adesogan, 2005) to the hay such that the initial attachment caused the released of sugars to be utilized by epiphytic microbes, which in turn increased and weakened the fibrous cells of the forage. The initial hydrolysis and released of soluble carbohydrates may explain the proportional rise in NDF and hemicellulose, while the weakened fibrous cells may explain their increased

digestibilities for Eb. However, for EIb, the preservative effect of the bacterial inoculant in it may have curtailed the growth of spoilage organisms that depended on soluble carbohydrates for their growth (as may have been the case for Eb); such that there was no proportional rise in fiber, allowing lambs to efficiently increase intake of the digestible OM (soluble carbohydrates) in the forage for an increase ADG. The same effect of initial hydrolysis of soluble carbohydrates may have been seen with the Ef treatment, since enzyme applied to feed prior to ingestion increase the rate of digestion but not the extent of digestion (Colombatto et al., 2003; Krueger and Adesogan, 2008). Thus, Ef produced an increase in the DM and OM digestibilities but not fiber digestibility because of the short period of attachment of the enzymes on the feed.

Future research

Future research using large ruminants (beef cattle) is needed to ascertain the benefits of applying enzymes at baling relative to at feeding and also gain more insights into the interaction between FAE producing bacterial inoculant and enzyme products. Also, the economical (cost:benefit analysis) use of exogenous fibrolytic enzyme products applied with or without an inoculant either at baling or at feeding may be essential for large scale adoption by farmers. Again, the economical use of these products and the timing of application to improving the environment in terms of non-CO_2 (methane and nitrous oxide) per animal product would be essential for its acceptance.

Conclusion and industry perspective

In Western Canada, alfalfa is the predominant forage used by the ruminant industry especially in the beef cattle industry, which is primarily a forage based industry, but high fiber content and low digestibility limit animal productivity and consequently profitability. The efficiency by which ruminants obtain energy from forages and, in turn, produce high quality meat or milk protein is increasingly important if the demands of an expanding population are to be met. Again if the target for reduction in methane production by farmers is to be met, then the efficiency by which ruminants utilize forage cell walls to produce milk or meat is even more important, as greater enteric methane production is associated with

higher forage diets compared with higher grain diets. Therefore, improving the nutritional quality of alfalfa is a high priority for the beef industry.

Though this study did not directly measure the environmental impact of the treatments in terms of methane emission, greater daily gain and improved efficiency of feed by lambs recorded for EIb shows that this treatment has the potential to reduce methane emission per unit of animal output (e.g., kilogram of gain). This method of application was in agreement with the hypothesis that FAE may enhance the efficiency of exogenous fibrolytic enzymes if applied together at baling.

In conclusion, application of fibrolytic enzymes with FAE producing bacterial inoculant at baling is a promising method of preserving baled forage to enhance performance of lambs. Thus, farmers can adopt this method of preserving hay so that ruminants consume quality feed while reducing methane emission per performance.

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