USING BIOLOGICAL ADDITIVES TO IMPROVE DIETARY NUTRIENT CONSERVATION AND UTILIZATION BY LACTATING DAIRY COWS

By

KATHY GISELA ARRIOLA

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my lovely children, Wilhelm and Melanie

ACKNOWLEDGMENTS

I would like to thank my supervisory committee chair, Dr. Adegbola Adesogan, for his invaluable guidance and patience during my Ph.D. program. I will always be thankful to him for being such a wonderful person, professor, mentor, and friend. I appreciate his trust in me and everything he did to develop my critical thinking skills and professionalism. Thanks to Dr. Charles Staples and Dr. Lokenga Badinga for all the advice and time that they dedicated to my Ph.D. program. I appreciate Dr. Carlos Risco's long-standing trust in my abilities. His advice and inspiration helped me to persist with my dream.

I thank my laboratory partners and supervisors (Sam-Churl Kim, Jamie Foster, Oscar Queiroz, Taewon Kang, Juan Romero, Max Huisden, Jan Kivipelto, Nancy Wilkinson, and Sergji Sennikov) for their help during my laboratory and field activities. Special thanks to my dear friends Sam and Jamie; you were the best friends I had at UF and our friendship will continue even though we are far apart. Thanks for her support to Miriam Garcia, who has been my friend since we were undergraduate students at the Universidad Nacional Agraria la Molina in Peru.

I would also like to thank my parents, Martha and Jackson Arriola for their continuous help, patience, and dedication through every important event in my life. I am grateful to my brothers, Jackson and Ricardo for their support and I specially appreciate my sister, Ines for her encouragement during tough times.

Special thanks to my husband, Julio Alberto, for being there unconditionally for me. Finally, I thank my children Wilhelm and Melanie for being my daily inspiration, my strength, and my reason for persisting with my studies.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Acid-detergent fiber
ADG	Average daily gain
BCS	Body condition score
BHBA	Beta-hydroxybutyrate
BUC	Buchneri 40788 inoculant
BUN	Blood urea nitrogen
BW	Body weight
B2	Biotal Plus II inoculant
B500	Buchneri 500 inoculant
CP	Crude protein
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
FA	Ferulic acid
FAE	Ferulic acid esterase
FCM	Fat-corrected milk
HC	High concentrate
HCE	High concentrate enzyme
HPLC	High-performance liquid chromatograph
LC	Low concentrate
LCE	Low concentrate enzyme
Meq	milliequivalent
NDF	Neutral-detergent fiber
NDFD	Neutral detergent fiber dibestibility

NH_3	Ammonia
NH_3-N	Ammonia nitrogen
PCR	Polymerase chain reaction
PUN	Plasma urea nitrogen
PEP	Phosphoenolpyruvate carboxylase
RF	Rumen fluid
SCC	Somatic cell counts
TMR	Total mixed ration
TMRH	High concentrate Total mixed ration
TMRL	Low concentrate Total mixed ration
W	Water
WSC	Water-soluble carbohydrate

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

USING BIOLOGICAL ADDITIVES TO IMPROVE DIETARY NUTRIENT CONSERVATION AND UTILIZATION BY LACTATING DAIRY COWS

By

Kathy Gisela Arriola

May 2010

Chair: Adegbola Adesogan
Major: Animal Sciences

Four experiments were conducted to evaluate the effect of adding biological additives to the diet or feeds of lactating dairy cows on their performance or the quality of the feeds or diets. The objective of Experiment 1 was to determine the effect of dietary addition of a fibrolytic enzyme preparation containing cellulase, xylanase and esterase activities on the performance of dairy cows fed low or high - concentrate diets. Sixty lactating Holstein cows in early lactation (22 $\pm$ 3 days in milk) were assigned to the following treatments: 1) Low concentrate (33%) diet (LC); 2) LC plus enzyme (LCE); 3) High concentrate (48%) diet (HC); 4) HC plus enzyme (HCE). The enzyme was sprayed at a rate of 3.4 mg/g of dry matter (DM) on the total mixed ration (TMR) daily for 63 d. The first 14 d were used for adaptation to diets and the last 49 d for measurements. In addition, four ruminally-fistulated cows were used to determine dietary treatment effects on indices of ruminal fermentation and in situ DM degradation in the rumen. Enzyme application did not affect milk yield or intake of DM, , but increased digestibility of DM, CP, NDF, and ADF,and increased the efficiency of milk production. Increasing the concentrate level reduced ruminal pH but increased intakes of DM and CP, digestibility of DM (DMD) and CP, and milk yield and milk protein yield.

Cows fed LCE instead of HC had less DMI, similar milk yield and greater efficiency of milk production. Enzyme application did not affect ruminal pH or ruminal degradation of the diets. However, increasing the level of concentrate supplementation decreased the pH, increased the immediately soluble dietary fraction, and tended to decrease the potentially degradable fraction. In conclusion, application of the enzyme increased nutrient digestion and the efficiency of milk production by the cows.

Experiment 2 was designed to determine if the enzyme used in Experiment 1 primarily exerted its hydrolytic effect prior to ingestion or within the rumen. A second objective was to determine if the enzyme was more effective on specific components of the diet. Substrates were incubated in a buffer or a buffer - enzyme solution in triplicate for up to 24 h and chemical composition and DM disappearance were measured. In addition, DMD and NDFD were determined after untreated or enzyme-treated substrates were incubated in water (W) or ruminal fluid (RF) for a further 24 h after the initial incubation in the buffer or buffer-enzyme solution. Application of the enzyme reduced concentrations of NDF and hemicellulose, and increased water-soluble carbohydrate (WSC) concentration and DM disappearance. Incubation of enzyme-treated substrates in RF resulted in greater DMD than incubation in W except for AH, which had similar DMD in both media. Enzyme addition increased DMD and NDFD in W by 10 and 84% respectively, but had no effect on DMD and NDFD in RF; suggesting that preingestive effects of the enzyme were greater than ruminal effects. Enzyme effects on NDF, WSC, and hemicellulose concentration or DMD and NDFD in W or RF did not depend on the substrate. Therefore, this study provided no evidence that the enzyme preferentially hydrolyzed specific substrates and it suggested that preingestive

effects of the enzyme were greater than ruminal effects under the conditions of the study.

Experiment 3 determined the effect of bacterial inoculants on the fermentation and quality of corn silages. A corn hybrid Vigoro 61R36 (Royster Clark, Inc.) was grown and harvested at 35% DM. Chopped corn forage was treated with 1) deionized water (CON); 2) Biotal Plus II (B2) inoculant containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); 3) Buchneri 40788 (BUC) inoculant containing *Lactobacillus buchneri*; and 4) Buchneri 500 (B500) inoculant containing *P. pentosaceus* and *L. buchneri*. Four replicates of each treatment were weighed into polyethylene bags within 20-L mini silos, which were stored for 575 d at ambient temperature (25ºC) in a covered barn. After silos were opened, aerobic stability, chemical composition, and yeast and mold counts were determined. The DNA from treated and untreated silages was isolated using a lysozyme/sodium dodecyl sulfate lysis and phenol/chloroform extraction method. The DNA was used as a template for a conventional PCR with primers designed on the 16S rRNA gene to detect the presence of *L. buchneri* in silage samples. The WSC concentrations of all silages were reduced during the fermentation. However, B500 had the greatest residual WSC concentration, suggesting that plant sugars were less extensively fermented by the bacteria in this inoculant compared to those in other treatments. Dry matter loss was lower in BUC silages compared with Control and B2 silages. Control and B2 silages had higher pH and propionic acid concentration and lower lactic acid concentrations than other treatments. The greater lactate concentration and lower pH of BUC silages explain the lower DM loss from this silage. Acetate concentration was greatest in B2 silage,

intermediate in Control and BUC silages, and lowest in B500 silage. However, aerobic stability was generally high (> 250 h) and was not improved by inoculant application. The PCR analysis confirmed the presence of similar populations of *L. buchneri* in all treatments, perhaps explaining why aerobic stability was high in all silages. The inoculants had differing effects on the fermentation of the silages with BUC producing the most desirable fermentation and least DM losses. However, none of the inoculants improved aerobic stability, probably because all treatments had high populations of *L. buchneri.*

Experiment 4 determined the effect of applying three different bacterial inoculants to corn silage on the performance of lactating dairy cows. Corn plants were harvested, chopped, and ensiled in 2.4-m wide bag silos after application of the same treatments as in Experiment 1. Each of the 4 silages was mixed into separate TMR consisting of 44% corn silage, 50% concentrate, and 6% alfalfa hay (DM basis). Fifty-two lactating Holstein cows in early lactation (22 DIM) were fed for 49 d. Chemical composition and yeast and mold counts of silages did not differ among treatments. Treatment with BUC improved silage aerobic stability by 200% and numerically resulted in the least losses compared with other treatments. Inoculant treatment did not affect DMI or digestibility of DM or CP. However, cows fed B2 had lower NDF and ADF digestibility than cows fed the control diet. Consequently, cows fed B2 had lower digestible NDF and ADF intake than cows fed the control diet. Nevertheless, milk yield, milk composition, and feed efficiency were not affected by treatment. Therefore, the inoculants did not affect the performance of the cows, but application of *L. buchneri* improved the aerobic stability of corn silage.

These experiments indicate that fibrolytic enzyme application can improve nutrient digestion and efficiency of milk production by lactating dairy cows. Application of the bacterial inoculants improved the fermentation of silages in one study and improved aerobic stability in another study, but feeding inoculated silages did not affect the performance of lactating dairy cows.

CHAPTER 1
INTRODUCTION

Forages provide energy and nutrients that are vital for the growth and productivity of cattle and therefore represent an important feed resource for cattle production. However, the relatively high lignin and fiber concentrations in forages limit the extent to which they are digested by cattle. Therefore, various methods of improving the digestibility of forages have been explored. One of the most common and successful forage treatment methods is ammonia treatment. Ammoniation typically increases forage crude protein concentration (CP), reduces the concentration of neutral detergent fiber (NDF), and increases the digestibility of NDF in forages. However, the use of ammonia and other alkalis for forage improvement has been limited by their corrosive nature and the hazards they pose to humans.

Recently, interest in using fibrolytic enzymes to improve forage quality has increased. Fibrolytic enzymes increase fiber hydrolysis and enhance feed colonization by increasing the number of ruminal fibrolytic microbes (Nsereko et al., 2000a), such that the rate of degradation of feeds in the rumen is increased (Yang et al., 1999). However, results of applying such enzymes to feeds have been equivocal. Some studies have reported that dietary addition of exogenous fibrolytic enzymes increased dry matter (DM) digestion and DM intake (DMI; Feng et al., 1996; Yang et al., 1999), but others found no improvements in animal performance (ZoBell et al., 2000). Some authors reported an increase in milk yield when enzymes were added to the diet of dairy cattle (Lewis et al., 1999; Beauchemin et al., 2000; Zheng et al., 2000) but others did not (Kung et al., 2003a; Sutton et al., 2003; Vicini et al., 2003). The principal enzymes that have been used in such animal performance trials are cellulases and xylanases.

Little is known about potential benefits on animal performance of incorporating other fibrolytic activities like esterases in such enzyme preparations.

Silage is a forage conservation method widely used in temperate livestock production systems. In many US dairy systems, silage represents up to 45% of the total mixed ration. Ensiling involves anaerobic bacterial fermentation of plant sugars to primarily lactic and acetic acids (Muck, 1988). Rapid achievement of a low pH during the process is vital because inadequate fermentation can increase the population of undesirable microorganisms in silage leading to deterioration, nutrient and DM losses, and growth of spoilage and pathogenic organisms. Several types of silage additives such as acids, alkalis, and microbial inoculants have been evaluated to improve the fermentation and nutritive value of forages. Though effective, chemicals such as sulfuric acid, ammonia and unbuffered propionic acid are not widely used because of their caustic nature (Kung, 2009). Bacterial inoculants are the most common type of additive in the US. They supplement the natural lactic acid bacteria on the crop in order to guarantee a fast and efficient fermentation in the silo (Muck, 1993). The species most widely used include *Lactobacillus plantarum*, *L. acidophilus*, *Enterococcus faecium*, *Pediococcus acidilacti*, *P. pentosaceus* (McAllister and Hristov, 2000), and *L. buchneri* (Weinberg and Muck, 1996). However, these bacteria have different roles and effects on the fermentation. Some rapidly increase the rate of acidification at the onset of the fermentation, others increase acidification in a more gradual manner whereas others increase aerobic stability after the silo is opened. Various inoculants containing one type or multiple types of these bacteria exist, but few studies have compared effects of different types of inoculants on the fermentation, nutritive value and aerobic

stability of silage. Even fewer studies have examined inoculant effects on animal performance.

The objectives of the first two experiments were to determine effects of adding a fibrolytic enzyme containing esterase, cellulase, and xylanase activity on the hydrolysis of various dietary ingredients and the performance of lactating dairy cows. The objectives of the last two experiments were to compare effects of different bacterial inoculants on the fermentation, nutritive value, and aerobic stability of silages and the performance of lactating dairy cows.

CHAPTER 2
LITERATURE REVIEW

Importance of Forages in Dairy Nutrition

Forages represent an important cost effective feed resource in ruminant nutrition (Jung and Allen, 1995). Leguminous forages like alfalfa (*Medicago sativa*) provide an important source of protein for ruminant livestock, whereas grass and small-grain cereal silages are important sources of dietary energy. However, fiber is perhaps the most important contribution of forages to animal feeds. An adequate fiber supply is necessary to maintain the rumen mat, which slows the passage of feed through the digestive tract and increases the amount of nutrients that can be digested and absorbed from the feed. Forage fiber also increases chewing and rumination and therefore increases the production of saliva, which maintains normal ruminal pH and reduces the incidence of ruminal acidosis. National Research Council (2001) recommends that dairy cow rations should contain at least 25% of neutral detergent fiber (NDF) and at least 19% of NDF from forage when the diet is fed as a TMR providing the forage has adequate particle size and the predominant starch source is ground corn (*Zea mays*). Numerous studies have shown the importance of an optimal ratio of forage to concentrate for increasing the productivity of dairy cows (Miller and O'Dell, 1969; Weiss and Shockey, 1991). Diets with low forage to concentrate ratios generally have high starch concentrations that decrease milk fat yield because they reduce the acetate to propionate ratio in the rumen (Nocek and Tamminga, 1991). Appropriate levels of forages in the diets of dairy cows promote production of ruminal acetate and butyrate, which are the major carbon sources for de novo synthesis of milk fatty acids.

Plant Cell Wall Structure in C_3 and C_4 Forages

Cell walls are the main plant fraction that is resistant to enzymatic degradation in mammalian gastrointestinal tracts. Cell wall concentration and chemical composition differs between forage species and parts but for most species, stems have a greater concentration of cell walls than do leaves and cell walls of stems usually contain a greater lignin concentration (Albrecht et al., 1987; Buxton and Hornstein, 1986).

Photosynthetic Pathways of C_4 and C_3 Forages

Photosynthesis occurs mainly in leaf chloroplasts, but the pathway involved varies between C_3 and C_4 forages. Ribulose biphosphate carboxylase/oxygenase (rubisco) is an abundant photosynthetic enzyme in plants, accounting for 20 to 30% of leaf N in cool-season or C_3 plants (Long, 1999). During the Calvin Benson photosynthetic pathway in C_3 forages, atmospheric CO_2 enters the leaf through the stoma and diffuses into the mesophyll cells. Rubisco, a carboxylase adds CO_2 to ribulose 1,5 phosphate (RuBP) to form a highly unstable 6-carbon compound that immediately forms two molecules of the 3-carbon compound, 3-phosphoglycerate, which is ultimately converted into glucose. However, rubisco is also an oxygenase with a high affinity for O_2. Photorespiration occurs when rubisco fixes O_2, leading to a substantial reduction in the efficiency of C fixation (Moore et al., 2004). The unique anatomical features of C_4 plants reduce photorespiration and make the C_4 photosynthetic pathway more efficient. In such plants, photosynthesis occurs in mesophyll cells as well as the bundle sheath within the Kranz structure. The CO_2 entering the leaf through the stomata diffuses into the mesophyll cells where it is fixed by phosphoenolpyruvate carboxylase (PEP) into oxaloacetate, a C_4 compound, which is converted to malate or aspartate, and transported to the bundle sheath cells where it is decarboxylated . The CO_2 released is

refixed by rubisco in the bundle sheath and the Calvin Benson photosynthetic pathway proceeds as in C_3 plants. The presence of the 'CO_2 shuttle' in C_4 plants allows them to increase the concentration of CO_2 around rubisco, which reduces the occurrence of photorespiration and increases the efficiency of C fixation relative to that in C_3 plants. Consequently, C_4 species grow at a faster rate than C_3 species particularly under warm temperature and full sunlight (Long, 1999).

Anatomical Differences between C_3 and C_4 Forages

The Kranz anatomy is a unique characteristic of C_4 grasses which consists of many vascular bundles surrounded by specialized parenchyma bundle sheath cells (Dengler and Nelson, 1999). This thick-walled, lignified parenchyma bundle sheath occupies about 18% of the leaf volume of C_4 plants, with a corresponding decrease in proportion of thin-walled mesophyll tissue (Coleman et al., 2004). Consequently, the Kranz structure resists bacterial digestion and slows access to the digestible content within parenchyma bundle sheath cells (Coleman et al., 2004).

In legumes and most cultivated C_3 grasses, the epidermis is lost from the leaf quickly with chewing and digestion. This process is much slower and less complete in tropical C_4 grasses (Pond et al., 1984) because their epidermis is attached to the leaf at the major and intermediate-sized vascular bundles through thick-walled sclerenchyma cells (Wilson et al., 1989). This attachment can be either directly to the vascular bundle or indirectly to thick-walled bundle sheath cells and then to the vascular tissue. In both cases, the attachment reduces epidermal degradation during mastication thus limiting microbial access to other anatomical features in the cell wall (Wilson et al., 1989).

In leaves of legumes and C_3 grasses, the mesophyll cells are more loosely arranged than in C_4 grasses. Byott (1976) stated that the percentage of intercellular air

space in C_3 grasses (10 - 35%) and legumes (41 - 51%) is much higher than in C_4 grasses (3 - 12%). This allows more rapid penetration of bacteria into the C_3 leaf and hence quicker digestion of leaves (Hanna et al. 1973) than occurs in C_4 grasses. In addition, Wilson and Hattersley (1989) reported that C_3 grasses have greater proportions of mesophyll (53 to 67 versus 28 to 47%), lower proportions of bundle sheath cells (5 to 20 versus 12 to 33%), and lower portions of vascular tissue (3 to 9 versus 6 to 12%) than C_4 grasses. Consequently, greater proportions of thin-walled, non-lignified easily digestible cells are present in C_3 grasses.

The foregoing illustrates anatomical characteristics of C_4 forages that make them less susceptible to physical and microbial breakdown in animals than C_3 forages (Wilson et al., 1989). However, the magnitude of these differences varies between species, maturity stage, and growth conditions.

Cell Wall Chemical Composition

Cellulose

Cellulose and hemicellulose are the main structural polysaccharides in plants. Cellulose, hemicelluloses, and lignin occupy about 40 to 45%, 30 to 35%, and 20 to 30% of the plant cell wall (Ladish et al., 1983). Cellulose is a linear polymer of glucose linked by β-1,4 glycosidic bonds with a simple primary structure and a complex tertiary structure (Bhat and Hazlewood, 2001). The primary structure reflects the pattern of covalent bonding in cellulose molecules. The secondary structure is the conformation of individual molecules, which defines the relative organization in space of the repeating units of an individual molecule. The tertiary structure reflects the arrangement of the molecules relative to each other in a particular state of aggregation (Atalla, 1990). In some regions, cellulose chains are highly ordered and linked by strong hydrogen bonds

to form crystallites, whereas loosely arranged cellulose molecules form the amorphous regions (Bhat and Hazlewood, 2001). Pure cellulose is quickly and completely degraded ruminally as is cellulose cross-linked to hemicellulose alone (Hatfield et al., 1999).

Hemicellulose

Hemicellulose is the second most abundant plant structural polysaccharide. Hemicellulose is composed of a range of heteropolysaccharides, which contain different types of linkages and sugars (Hatfield et al., 2007). Xylans are the main heteropolysaccharides and their backbone structure is comprised of β-1,4-linked xylose residues (Chesson et al., 1983). Xylan polymers may be cross-linked to other hemicellulose backbones (β-1,4-linked-D- pyranosyl residues such as glucose, mannose and xylose) or to lignin through ferulic acid or 4-*O*-methyl-α-D-glucoronic acid residues (Hatfield and Ford, 1989; Lam et al., 1992). Grasses produce xylans with more complex structures than legumes and contain substitutions of arabinose, glucuronic acid, or both (Hatfield et al., 2007). In addition, some of the arabinose residues contain ferulic acid and to a much lesser extent *p*-coumaric acid ester linked to the C5 hydroxyl group (Hartley, 1972). Hemicellulose is soluble in dilute alkali and is linked to cellulose by multiple hydrogen bonds (Albersheim et al., 1984). Hemicellulose is completely digestible when removed from the cell wall matrix but its association with lignin in the cell wall limits its digestion (Hatfield et al., 2007).

Lignin and Phenolic Acids

Lignin is a key component of plant cell walls and its functions include imparting strength to cell walls, facilitating water transport, and acting as a major line of defense against pathogens, insects, and other herbivores (Hatfield and Vermerris, 2001). Lignin also acts as a physical barrier to microbial digestion of fiber polysaccharides. Sarkanen

and Ludwig (1971) described lignin as a polymeric natural product arising from an enzyme initiated dehydrogenative polymerization of three primary precursors: coniferyl, sinapyl, and *p*-coumaryl alcohols (Figure 2-1). The shikimic acid pathway and phenylpropanoid metabolism lead to synthesis of lignin intermediates like *p*-coumaric acid, ferulic acid, diferulic acid, sinapic acid, cinnamic acid, and *p*-hydroxybenzoic acid (Bidlack et al., 1992; Humphreys and Chapple, 2002), which are converted into coniferyl, sinapyl, and *p*-coumaryl alcohols and ultimately to guaiacyl, syringyl, or *p*-hydroxyphenyl lignin, respectively (Figure 2-2). The hydroxycinnamates are structurally related to lignin precursors and they may be attached to lignin, playing an important role during regulation of wall matrix organization (Hatfield et al., 1999). Ferulate and *p*-coumarate molecules are esterified to arabinoxylan in grasses; however, the majority of *p*-coumarates are ester linked to lignin (Ralph et al., 1994). As forages mature and lignin concentration increases, ferulates that were esterified to arabinoxylan become etherified to lignin via cross-links between lignin and the cell wall polysaccharides (Iiyama et al., 1990). The degree of lignin/arabinoxylan cross-linking by ferulates influences cell wall digestibility (Grabber et al., 1998). Increases in the ratio of syringyl to guaiacyl lignin has also been associated with poorer cell wall digestibility due to accumulation of poorly digested, lignified secondary cell walls that are intrinsically higher in syringyl lignin content (Jung and Engels, 2002).

Akin (1986) reported that C_4 grasses contained greater concentrations of phenolic compounds like *p*-coumaric acid and greater ratios of *p*-coumaric acid to ferulic acid than did C_3 grass species. The authors suggested that these compounds might be responsible for limiting the extent of degradation in C_4 grasses.

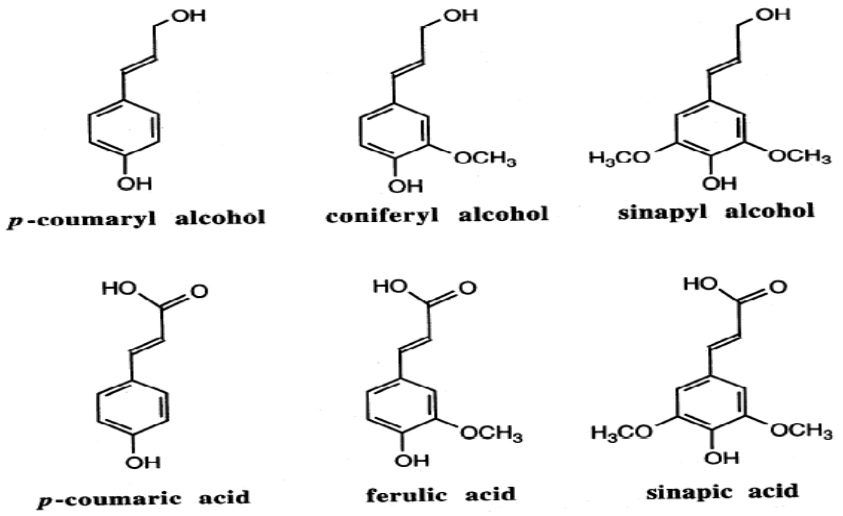

Figure 2-1. Chemical structural characteristics of typical lignin precursors (coniferyl, sinapyl, and p-coumaryl alcohols) and hydroxycinnamic acids (ferulate and p-coumarate) found in forage cell walls (Hatfield et al., 1999).

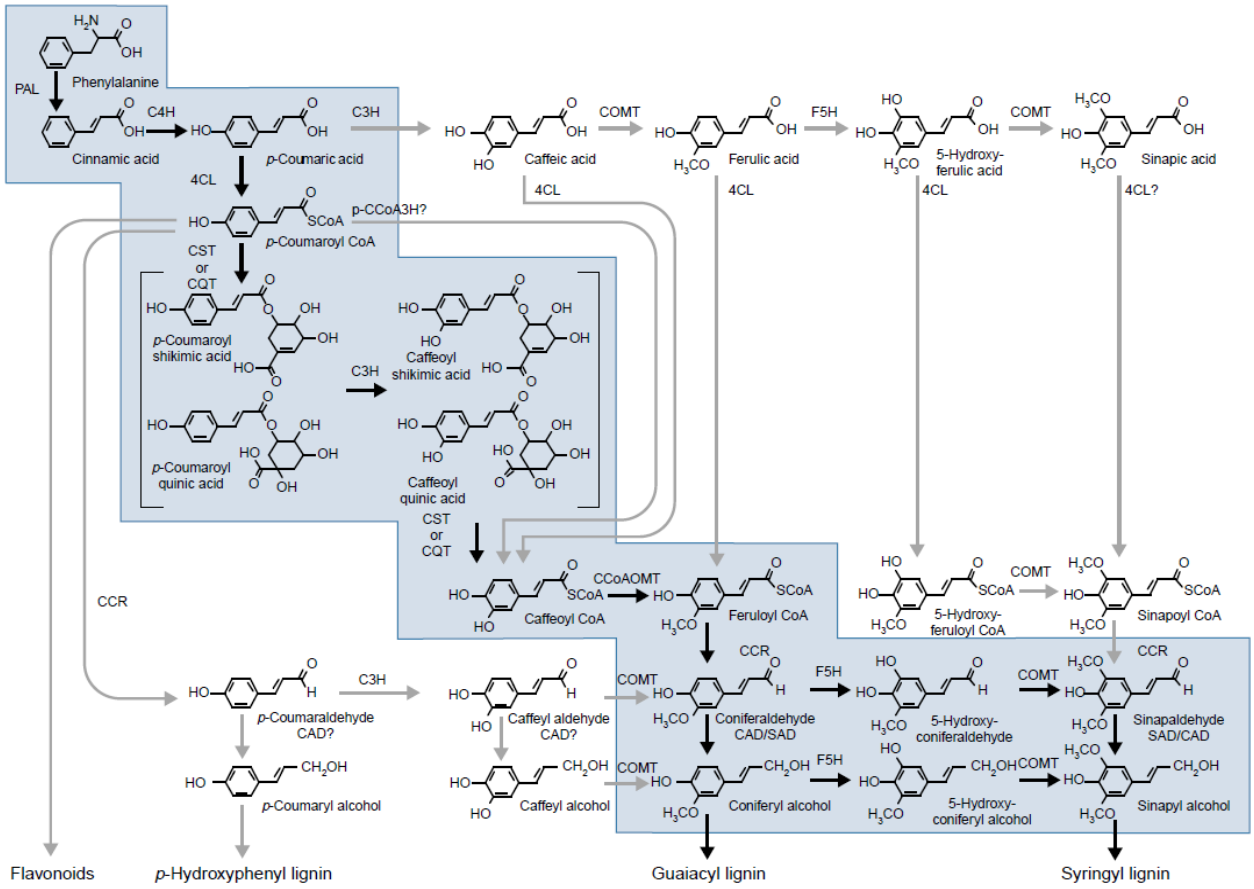

Figure 2-2. Phenylpropanoid pathway leading to lignin biosynthesis (adapted from Humphreys and Chapple, 2002).

Others have also attributed the poorer digestibility of C_4 grasses to greater concentrations of phenolic acid precursors of lignin (Chaves et al., 1982; Burritt et al., 1984; Akin and Benner, 1988).

Chemical Methods of Cell Wall Degradation

Several chemical treatment methods have been developed to improve the nutritive value of low quality forages by reducing the concentration of cell walls or increasing their digestion.

Alkali Hydrolysis of Cell Walls

Alkali treatment is the most widely used method for increasing the degradation of forage cell walls and the main alkalis used are ammonia and NaOH. The underlying principle of the method is partial solubilization of hemicellulose, lignin, and silica and hydrolysis of uronic and acetic acid esters by the alkali (Chesson, 1981; Chesson et al., 1983). The alkalis disrupt intermolecular H bonding within cellulose and hydrolyze covalent ester linkages between arabinoxylans and phenolic acids that are cross-linked to lignin (Chesson et al., 1983; Mueller-Harvey et al., 1986). Alkali treatment reduces concentrations of phenolic compounds, hemicellulose, and acetyl groups in forages and thereby improves digestibility (Sawai et al., 1983).

Several studies have demonstrated increased performance of animals due to forage ammoniation. Kunkle et al. (1983) reported that treating poor quality, tropical forages with anhydrous ammonia (3 to 5%) increased the crude protein (CP) concentration (4.2 to 11.7%) and in vitro dry matter (DM) digestibility (41 to 52%). Horton and Steacy (1979) reported an increase in apparent digestibility of DM, organic matter (OM), crude fiber and CP when steers were fed ammoniated barley (*Hordeum vulgare*), oat (*Avena sativa*) and wheat (*Triticum aestivum*) cereal straws. In addition,

Solaiman et al. (1979) reported that treating wheat straw with ammonium hydroxide increased in vitro DM digestibility. Ammoniation of bermudagrass (*Cynodon dactylon*) increased intake (Wyatt et al., 1989) and average daily gain (ADG) of heifers (Ocumpaugh et al., 1984). Brown (1988) conducted laboratory, digestion and growth trials to evaluate ammoniation effects on 'Ona' stargrass (*Cynodon nlemfuensis* Vanderyst) hay. Ammoniation increased in vitro total N and in vitro OM digestibility (IVOMD) of stargrass hay, but only increased in vivo apparent OM, NDF, and acid detergent fiber (ADF) digestibilities when the hay was mature (6 versus 12 week regrowth). During the growth trial, ammoniation improved ADG and intake by cattle fed the more mature hay. Vagnoni et al. (1995) reported that ADG was increased when steers were fed ammoniated bermudagrass hay. In a second study, treating mature bermudagrass hay with ammonia increased both the *in situ* rate and the potential extent of forage DM and NDF disappearance in Holstein steers. Therefore, the literature clearly demonstrates the nutritional benefits of ammoniation.

Treating hay with ammonia also has non-nutritional benefits. Cattle waste less ammoniated hay than non-treated hay (10% vs. 25% loss, respectively) and ammoniation allows proper conservation and utilization of wet (25 to 60% moisture) forages that would otherwise deteriorate rapidly when stored as hay due to mold growth (Brown and Kunkle, 1992). Despite the many benefits of ammoniation, the use of this forage improvement method has been limited due to the hazards it poses to humans and its corrosive effect on equipment.

Another widely used chemical method for improving forage nutritive value is NaOH treatment. Two treatment methods are available. In the wet or "Beckmann" method, the

forage is soaked in a dilute solution of NaOH for several days and then washed to remove the residual chemical. Sundstol (1988) reported that using this method increased the in vivo OM digestibility of rye (*Secale cereal* L.) straw from 460 to 760 g/ kg. However, there are two major drawbacks with this procedure. Firstly, the wastewater was contaminated with residual NaOH, which could pollute the environment. Secondly, there is considerable loss of DM due to rinsing the treated material prior to feeding. Consequently, a dry treatment method was developed. This involves spraying the forage with a solution of NaOH without rinsing the treated forage prior to feeding. The disadvantage of the dry method is the possibility of increasing toxicity if the samples are not uniformly treated. Various studies have demonstrated the effectiveness of dry and wet NaOH treatment processes at improving the nutritive value of low quality forages and crop residues (Wanapat et al., 1985; Moss et al., 1990). However, NaOH has the similar caustic properties to ammonia, which make it hazardous to handle.

Other chemicals such as hydroxides of calcium, ammonium and potassium have also been used to treat low quality forages. Two experiments were conducted by Haddad et al. (1994) to compare different alkali treatments. In Experiment 1, ground wheat straw was treated with NaOH, NH_4OH, urea, or Ca $(OH)_2$ or certain mixtures of these alkalis. The NDF concentration of wheat straw was decreased by all alkali treatments whereas ADF concentration was not affected. The greatest in vitro NDF digestibility was obtained when wheat straw was treated with 5% NaOH or with 2.5% NaOH and 2.5% Ca $(OH)_2$. In Experiment 2, chopped wheat straw was fed to Holstein heifers after treatment with 1) nothing (control); 2) 2.5% NaOH; 3) 5% $Ca(OH)_2$; 4) 2.5%

$Ca(OH)_2$ + 2.5% NaOH; and 5) 5% Ca $(OH)_2$. The greatest in vivo NDF digestibility occurred when NaOH alone or NaOH plus $Ca(OH)_2$ was applied.

Calcium hydroxide is safer to handle than NaOH or NH_3. However, few studies have used it to treat low quality forages (Owen et al., 1984) because it is a weaker alkali than NaOH and is therefore less effective at improving digestibility (Wilkinson and Gonzalez-Santillana, 1978). In addition, $Ca(OH)_2$ treatment has increased mold growth in some forages (Patterson et al., 1980; Bass, et al., 1982).

Enzymatic Hydrolysis of Cell Walls

Fibrolytic Enzymes

Ruminant feed enzyme additives, primarily xylanases and cellulases, are concentrated extracts resulting from bacterial or fungal fermentations that have specific enzymatic activities (Beauchemin et al., 2003). Several digestive enzymes are used regularly and successfully to enhance the performance of poultry and swine diets but enzymes have not routinely been used in diets fed to ruminants (Kung, 2001). This was partly due to the perception that enzymes are proteins that are subject to degradation by microbial proteases in the rumen and or inactivation by proteases in the small intestine. Kopecny et al. (1987) reported that a cellulase enzyme complex was rapidly degraded by ruminal bacterial proteases and therefore had no effect on in vitro fiber digestion when added to substrates incubated in ruminal fluid. However, Fontes et al. (1995) compared the resistance of cellulases and xylanases to proteolysis and reported that several xylanases were resistant to several proteases but only one cellulase from a mesophilic organism was resistant to proteolytic attack. Other studies have also demonstrated that exogenous polysaccharide-degrading enzymes resist microbial proteolysis in the rumen long enough to escape the reticulorumen and some have been

active in duodenal fluid (Hristov et al., 1997, 1998). When applied to feeds, the close association of enzymes to feeds may also confer resistance to ruminal proteolysis (Beauchemin et al., 2004). The next section describes the main enzymes involved in cell wall digestion by ruminants.

Cellulase

Cellulose and hemicellulose are the main structural polysaccharides in plants (Van Soest, 1994) and they are converted to soluble sugars by cellulases and hemicellulases, respectively. Such enzymes are sourced from cultures of fungi such as *Trichoderma longibrachiatum*, *Aspergillus niger*, or *A. oryzae* or bacteria such as *Bacillus subtilis and Streptococcus faecium* (Pendleton, 2000, as cited by Beauchemin et al., 2004). The main enzymes involved in cellulose hydrolysis are as follows: 1) endoglucanase (EC 3.2.1.4), which hydrolyzes cellulose chains at random to produce cellulose oligomers of varying degrees of polymerization; 2) exoglucanase (EC 3.2.1.9.1) which hydrolyzes the cellulose chain from the non-reducing end producing cellobiose; and 3) β-glucosidase (EC 3.2.1.21) which releases glucose from cellobiose and hydrolyzes short chain cello-oligosaccharides from both reducing and non-reducing ends (Bhat and Hazlewood, 2001; Beauchemin et al., 2003; Figure 2-3). Bhat et al. (1990) stated that microorganisms secrete multiple endoglucanases (I, II, II, IV, and V) with a wide range of substrate- specificity that allow efficient hydrolysis of complex cellulosic substrates. Likewise, two classes of exoglucanase (cellobiohydrolases; CBH) have been described (Barr et al., 1996). The first class includes one enzyme (CBH-I) from *T. reesei* and two exoglucanases (E4 and E6) from *T. fusca*. These hydrolyze the cellulose chain from the reducing end. The second class includes CBH-II from *T. reesei*

and E3 from *T. fusca*. These hydrolyze the cellulose chain and release cellobiose from the non-reducing end (Barr et al., 1996).

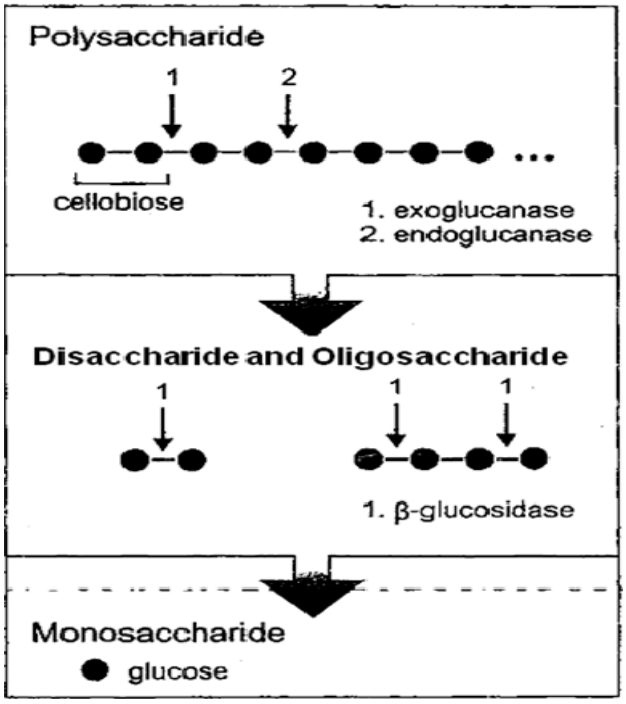

Figure 2-3. Schematic representation of the major enzymes involved in cellulose hydrolysis (Beauchemin et al., 2004)

Xylanase

The core polymer of xylan is degraded to soluble sugars by two main enzymes: xylanase (EC 3.2.1.8) and β-1,4 xylosidase (EC 3.2.1.37) (Bhat and Hazlewood, 2001). Xylanases are commonly called endoxylanases and they yield xylooligomers when they hydrolyze xylan whereas, β-1,4-xylosidases yields xylose (White et al., 1993). In addition, other hemicellulase enzymes involved primarily in the digestion of side chains include β-mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), α-D-galactosidase (EC 3.2.1.22), acetyl- xylan esterases (3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) (Biely et al., 1992). Xylans containing

branching involving linkages with arabinose and glucuronic acid are more soluble than those that do not (Van Soest 1994). Xylanases efficiently degrade linear xylan but branched chains are more slowly or incompletely degraded (Van Soest, 1994). Xylans from annual plants are more heterogeneous than xylans from perennial plants (Bhat and Hazlewood, 2001). Some arabinoxylans such as those in cereal endosperm are highly branched and lack uronic acid substitution for xylose, whereas others found in lignified tissues, have much less branching and have substitutions of uronic acid, 4-*O*-methyl ether and galactose for xylose (Bhat and Hazlewood, 2001).

Synergistic Action of Cellulases and Xylanases

Some authors have demonstrated the existence of synergy between endo and exoglucanases during solubilization of crystalline cellulose (Wood et al., 1989; Klyosov, 1990). The most common types of synergy are between endoglucanase and exoglucanase (CBH) or different types of exoglucanases (CBH) or endoglucanases. Complete and efficient hydrolysis of xylan requires the synergistic action of main and side-chain-cleaving enzymes with different specificities (Coughlan et al., 1993). These authors described the following types of synergy between enzymes: 1) Homeosynergy, the synergistic interaction between two or more different types of side-chain-cleaving enzymes or between two or more types of main-chain-cleaving enzymes; 2) Heterosynergy, the synergistic interaction between side-chain and main-chain cleaving enzymes; 3) Antisynergy, the action of one type of enzyme that prevents the action of a second enzyme (Coughlan et al., 1993). Combining cellulases and xylanases has led to synergistic degradation of corn fiber (Kosugi et al., 2002; Murashima et al., 2003; Koukiekolo et al., 2005) oat hulls (Yu et al., 2005), and bahiagrass (*Paspalum notatum*)

hay (Krueger and Adesogan, 2008) but the specific types of synergistic interaction involved in these studies was not disclosed.

Esterase

Polysaccharides may be linked to lignin by glycosidic, ether, and ester cross-linkages, or cinnamic acid bridges (Figure 2-4; Lam et al., 1990). Yet xylanases and cellulases can only hydrolyze glycosidic linkages. Ferulic acid (FA), the most abundant hydroxycinnamic acid in the cell wall of cereal grains (Kroon et al., 1996; Bartolome et al., 1997a, b) is covalently cross-linked to polysaccharides by ester bonds and to components of lignin mainly by ether bonds (Scalbert et al., 1985; Borneman et al., 1991). Ferulic acid esterase (FAE), can hydrolyze the ester linkage between FA and the attached sugar and release FA from complex cell walls like wheat bran (Bartolome et al., 1997a), sugar beet pulp (Kroon et al., 1996), and spent barley grain (Bartolome et al., 1997b) thus opening the remaining cell wall polysaccharides to further hydrolytic attack by other enzymes (Yu et al., 2005).

Xylan exists in many plants in an acetylated, poorly digestible form, a circumstance largely neglected in studies of its breakdown by microbial enzymes (Biely et al., 1986). Biely et al. (1986) demonstrated that fungal esterase and xylanase activities act cooperatively to hydrolyze acetyl xylan. In the absence of esterase, xylanases break only a limited number of glycosidic bonds in xylans. Both the extent and rate of breakdown increase when esterase is present. The two activities also act synergistically to liberate acetyl residues. Yu et al. (2002) studied the ability of FAE from Aspergillus to release FA from oat hulls in the presence or absence of xylanase from Trichoderma xylanase. Little release of FA by FAE occurred in the absence of the

xylanase, whereas a significant release in occurred in the presence of the xylanase, indicating a synergistic interaction between the enzymes.

Several authors have suggested a possible role for supplemental esterases in enhancing forage degradation in the rumen (Nsereko et al., 2000b; Tricarico and Dawson, 2001; Dhiman et al., 2002). However, esterase activities of fibrolytic enzymes have not been reported in most enzyme studies and few studies have examined supplementary esterase effects on animal performance. Dhiman et al. (2002) compared adding xylanase and cellulase enzymes with or without FAE to a diet fed to lactating dairy cows, but no effect on DMI or milk production response was reported. Therefore, beneficial responses of esterase addition have only been demonstrated in vitro. For instance, Eun and Beauchemin (2006) reported that combining xylanase, cellulase, and esterase enzymes improved DM and NDF degradability of alfalfa hay and corn silage by 25% relative to the untreated control. Krueger et al. (2008a) applied an esterase enzyme at different rates to three tropical grasses and reported that enzyme application enhanced NDF hydrolysis and 96 h IVDMD. Increasing the rate of application also increased the in situ washout fraction and enhanced release of water-soluble carbohydrates (WSC) and ether-linked ferulic acid from Tifton 85 bermudagrass, and ester-linked ferulic and *p*-coumaric acids from Pensacola bahiagrass. These in vitro studies reveal the important contribution of FAE to cell wall hydrolysis and emphasize the need for in vivo experiments to evaluate performance responses of ruminants to dietary esterase addition.

Etherase

As forages mature, and become increasingly lignified, ferulates that were esterified to arabinoxylan become etherified via cross-links between lignin and the cell wall

polysaccharides (Iiyama et al., 1990). Ether linkages are highly resistant to biodegradation due to their high bond energy and chemical inertness (White et al., 1996). Etherases are a group of enzymes that catalyze the scission of such linkages by the following mechanisms: oxygenative cleavage via monooxygenases, oxidation of the C atom linked to the ether bond followed by hydrolysis of the resultant ester, cleavage of methyl-aryl ethers, direct hydrolysis of the C - O bond, and C - O lyase-mediated cleavage (Cain, 1981). Ruminal fungi such as *Neocallimastix patriciarum* may solubilize lignin via dissolution of the xylan in the lignin – xylan matrix (McSweeney et al., 1994) but they do not cleave the ether linkage that cross links lignin and polysaccharides in hydroxycinnamic acid bridges. Actual lignin depolymerization and ether linkage scission involves cleavage of the arylglycerol- β –aryl ether linkage, which has been achieved with soil bacteria such as *Pseudomonas paucimobilis* SYK-6 (Masai et al., 1989) but not with ruminal or commercial exogenous fibrolytic enzymes.

Ligninase

Ligninase is a fungal enzyme discovered in the extracellular fluid of lignolytic cultures of White-rot fungi (*Phanerochaete chrysosporium;* Tien and Kirk, 1983). Some species of White-rot fungi have improved the biodegradation of grasses by attacking the aromatic constituents of lignin (Akin, 1993). These fungi are capable of degrading lignin due to the presence of several enzymes like ligninases, phenol oxidases, Mn-dependent peroxidases, and H_2O_2-producing enzymes (Kirk and Farrell, 1987). To test if ligninase enzymes from white rot fungi can be used to improve forage quality, Khazaal et al. (1993) examined the effect of treating barley straw with *P. chrysosporium* with or without addition of ligninase. No effect on chemical composition and digestibility was reported. In contrast, several studies have shown that addition of strains of white-rot

fungi to forage increases in vitro digestibility (Agosin and Odier, 1985; Gamble et al., 1994; Zadrazil et al., 1995). However, the growth substrates of the fungi are C sources like carbohydrates (Fahey et al., 1993), which no longer available to animals after fungal hydrolysis. This has limited the use of white-rot fungi for forage improvement. Further research on optimizing lignin degradation by cell-free ligninases is required.

Modes of Enzyme Action

Mechanisms of fibrolytic enzyme action on forages that have been postulated include direct hydrolysis (Sheperd and Kung, 1996; Colombatto et al., 2003a), stimulation of microbial numbers and microbial attachment to substrates (Morgavi et al., 2000), and improvement of palatability (Adesogan, 2005). These effects could be initiated before or after ingestion of the forage.

Preingestive effects

The preconsumptive effect of exogenous enzymes on feeds may be as simple as release of soluble carbohydrates or as complex as removal of structural barriers that limit the microbial digestion of feed in the rumen (McAllister et al., 2001). Cell wall hydrolysis in the rumen proceeds in an erosive manner (White et al., 1993) and the major restriction to digestion is limited colonization and penetration by cellulolytic microbes and their hydrolytic enzymes onto the exposed surfaces of feed particles (Beauchemin et al., 2003). Applying exogenous enzymes directly to feed causes a release of reducing sugars (Hristov et al. 1996a), and in some cases, partial solubilization of neutral detergent fiber and acid detergent fiber (Krause et al., 1998). Forsberg et al. (2000) suggested that the sugars released would provide sufficient additional available carbohydrates to encourage rapid microbial growth, shortening the lag time required for microbial colonization. The degree of sugar release depends on

both the type of feed and the type of enzyme. Hristov et al. (1996a) reported that only two of 11 fibrolytic enzyme preparations tested released significant amounts of reducing-sugars from barley silage. The authors suggested that the release of sugars arises at least partially from the solubilization of NDF and ADF. Some studies have attempted to simulate preingestive effects of enzymes by examining their hydrolytic action on substrates in the absence of ruminal fluid or in buffers or water. Colombatto et al. (2003a) examined the effect of adding a commercial fibrolytic enzyme on hydrolysis and fermentation of cellulose, xylan and a mixture of both polymers. They reported increased release of reducing sugars after 20 h of incubation in the absence of ruminal fluid and concluded their enzyme released sugars from cell wall polysaccharides prior to ruminal digestion. Krueger and Adesogan (2008) noted that combinations of ferulic acid esterase, cellulase and xylanase improved 24 h DM disappearance of mature bahiagrass in the absence of ruminal fluid. Yu et al. (2005) also demonstrated that xylanase and cellulase enzymes synergistically increased the DM disappearance of oat hulls in the absence of ruminal fluid.

To determine if activity of exogenous enzymes in the rumen is a prerequisite for ruminal fiber digestion, Nsereko et al. (2000b) pretreated alfalfa hay with buffer alone or with fibrolytic enzymes diluted in the buffer for 0 or 2 h. The hay was then autoclaved to denature enzymic activities, washed to remove hydrolysis products, dried, and incubated in ruminal fluid for 12 or 48 h. Pretreatment with the enzyme increased NDF degradation at 12 and 48 h in the absence of active enzymes or soluble hydrolysis products. This indicated that hydrolytic action prior to incubation with ruminal microorganisms was the prevailing mode of action of the enzyme. The authors

concluded that activity of exogenous enzymes in the rumen is not a prerequisite for improved ruminal fiber degradation but interactions between exogenous enzymes and ruminal bacteria or endogenous could further enhance fiber degradation. These studies demonstrate the existence of preingestive enzyme effects but their relative importance compared to ruminal effects has not been documented.

Post-ingestion effects

Within the rumen, exogenous enzymes could directly hydrolyze feeds or indirectly stimulate digestive activity through synergistic effects with ruminal microorganisms (McAllister et al., 2001). Exogenous enzymes have increased the hydrolytic capacity of the rumen by exhibiting synergy with ruminal microbial enzymes (Morgavi et al., 2000) and increasing bacterial attachment (Morgavi et al., 2000; Wang et al., 2001) and numbers (Nsereko et al., 2002). Postconsumptive effects of exogenous enzymes can potentially increase the rate (Adesogan, 2005) and extent of digestion or passage (McAllister et al., 2001; Adesogan, 2005). Enzymes can change the viscosity of ruminal fluid (Hristov et al., 2000; McAllister et al., 2001) and modify the site of digestion of nutrients (Hristov et al., 2000). Beauchemin et al. (2003) suggested that supplementing the diet with exogenous enzymes should increase total enzyme activity within the rumen. Based on the enzymic activity of ruminal fluid and the amount of enzyme product consumed daily, Beauchemin and Rode (1996) calculated that adding exogenous enzymes to feed could potentially increase cellulase activity in the rumen by up to 15%. Recent studies revealed that enzyme addition (0.5 and 2.55 µL/g of DM) increased ($P < 0.05$) the initial (up to 6 h) xylanase, endoglucanase, and ß-D-glucosidase activities in the liquid fraction of ruminal fluid by an average of 85% (Colombatto et al., 2003c). Hristov et al. (2000) reported that once daily intraruminal

dosing of exogenous polysaccharide-degrading enzymes into heifers increased xylanase and cellulase activity in the rumen. However, over time, the activity of exogenous enzymes can decline due to inactivation and outflow with the fluid phase of ruminal contents (Hristov et al., 1996b).

Several studies have been conducted to examine the existence of synergy between ruminal and exogenous enzymes. Colombatto et al. (2003b) examined the effect of two commercial fibrolytic enzyme products on the hydrolysis of alfalfa stems and leaves in ruminal fluid after a pretreatment period consisting of incubation in an enzyme solution or water alone for 20 h before ruminal fluid was added. Solubilization of substrates by the enzymes or water alone during the pretreatment period was separately quantified. The enzymes increased OMD in ruminal fluid of stems and leaves by up to 8 and 15%, respectively. However, direct hydrolysis of forage fractions during the pretreatment period did not fully account for the magnitude of the increases in OMD, suggesting that synergy between the exogenous and endogenous enzymes occurred. Wood et al. (1994) reported synergy in the solubilization of crystalline cellulose between cellulase from *Trichoderma koningii* and the cellulosome-type complex of the ruminal anaerobic fungus *Neocallimastix frontalis*. Morgavi et al. (2000) demonstrated synergy between exogenous and ruminal enzymes, by showing that the combined hydrolytic effect of both types in the rumen was much greater than that estimated from the individual enzymes. These studies confirm that exogenous enzymes act synergistically with endogenous ruminal enzymes to increase the hydrolytic capacity of the rumen.

Factors Affecting Enzyme Efficacy in Dairy Cattle Rations

Responses of dairy cattle to dietary addition of exogenous enzymes have been variable and in many cases small (Rode et al., 1999; Schingoethe et al., 1999; Yang et

al., 1999). Beauchemin et al. (1999) examined the effect of applying a cellulase - xylanase enzyme to barley and hull-less barley-based diets of dairy cows. Fibrolytic enzyme supplementation tended to improve 4% FCM and increased total tract digestion of OM, starch, N, NDF, and ADF. In contrast, Elwakeel et al. (2007) reported that a cellulase - xylanase enzyme increased in vitro DM digestibility but did not affect DMI and milk production by dairy cows. Several factors contribute to the variability in animal response to fibrolytic enzyme addition to diets. These include the composition, activity and application rate of the enzyme (Dawson and Tricarico, 1999; Siciliano-Jones, 1999), the time and method of enzyme application (Sutton et al., 2003), and the prevailing temperature, pH, ionic strength, and substrate concentration and type (Beauchemin et al., 2003). In addition, the effectiveness of a particular enzyme depends on the composition of the diet (Beauchemin et al., 1995). For instance, enzymes that increased the DM degradation of alfalfa had minimal effects on corn silage and vice versa (Colombatto et al., 2003 a, b). Some of these factors are discussed in the ensuing sections.

Effect of the form or component of the diet to which the enzyme is applied

Several studies show that effects of enzyme application depend on the part or portion of the diet to which the enzyme is applied, reflecting enzyme-feed specificity. Application of enzymes to different forms of the same ingredient has also produced different results. Feng et al. (1996) reported that cellulase and xylanase enzymes were more effective when applied to dried smooth bromegrass (*Bromus inermis*) at feeding than to freshly cut wilted grass at harvest or to untreated grass. The authors suggested that greater cuticular disruption of the dried grass occurred at feeding and this may have

enhanced ruminal microbial colonization and therefore increased feed degradation and particle size reduction.

Krueger et al. (2008b) applied a fibrolytic enzyme to bermudagrass hay immediately after it was cut, just before it was baled (after wilting) or just before it was fed and compared these to untreated hay in diets of growing steers. Steers fed hay treated with the enzyme after cutting had greater intakes of total DM, hay DM and total NDF and greater digestibilities of hay NDF, total CP and CP intake than those fed the untreated hay. The superiority of the 'enzyme at cutting' treatment was attributed to greater forage moisture availability at the time of enzyme application. Based on the results of several studies, Beauchemin et al. (1999) suggested that fibrolytic enzymes are most effective when added to the dry components of the feed as this allows greater opportunities for the enzyme to adhere to the feed and therefore enhance digestion. However, the ideal moisture concentration that optimizes the activity of enzymes without jeopardizing their adherence to feeds has not been determined.

Bowman et al. (2002) studied the effect of applying a fibrolytic enzyme (cellulose and xylanase; Promote N.E.T. Agribrands International, St. Louis, MO) product to the following components of the TMR: 1) nothing (control); 2) the concentrate (45% of TMR); 3) the supplement (4% of TMR), or the premix (0.2% of TMR). Enzyme supplementation did not affect milk yield or DMI, but digestibility of DM, OM, NDF, and ADF were greater in cows fed the concentrate-treated diet than the control diet. Because the concentrate accounted for the greatest proportion of the ration, the authors suggested that exogenous fibrolytic enzymes should be applied to a substantial portion of the diet to ensure their effectiveness. Rode et al. (1999) also reported that DMI was

unaffected but DM, NDF, and ADF digestibility and milk production increased when a xylanase and cellulase enzyme was applied to the concentrate portion of a TMR. Furthermore, Yang et al. (2000) reported that OM digestibility and milk yield increased when a xylanase-cellulase enzyme was applied to the concentrate portion of a TMR. These studies support enzyme addition to the concentrates but contradictory studies exist. No differences in milk yield or DMI occurred when enzymes were applied to a TMR versus a concentrate or forage (Dean et al., 2005b), to a TMR versus a concentrate (Phipps et al., 2000), or to alfalfa cubes alone versus a mixture of alfalfa cubes and a concentrate (Yang et al., 1999). To our knowledge, only one study has examined enzyme addition to the forage instead of the TMR and no effects on DMI or milk production by dairy cows were reported in that study (Dean et al., 2005b). Therefore, the ideal dietary component to which fibrolytic enzymes should be applied remains elusive.

Effect of the site of enzyme delivery

The idea that exogenous polysaccharide-degrading enzymes could not survive the ruminal proteolysis (Chesson, 1994) has been refuted by studies demonstrating that exogenous polysaccharide-degrading enzymes could resist proteolysis by ruminal microorganisms and remain active in the duodenum (Hristov et al., 1997). Hristov et al. (2000) reported that once daily intraruminal dosing of exogenous polysaccharide-degrading enzymes into heifers increased xylanase and cellulase activity in the rumen. These studies suggest that ruminal dosing is a potentially useful strategy for administering enzymes but it has not improved the performance of dairy cattle. Sutton et al. (2003) applied a xylanase-cellulase enzyme to either the concentrate or TMR or infused it into the rumen of dairy cows. Milk yield and DMI were not affected by

treatment but total tract DM digestibility was greater when the enzyme was applied to the TMR. Lewis et al. (1996) studied the effect of applying cellulases and xylanases via the diet of steers or by ruminal infusion. Compared to the control treatment, enzyme treatment decreased ruminal pH and increased total VFA concentration 16 h after feeding. However, ruminal infusion produced lower DM disappearance and lower total tract digestibility of DM, NDF, and ADF than dietary application. Similar results were also reported by Sutton et al. (2003). These studies demonstrate that dietary enzyme addition is more effective than ruminal infusion.

Effect of time of enzyme application to the diet

Beauchemin et al. (2004) suggested that a close feed-enzyme association might enable some form of preingestive hydrolysis of plant fiber leading to creation of binding sites between the enzyme and feed. Adding certain cellulase - xylanase enzyme mixtures to the diet just prior to feeding was as effective as treating the forage 2 wk (Yang et al., 1999) or 1 to 3 d before feeding (Lewis et al., 1996; Nussio et al., 1997). Sutton et al. (2003) reported that adding a cellulase - xylanase enzyme 1 h prior to feeding improved total tract DM, OM, and starch digestibility compared to adding the enzyme 24 h before feeding but milk yield and intake of nutrients did not differ. Allowing adequate time for the enzyme-feed interaction is important in order to optimize hydrolysis of cell wall components and guarantee further interaction of exogenous enzymes and ruminal microbes. However, studies on the effects of the time of enzyme application have produced equivocal results. Therefore, the ideal duration of the enzyme – substrate interaction period has not been established.

Effect of the enzyme application rate

Theoretically, increasing the enzyme application rate should result in greater cell wall digestion. To examine this theory, Beauchemin et al. (1995) studied the effect of applying a cellulase - xylanase enzyme at different rates to alfalfa hay, timothy hay, and barley silage in diets of steers. The enzyme was applied at six incremental levels of xylanase (international units, IU) and cellulase (filter paper units, FPU; 1) 0, 0; 2) 1000, 40; 3) 2000, 80; 4)3900, 156; 5) 7900, 316; 6) 15800, 632 IU, FPU kg^{-1} of forage DM. Enzyme application had no effect on the performance of steers fed barley silage. Low application rates (treatments 2 to 4) increased (24 to 30%) the ADG of steers fed alfalfa due to greater digestible DMI but higher rates (treatments 5 and 6) were not effective. In contrast, higher application rates increased ADG and ADF digestibility of steers fed timothy hay by 36 and 17%, respectively but lower rates did not. Therefore, the relationship between enzyme concentration and animal response was non-linear and it differed for legumes and grasses.

Lewis et al. (1999) reported that cows fed alfalfa hay and silage treated with a medium dose (2.5 ml/kg of forage DM) of a cellulase and xylanase enzyme produced 4 kg more milk than cows receiving the control, low, and high doses (0; 1.25; 5 ml/kg of forage DM; respectively) of the enzyme. Kung et al. (2000b) investigated effects of adding increasing levels (0, 1, 2.5 ml/kg of TMR) of a cellulase – xylanase enzyme to the forage (corn silage and alfalfa hay) in a TMR fed to lactating cows. Cows fed forage treated with the low dose tended to have greater milk yield (39.5 kg/d) than those fed the control diet (37.0 kg/d) or the high dose (36.2 kg/d) but intake or efficiency of milk production was not improved by any treatment. These studies demonstrate that

application rate is an important factor affecting enzyme action but that the most appropriate rate is dependent on the composition of the enzyme and diet.

Effects of stage of lactation and parity of dairy cattle

Enzyme supplementation should be most effective when ruminal fiber digestion is compromised due to factors like acidosis, or when dietary glucose supply is inadequate to meet the needs of the cow such as in early lactation (Adesogan, 2005). In support, Beauchemin et al. (2000) reported that when cows in positive energy balance were fed enzyme-supplemented diets, intake of digestible energy increased but milk yield did not. Schingoethe et al. (1999) studied the response to adding a cellulase - xylanase enzyme to diets of lactating dairy cows for 12 weeks. Data from early (64 DIM) and midlactation (178 DIM) cows were analyzed separately to determine when it was most effective to feed the enzyme. Milk production was increased by 10.8% in early lactation, but no response occurred for cows in midlactation. Knowlton et al. (2002) reported that enzyme treatment of a dairy cow diet numerically increased milk production by early lactation (30 ± 10.6 DIM) cows but decreased milk production by cows in late lactation (194 ± 9.7 DIM). Lewis et al. (1999) reported that more milk was produced by midlactation (213 DIM) cows fed a cellulase - xylanase enzyme treated diet than cows fed the control diet (27.2 vs. 25.9 kg/d). The discrepancy between this and other studies could be due to differences in enzyme activity, diet composition, and trial duration.

To determine effects of the time of initiating enzyme dosing on efficacy, Zheng et al. (2000) applied a cellulase - xylanase enzyme to the corn silage and alfalfa hay in a TMR beginning at parturition (wk 0 to 18 postpartum), or before (wk -4 to 18 postpartum) or after (wk 6 to 18 postpartum) parturition. Cows fed enzyme-treated forage had greater 3.5% FCM than cows fed the untreated forage. The time of initiating

enzyme dosing had no effect on milk production but numerically, the greatest values occurred when enzyme addition began at parturition.

Most of these studies suggest that responses to dietary enzyme addition are greater in early lactation than at later stages of lactation. However, as outlined above, several other factors determine the outcome of enzyme application to feeds. More research is needed to determine conditions that optimize and guarantee efficacy when fibrolytic enzymes are added to diets of dairy cows.

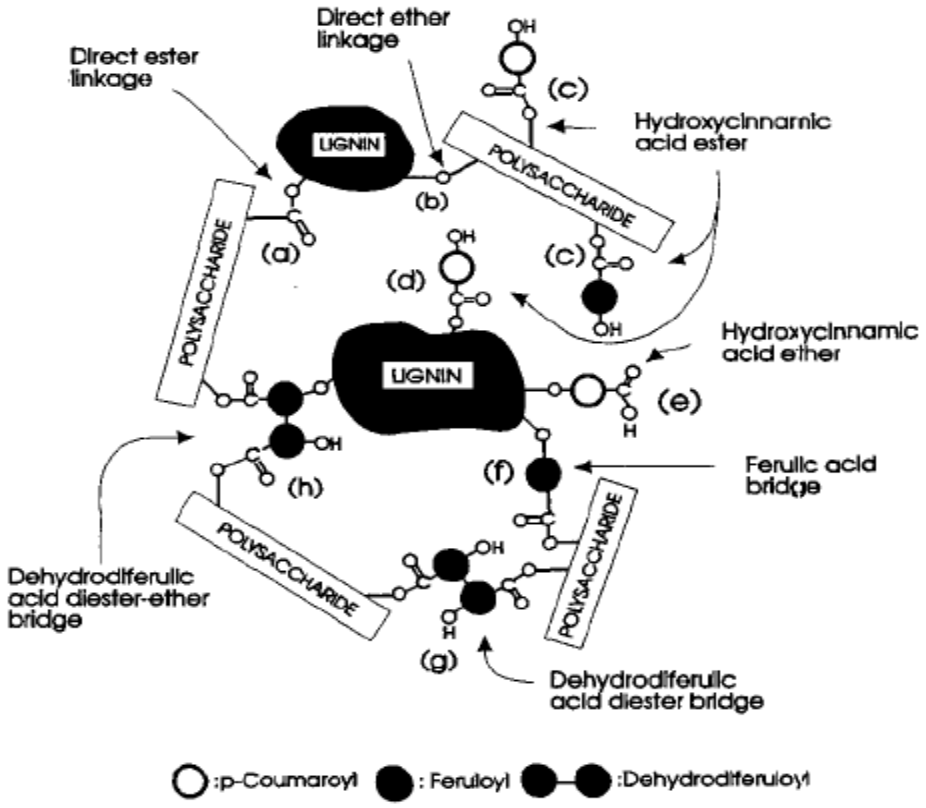

Figure 2-4. Schematic diagram showing possible covalent cross links between polysaccharides and lignin in walls. ○, PCA; ●, FA; ●—●, dehydrodiferulic acid. a, Direct ester-linkage; b, direct ether-linkage;c, hydroxycinnamic acid esterified to polysaccharides; d, hydroxycinnainic acid esterified to lignin; e, hydroxycinnamic acid etherified to lignin; f, FA ester-ether bridge; g, dehydrodiferulic acid diester bridge; h, dehydrodiferulic acid diester-ether bridge (Iiyama et al., 1994).

Silage Fermentation

The aim of silage fermentation is to produce sufficient lactic acid to inhibit both the growth of undesirable epiphytic microorganisms and the activity of endogenous plant catabolic enzymes, thus maximizing nutrient preservation (Bolsen et al., 1992). Plant respiration, plant proteolytic activity, clostridial activity, and aerobic microbial growth adversely affect silage fermentation and quality. Therefore, these processes need to be limited to ensure preservation of crop quality (Muck, 1988). The following section describes influences of these and other factors on the fermentation, quality, and aerobic stability of silages.

Factors Affecting Silage Fermentation

Several factors affect the rate of lactate production and hence the fermentation of silages. The most important factors include the epiphytic population of microorganisms on the plant and the WSC concentration, buffering capacity, and moisture concentration of the forage. However, environmental conditions, agronomic practices, and harvest conditions may increase the number and/or change the distribution of epiphytic microbes on plants and therefore affect the fermentation process.

Moisture Concentration and Maturity

Drier silages have higher pH values than wetter silages because of the greater osmotic pressure at high DM and these factors collectively inhibit the growth of LAB in drier silages (Woolford, 1984; McDonald et al., 1991). Excess moisture at ensiling predisposes crops to effluent production, whereas inadequate moisture at ensiling predisposes to heating and spoilage (Muck and Kung, 2007). Excess moisture encourages the growth of undesirable bacteria such as clostridia, which can dominate the ensiling process in grasses and alfalfa (*Medicago sativa*), resulting in a low quality

product (Muck and Kung, 2007). Clostridia can ferment lactate to butyrate resulting in significant loss of DM and energy. This secondary fermentation can be associated with substantial decarboxylation and deamination of amino acids from plant protein (McDonald et al., 1991).

Moisture concentration typically decreases with plant maturity and different studies have shown that fermentation is decreased with advancing plant maturity. Bal et al. (1997) observed a decline in lactate, acetate, and ethanol concentration of corn (*Zea mays*) silage as maturity advanced. Similarly, Neylon and Kung (2003) reported an increase in pH and a decline in lactate and acetate concentrations as maturity of corn silage increased.

Epiphytic Bacteria

The total microflora population on grasses and herbages varies between 10^5 and 10^9 colony forming units (cfu) / g of the crop to be ensiled (Langston and Bouma, 1960 a, b). The epiphytic lactic acid bacteria (LAB) are the essential microflora for spontaneous silage fermentation (Pahlow et al., 2003). The number of LAB varies with the type of forage and populations (cfu/g of fresh matter) of 10^5 on alfalfa, 10^6 on perennial grasses, and 10^7 on corn and sorghum (*Sorghum bicolor*) were reported by Pahlow et al. (2003). However, the LAB population also varies with stage of maturity, and prevailing weather and is typically greatest on second to third cuts of alfalfa and grasses and on early maturing varieties of corn (Bolsen et al., 1988; Muck, 1989). Whereas, the lowest numbers of LAB usually occur during cool weather (Pahlow et al., 2003) because the optimal growth temperature range for LAB is between 25 and 40°C (Pahlow et al., 2003).

Enterobacteria are the second largest group (10^3 – 10^6 cfu g^{-1}) of epiphytic microflora (Pahlow et al., 2003). They are active in the silo and are important due to their competition with LAB. They ferment sugars into acetic acid, hydrolyze proteins, and deaminate peptides and amino acids (Rooke and Hatfield, 2003). They also reduce NO_3 resulting in the production of nitrites and nitrogen oxide gases (Pahlow et al., 2003).

Yeasts are frequently also present on growing forages. Yeasts ferment sugars such as glucose, maltose, and sucrose mainly into ethanol and CO_2 and small amounts of propanol, 2-butanediol, 2-methylpropanol, pentanol, 3-methylbutanol, acetate, propionate, butyrate, and lactate (Schlegel, 1987; McDonald et al., 1991). When air penetrates the silo during fermentation, lactate-fermenting yeasts of the genera *Candida* and *Hansenula* dominate, whereas when anaerobic conditions are achieved, the population of the latter yeasts is reduced to about 15% and *Saccharomyces* spp., which do not utilize lactate dominate the population (McDonald et al., 1991).

Members of the genera clostridia and bacilli appear to be scarce on fresh forage (Pahlow et al., 2003). They principally arise from contamination from soil and farmyard manure (Ostling and Lindgren, 1991; Rammer et al., 1994). When low pH silage is not achieved, clostridia ferment lactate into butyric acid and degrade amino acids to a variety of products, which are of poor nutritional value (McDonald et al., 1991). Bacilli can also be important facilitators of aerobic deterioration after other aerobic microorganisms have raised silage pH (Pahlow et al., 2003). Bacilli may be implicated in initiating aerobic deterioration in some silages as they possess an electron transport chain and can therefore metabolize lactic acid aerobically (Rooke and Hatfield, 2003).

Water-Soluble Carbohydrates

The main nonstructural carbohydrates in temperate grasses are glucose, fructose, sucrose, and fructans (McDonald et al., 1991). These carbohydrates are soluble in water and are known as WSC. Fructans are the most abundant WSC in temperate grasses, whereas subtropical and tropical grasses accumulate starch instead of fructans (Nelson, 1995). Glucose is the most fermented WSC by various species of LAB found on plants and it is fermented into lactic acid, VFA, and alcohols by such bacteria (Muck and Kung, 2007). Ensiling crops with high WSC concentrations accelerates the fermentation by enhancing substrate availability for LAB. Minimal WSC concentrations required to optimize the fermentation vary with forage type and DM concentration and are 14, 7, and 5 % (DM basis) for alfalfa, cool-season grasses, and corn forage harvested at 35% DM (Leibensperger and Pitt, 1988). Excessively high forage WSC concentrations may provide a source of readily available nutrients for aerobic spoilage organisms such as yeasts (Mahanna and Chase, 2003), thus predisposing the silage to spoilage.

Buffering Capacity

The buffering capacity of plants is their ability to resist a change in pH. It is expressed as milliequivalents (meq) of alkali required to change the pH of 1 kg of the substrate from 4 to 6 (McDonald et al., 1991). The anionic fraction of forage crops, the organic acids, sulfates, nitrates, and chlorides, represent 75 to 90% of the total buffering constituents, and plant proteins account for 10 to 20% (Woolford, 1984). Forages with high buffering capacity require more acid to reduce their pH. Woolford (1984) noted that forages with low buffering capacity (250 to 350 meq NaOH kg^{-1} DM) are relatively easy to ensile, whereas forages with high values (400 to 600 meq NaOH kg^{-1} DM) are

harder to ensile. Corn forage has a low buffering capacity of approximately 200 meq NaOH kg^{-1} DM (McDonald et al., 1991), therefore it is relatively easy to ensile.

Climatic Factors

The environmental temperature at and during ensiling may affect silage fermentation by influencing bacterial activity. Ensiling at high temperatures reduces lactic acid concentration and aerobic stability, and increases pH and DM losses (Weinberg et al., 2001; Ashbell et al., 2002). High ensiling temperatures reduce numbers of LAB (Weinberg et al., 1998, 2001), enhance proteolysis (Muck and Dickerson, 1988, Weinberg et al., 2001), and make the fermentation less homolactic (McDonald et al., 1996). Kim and Adesogan (2006) reported that fermentation of corn silage was adversely affected by wet conditions at harvest and high ensiling temperatures which resulted in greater pH and concentrations of residual WSC and ammonia-N, greater proteolysis, lower lactic to acetic ratio, and increased secondary fermentation.

Most homofermentative lactobacilli grow optimally at 30 to 35°C but *Pediococcus* spp. and some heterofermentative bacteria tend to be more thermotolerant and prefer temperatures of 40 to 45ºC (Woolford, 1984). Mulrooney and Kung (2008) reported that inoculant LAB were relatively stable when exposed to temperatures of 30 to 35ºC for 3 to 6 h; however, exposure to 40 and 45ºC resulted in marked reductions in viable cell counts of several inoculant bacteria within 3 h. Therefore, the authors suggested that thermotolerant bacteria should be used in inoculants and care should be taken to avoid exposing these bacteria to temperatures exceeding 35 to 40ºC.

Factors Affecting Aerobic Stability

Aerobic stability is the length of time it takes for silage to begin to heat during feedout. When silage is exposed to air, certain opportunistic microorganisms become metabolically active, produce heat, and consume nutrients from the silage resulting in spoilage. Aerobic instability increases nutrient losses from feeds and reduces feed intake and productivity of dairy (Hoffman and Ocker, 1997) and beef cattle (Whitlock et al., 2000).

Yeasts

Yeasts are facultative, anaerobic, heterotrophic microorganisms and are considered undesirable in silages. Silage yeasts ferment sugars to ethanol and CO_2 under anaerobic conditions (Schlegel, 1987; McDonald et al., 1991). This decreases the amount of sugar available for lactic acid production. Intake of ethanolic silage by dairy cows can taint the flavor of milk (Randby et al. 1999).

Many yeasts species in silages degrade lactic acid to CO_2 and H_2O under aerobic conditions, thereby, causing a rise in silage pH, and promoting the growth of other spoilage organisms (McDonald et al., 1991). Woolford et al. (1982) reported that yeasts are essentially responsible for the aerobic instability of corn silage. Some authors reported that during the first weeks of ensiling, yeast populations can increase up to10^7 cfu/g, though prolonged storage will lead to a gradual decrease in yeast numbers (Jonsson and Pahlow, 1984; Middelhoven and van Baalen, 1988). Lactate-assimilating yeast such as *Candida*, *Cryptococcus*, and *Pichia* have been implicated as the primary causes of aerobic deterioration in silages (Jonsson and Pahlow, 1984).The presence of oxygen enhances the growth of yeasts during storage, whereas high concentrations of formic or acetic acid reduce their growth (Oude Elferink et al., 1999). Yeast counts

greater than 10^5 cfu/g are usually indicative of aerobic instability (Pahlow and Zimmer, 1985; O'Kiely et al., 1987).

Molds and Mycotoxins

Molds are eukaryotic microorganisms that develop in silage when oxygen is present. Silage infested with molds are usually easily identified by the large filamentous structures and colored spores that many molds produce though some molds are not visible to the naked eye. Mold species regularly isolated from silage belong to the genera *Penicillium, Fusarium, Aspergillus, Mucor, Byssochlamys, Absidia, Arthrinium,* and *Trichoderma* (Woolford, 1984; Jonsson et al., 1990; Nout et al., 1993). Molds cause a reduction in feed value and palatability, and have a negative effect on human and animal health due to the mycotoxins they produce.

Silages that are heavily infested with molds do not necessarily contain high levels of mycotoxins (Nout et al., 1993) and mycotoxins could be present when molds are not visible. Therefore, the relationship between visible molds and occurrence of mycotoxins is not clear. Scudamore and Livesey (1998) reported that mycotoxicoses caused by mold mycotoxins range from digestive upsets, fertility problems, and reduced immune function to serious liver or kidney damage and abortions, depending on the type and amount of toxin present in the silage. Some important mycotoxin-producing mold species are *Aspergillus fumigatus, Penicillum roqueforti*, and *Byssochlamys nivea. Penicillium roqueforti* is acid tolerant and it can grow under low oxygen, high CO_2 conditions. It is the predominant mold species in different types of silages (Lacey, 1989; Nout et al., 1993; Auerbach et al., 1998).

Two species of *Aspergillus, A. flavus* and *A. fumigatus* and their aflatoxins are often reported in silages from warm areas. *Aspergillus flavus* is common in hot and dry

regions where it colonizes corn plants in the field and produces aflatoxins and cyclopiazonic acid (Munkvold, 2003). Cyclopiazonic acid (CPA), which is also produced by Penicillium molds, is a potent specific inhibitor of the endoplasmic reticulum Ca^{++} - ATPase (Goeger et al., 1988). *Aspergillus fumigatus* is a thermotolerant fungus that produces several different mycotoxins including fumitremogens B and C, and gliotoxin (Cole et al., 1977).

Molds of the genus Fusarium produce several classes of important mycotoxins including the fumonisins, thichothecenes and zearalenone (D'Mello et al., 1999). Fumonisins are produced by *Fusarium proliferatum* and *F. verticillioides* as well as numerous other related Fusaria. These two species are extremely common on corn plants and cause ear and stalk rot diseases (Payne, 1999). In addition, these fungi can grow inside the corn plant without causing disease symptoms (Bacon and Hinton, 1996).

Maintenance of an anaerobic environment in the silo during the fermentation and storage phases and maintenance of aerobic stability during the feedout phase are important in silage preservation (Bolsen et al., 1996). Failure to achieve such conditions may cause lower recovery of nutrients, and the production of poor quality silage, which can reduce DMI and animal performance (Chen et al., 1994). Oxygen is the ultimate enemy of the ensiling process because most molds and yeasts require oxygen for growth. Thus, any management practice that helps exclude oxygen from the silage mass is helpful. Such practices include harvesting at proper moisture concentrations, rapid filling, adequate packing, and covering with plastic. This exclusion of oxygen from the silage promotes rapid fermentation by anaerobic hetero and

homofermentative bacteria, thereby reducing the growth of yeasts and molds during the initial stages of fermentation.

Bacilli

Bacilli belong to the family *Bacillaceae* and they grow aerobically, however, some are facultative anaerobes (Pahlow et al., 2003). They can ferment a wide range of carbohydrates to acetate, lactate, butyrate, ethanol, 2,3-butanediol, and glycerol (Woolford, 1977). The main habitat for *Bacillus* spp. is the soil (Claus and Berkeley, 1986) and they are scarce in fresh forage (Lindgren et al., 1985a) except when contaminated with soil. The number of *Bacillus* spores in cattle manure can range from 10^4 up to 10^6 cfu g^{-1} FM (Ostling and Lindgren, 1991; Rammer et al., 1994). Therefore, fertilizing with manure can increase the number of *Bacillus* spores on the crop and consequently increase the number of *Bacillus* spores in silage. *Bacillus* spp. may be able to initiate aerobic deterioration of silage, but more commonly, they contribute later to deterioration after it is initiated by yeasts (Lindgren et al., 1985b, Spoelstra et al., 1985).

Moisture

Moisture stimulates the growth of LAB but excess moisture encourages the growth of undesirable bacteria like clostridia. High moisture concentrations are also undesirable because compaction in the silo may produce seepage (effluent) losses, which contains high levels of soluble nutrients (Muck and Kung, 2007). Moisture concentration of crops affects O_2 supply to spoilage microorganisms and the temperature rise produced from the heat generated by aerobic microbial growth (Muck et al., 2003). Moisture concentration also affects the amount of heat needed to raise silage temperature (i.e. the specific heat of the silage). The specific heat of water is 4.19

J /g H_2O ºC^{-1}, whereas that of forage DM is only 1.89 J /g DM ºC^{-1} (McDonald et al., 1991). Consequently, the heat released by microbial respiration of a given amount of DM in silage containing 50% water produces more than a threefold higher increase in temperature than the same loss in silage containing 80% water (Muck et al., 2003). This partly explains why drier silages are more susceptible to heating and deterioration.

Packing Density

Achieving high density in a silo reduces DM and nutrient losses resulting from plant respiration during filling or aerobic microbial growth at filling, storage, or feed-out (Muck et al., 2003). Porosity, which is determine by density and moisture concentration, determines the rate at which air moves into the silo and subsequently the amount of spoilage which occurs during storage and feed-out (Holmes and Muck, 1999). Inadequately packed silos or bags often have passageways for air to move back rapidly from the open face. This exposes more of the silage to oxygen soon after opening, increasing the opportunity for spoilage and heating (Muck and Kung, 2007). In bunker silos, density is determined primarily by the weight and number of passes used by the tractor packing the silage into the bunker. In tower silos, density is increased by gravitational self compaction and therefore depends on depth of silage and silo factors such as height, diameter, and wall material (Muck et al., 2003).

Silage Additives

Additives are added to silages to direct the fermentation and prevent adverse effects of plant respiration, plant proteolytic activity, clostridial activity, and aerobic microbial growth on silage quality. Various biological, chemical, and nutrient additives are used for these purposes.

Chemical Additives

Organic acids

Several European studies have reported that formic acid treatment reduces NPN formation in direct-cut grass silages and improves their nutritive value for ruminants (McDonald et al., 1991). Formic acid-treated alfalfa silage had lower pH and NH_3 concentrations than untreated controls and greater water-insoluble N (Lancaster and Brunswick, 1977; Barry et al., 1978). Nagel and Broderick (1992) showed that formic acid treatment of wilted alfalfa silage was more effective at reducing pH and had the lowest concentrations of lactic and acetic acid compared to untreated silage and formaldehyde treated silage. In addition, formic acid treatment decreased NPN formation and substantially improved N utilization when fed to lactating dairy cows. Rooke et al. (1988) reported that formic acid-treated ryegrass (*Lolium perenne*) silage had lower concentrations of ammonia-N and acetic and lactic acids and higher concentrations of WSC and ethanol than the control silage. Furthermore, when the silage was fed to sheep, N retention and silage DMI were improved by formic acid treated-silage. Despite its efficacy, application of formic acid to forage is challenging because of its corrosive nature. Therefore, formic acid is not a widely used additive for silage preservation.

Propionic acid-based preservatives have also been used to improve the aerobic stability of corn silages (Leaver, 1975) because of the antifungal nature of the acid (Britt et al., 1975; Leaver, 1975). These have been largely replaced by buffered propionic acid additives (Kung et al., 2000a) due to the corrosive nature of propionic acid. Kung et al. (1998) reported substantial improvements (120 – 160 h) in the aerobic stability of corn silage treated with relatively low concentrations (0.1 to 0.2% of fresh forage weight)

of several additives that contained buffered propionic acid as their primary active ingredient. Kung et al. (2000a) examined the effect of various levels (0.1, 0.2, and 0.3%) of ammonia-N or a buffered propionic acid preservative on the fermentation of whole-plant corn. They reported that high levels (0.3%) of ammonia-N or buffered propionic acid markedly improved aerobic stability of corn silage compared with the control; however, the number of yeasts and concentrations of lactate and acetate were similar in all treatments. Ammoniated silages had greater pH than buffered propionic acid and untreated silages, but propionic acid concentration was only increased in silages treated with buffered propionic acid.

Ammonia

Moderate concentrations of ammonia (0.1 to 0.3%) have increased concentrations of lactic and acetic acids (Muck and Kung, 1997), decreased proteolysis (Huber et al., 1979, 1980), improved DM recovery (Goering and Waldo, 1980), and improved the aerobic stability of corn silage (Britt and Huber, 1975). Many researchers have suggested that addition of ammonia to silage improves aerobic stability because of its fungicidal properties (Depasquale and Montville, 1990). Kung et al. (2000a) studied the effect of ammonia hydroxide application at the rate of 0.3 % of fresh forage on corn silage. They reported that the number of enterobacteria were less than 2.00 log cfu/g after 4 d of ensiling in control silages but remained high (>5 log cfu/g) in ammoniated silages through 6 d of ensiling. The persistence of enterobacteria and subsequent growth of heterofermentative LAB contributed to higher concentrations of acetic acid in ammoniated silages during their study. The number of yeasts in control silages increased rapidly whereas that in ammoniated silages remained low for 144 h after aeration. Alii et al. (1983) reported that numbers of yeasts decreased immediately in

high moisture corn after treatment with ammonia (1% of fresh forage). However, ammoniation is not widely used because it is corrosive on machinery, toxic at high doses, and hazardous.

Biological Additives

Homolactic Bacterial Inoculants

The earliest known use of LAB cultures is by French workers on sugar beet pulp at the beginning of this century (Wilkinson and Phipps, 1979). Inoculants containing selected strains of LAB have been developed to reduce dependence of the ensiling process on epiphytic LAB and on chemical additives. The principal function of these homofermentative inoculants is to ensure a rapid and efficient fermentation of WSC into lactic acid, a rapid decrease in pH and improved silage conservation with minimal fermentation losses (Weinberg et al., 1993a). Fermentation of glucose by homolactic LAB via the Embden-Meyerhof-Parnas pathway is desirable because it yields high recovery of energy (99.3%) and DM (100%) and converts all of the glucose into lactic acid, a strong acid (McDonald et al., 1991). Inoculants containing homolactic LAB ($\geq 10^5$) can improve the fermentation quality of silages and reduce DM losses if the herbage contains sufficient fermentable carbohydrates and the inoculant bacteria dominate the epiphytic population of LAB (Spoelstra,1991). One of the most widely used LAB in inoculants is *Lactobacillus plantarum*. This bacterium is a facultative heterofermentative organism, which usually ferments hexoses homofermentatively into lactic acid but, when WSC concentrations are low, heterofermentative metabolism into lactic acid, carbon dioxide and ethanol (or acetic acid) occurs (Holzer et al., 2003).

Muck (1993) reviewed several studies from 1985 to 1992 and reported that inoculant application improved intake and body-weight gain in about 25% of studies,

milk production was increased in 40% of the studies and feed efficiency was improved in 45% of the studies. Adesogan et al. (2009) reviewed 38 studies from 1989 to 2009 and reported that inoculation with homofermentative bacteria increased digestibility of DM, organic matter (OMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) in 36, 47, 31, and 36% of studies, respectively. *Lactobacillus plantarum* was the most frequently used (82%) bacteria in the inoculants in these studies.

Ely et al. (1981) applied *L. plantarum* to alfalfa, corn, sorghum, and wheat (*Triticum aestivum*) silages and reported that treated silages had a lower pH, higher lactic acid concentration, and greater recovery of DM, and CP than untreated silages. Combinations of *L. plantarum* and other homolactic LAB have also generally improved the fermentation. Driehuis et al. (1997) applied *L. plantarum* and *Enterococcus faecium* to ryegrass silage ensiled in lab and farm scale silos. After 180 d ensilage, treated silages had lower pH, DM loss, and ammonia-N concentration, and higher lactic acid concentrations compared with control silages.

A summary of 14 lactation studies conducted in North America and Europe revealed that applying *L. plantarum* MTD1 to a variety of crops (grass, corn, and alfalfa) across a wide spectrum of DM contents (15 to 46% DM) resulted in a 4.6% increase in milk yield (Moran and Owen, 1994). Similarly, Moran and Owen (1995) summarized 19 comparisons among untreated and *L. plantarum* MTD1 - treated silages fed to beef cattle. Cattle fed the treated silage ate 7.5% more DM and gained 11.1% more weight.

Kung et al. (1993) examined the effect of applying microbial inoculants to corn silage on the performance of lactating dairy cows in two experiments. Cows were assigned to the following treatments: Control, *L. plantarum*, or *L. plantarum* and

Streptococcus faecium. Production of 3.5% FCM was greatest by cows fed silage treated with *L. plantarum* in both experiments. Muck (1993) reviewed several studies from 1985 to 1992 and reported that inoculant application increased milk production in 40% of the studies and feed efficiency was improved in 45% of the studies. Dry matter digestibility and animal performance were measured in 31 trials and reported that animal performance was improved in 9 of the 16 trials where the inoculant improved dry matter digestibility. When digestibility was not affected by the inoculant, only 2 of 15 trials had positive animal performance effects. The author suggested that increases in digestibility might be the key factor in explaining why inoculants improve animal performance.

These studies cited above show that in many cases, homofermentative LAB have improved the fermentation of forages and enhanced animal performance but their effect on aerobic stability is less consistent. Weinberg et al. (1993b) noted that addition of a LAB inoculant containing *E. faecium* to wheat silage impaired the aerobic stability of the silage. Addition of *L. acidophilus* had no positive effect on the fermentation of corn, sorghum, and wheat silages (Burghardi et al., 1980) and contributed to aerobic deterioration of these silages after opening (Moon et al., 1980). Muck and Kung, (1997) reported that inoculants, which were mainly homolactic improved aerobic stability in only 30% of 39 studies conducted between 1990 and 1995. The poor aerobic stability response to inoculation with homolactic LAB is attributable to the fact that the lactate they produce has a relatively low antifungal effect and is a growth substrate for certain spoilage yeasts (Pahlow et al., 2003). Consequently, inoculants containing other types of bacteria that inhibit spoilage have been developed.

Heterolactic Bacterial Inoculants

Lactobacillus buchneri

Heterolactic bacteria ferment glucose into a mixture of ethanol, CO_2, and acetic, butyric and lactic acids via a less efficient pathway than homolactic bacteria. However, the antifungal nature of the acetic and butyric acids they produce has led to their use for improving aerobic stability of silages. Several studies have demonstrated that treatment of forages and feeds at ensiling with *L. buchneri* improves aerobic stability (Taylor and Kung, 2002; Nishino et al., 2004; Kung et al. 2007). Muck (2004) indicated that inoculation with *Lactobacillus buchneri* 40788 at the rate of 4×10^5 cfu/g is one of the most consistent methods for improving aerobic stability of corn silage. Doubling the application rate has not been more effective (Huisden et al., 2009), but increasing the rate from 1×10^5 to 1×10^6 cfu/g of fresh material has (Ranjit and Kung, 2000). When applied at the rate of 10^6 cfu/g of fresh material *L. buchneri* increased aerobic stability of high moisture corn, corn silage, alfalfa silage, and small-grain silages relative to untreated controls (Muck, 2001; Taylor and Kung, 2002; Kleinschmit et al., 2005). Although the precise mechanism has not yet been determined, the beneficial impact of *L. buchneri* appears to be related to the production of acetic acid, which inhibits the growth of yeasts (Driehuis, et al., 1999a). The acetic acid can be further metabolized into propionic acid, a strong fungicidal agent by epiphytic *L. diolivorans* (Krooneman et al., 2002). Further inhibition of aerobic spoilage may be due to bacteriocins produced by *L. buchneri.* Yildirim (2001) and Yildirim et al. (2002) reported that buchnericin, a bacteriocin produced by *L. buchneri* had wide ranging bacteriocidal activity against spoilage bacteria such as *Listeria monocytogenes* and *Bacillus cereus*.

Yeast and mold counts of *L. buchneri* inoculated silages are generally lower at feedout and do not increase as rapidly as in untreated controls exposed to air (Kung and Ranjit, 2001). Driehuis et al. (1999a) showed that yeasts are affected in two ways by *L. buchneri*. Firstly, the survival of yeasts during the anaerobic ensilage phase is reduced, and secondly, growth of yeasts during exposure of silage to the air is inhibited.

As a result, the temperatures of silages inoculated with *L. buchneri* tend to remain similar to ambient temperature for several days, even in warm weather (Taylor et al., 2000). Inoculation with *L. buchneri* is most beneficial under circumstances where problems with aerobic instability are expected. Corn silage, small-grain silages, and high-moisture corn are more susceptible to spoilage once exposed to air than legume silages and therefore the former often respond more favorably to inoculation with *L. buchneri* (Muck, 1996).

Few studies have compared the spoilage inhibiting effects of *L. buchneri* to those of other heterolactic bacteria. Danner et al. (2003) evaluated the effect of homofermentative (*L. rhamnosus*, *Pediococcus pentosaceous*, and *L. plantarum*) or heterofermentative (*L. brevis* or *L. buchneri*) bacteria on the aerobic stability of silages. The silage inoculated with *L. buchneri* had the greatest aerobic stability and acetic acid concentration.

Few studies have examined effects of inoculating silage with heterolactic bacteria on animal performance. Taylor et al. (2002) reported that treating barley (*Hordeum vulgare*) silage with *L. buchneri* 40788 increased aerobic stability and reduced counts of yeasts and molds. However, when cows were fed the treated versus untreated silage, DMI, milk production, and milk composition were not affected by treatment. Driehuis et

al. (1999b) reported that treating corn silage with *L. buchneri* improved aerobic stability but DMI and milk production were similar to the untreated silage. Similarly, treating high moisture shelled corn with *L. buchneri* improved aerobic stability but milk production was decreased when cows were fed the TMR containing treated versus untreated high moisture shelled corn (Kendall et al., 2002). Therefore, *L. buchneri* application has improved aerobic stability of various forages but has not increased milk yield except in the following study. Treating alfalfa silage with *L. buchneri* 40788 improved aerobic stability and milk production was increased in cows fed treated versus untreated silages even though DMI and milk composition were unaffected (Kung et al., 2003a). Why milk production was improved in the latter study but not the others is unclear. More research evaluating effects of inoculating forages with *L. buchneri* on the performance of dairy cows are warranted.

Propionibacteria

Propionic acid bacteria can ferment glucose and lactate to acetate and propionic acid, which are two of the three main antifungal acids in silages (Moon et al., 1983). Inoculation with Propionibacteria has improved aerobic stability in some studies (Higginbotham et al., 1996; Bolsen et al., 1996; Dawson et al., 1998; Filya et al., 2006) but not others (Weinberg et al., 1995; Higginbotham et al., 1998; Pedroso et al., 2010). Dawson et al. (1998) evaluated the effect of Propionibacterium acidipropionici inoculation on fermentation and aerobic stability of corn silage. Inoculation improved aerobic stability, increased acetic and propionic acid concentrations, reduced pH, and reduced numbers of yeasts and molds. In contrast, Higginbotham et al. (1998) examined the effect of microbial inoculants containing Propionibacteria either alone or with *Pediococcus cerevisiae* and *P. cerevisiae* plus *L. plantarum*. The inoculants did

not affect the fermentation of corn silages but inoculated silages tended to heat more slowly and took a slightly longer time to reach their peak temperature than control silages. The authors concluded that the microbial inoculants evaluated did not prevent detrimental changes in quality when corn silage was exposed to air. The inconsistent effect of propionibacteria on aerobic stability is largely attributable to the slow growth of the organism at acidic pH (Weinberg et al., 1995; Higginbotham et al., 1998). Nevertheless, Propionibacteria is the main alternative antifungal bacterium to *L. buchneri* in commercially available inoculants in the US and studies comparing both types of antifungal inoculants are needed.

Inoculants containing Homolactic and Heterolactic Bacteria

Homofermentative LAB inoculants sometimes impair the aerobic stability of mature cereal silages by increasing the number of yeasts and fungi (Kennedy, 1990; Weinberg et al., 1993a). Adding a heterofementative LAB that produces fungicidal VFA has improved the stability of such silages (Weinberg et al. 1999). The combination potentially reduces DM losses that often occur when heterofermentative bacteria alone are applied to forages. Driehuis et al. (2001) reported that adding *Lactobacillus buchneri* alone or in combination with *P. pentosaceus* and *L. plantarum* improved aerobic stability of grass silage, reduced yeast and mold counts, and led to lower DM loss compared to the untreated silage. In addition, Weinberg et al. (1999) reported that wheat and sorghum silages were more stable when treated with *L. buchneri* alone or with *L. buchneri* and *L. plantarum*, whereas treatment with *L. plantarum* alone resulted in unstable silages. Filya (2003) reported that combination of *L. buchneri* and *L plantarum* reduced ammonia-N concentrations and fermentation losses in silages compared with *L. buchneri* alone. Huisden et al. (2009) reported that applying two

mixtures of homofermentative and heterofermentative bacteria resulted in similar reductions in lactate to acetate ratio, fewer yeasts and more stable silages than Control silages. Adesogan et al. (2008) reviewed 8 studies in which combination inoculants (mainly *L. buchneri* and either *L. plantarum* or *P. pediococcus*) were added to 16 forages and reported that DM recovery, lactate, acetate, and aerobic stability were improved 63, 25, 69, and 94% of the time, whereas pH and yeast counts were reduced 25 and 88 % of the time, respectively. Therefore, most of the studies demonstrate that combination inoculants have improved the aerobic stability of several types of forages but a few exceptions exist. Adesogan et al. (2004) showed that treatment with *P. pentosaceus* and *L. buchneri* improved the fermentation of bermudagrass (*Cynodon dactylon*) silage but did not improve aerobic stability because control silages had high concentrations of fungicidal butyric acid. Kleinschmit and Kung (2006b) examined the effect of adding *Lactobacillus buchneri* 40788 (4×10^5 cfu/g) and *P. pentosaceus* R1094 (1×10^5 cfu/g) to corn forage ensiled for different durations ranging from 14 to 361 d. Inoculation increased acetate and 1,2 propanediol concentrations in silages ensiled for 56 to 361 d, decreased yeast counts in silages ensiled for 42, 56, 70 and 282 days, and increased aerobic stability in silages ensiled for 14, 56, and 361 d. The reason for the inconsistent improvement of aerobic stability was not known. Most of studies on combination inoculants have been done in mini silos; consequently, animal responses to treatment of forages with such inoculants are unknown.

Enzymes

During the last two decades, enzymes have been evaluated as possible silage additives particularly those containing cellulolytic, hemicellulolytic and amylolytic activities. Their primary functions are to break down cell-wall constituents and grain

starch in the crop to improve silage fermentation and animal utilization (Muck and Bolsen, 1991). Enzyme addition has increased lactic acid production (Rauramaa et al., 1987; Jaakkola et al., 1991; Kung et al., 1991), reduced silage pH (Rauramaa et al., 1987; Kung et al., 1991; Stokes, 1992) reduced concentrations of acetic acid (Jaakkola et al., 1991; Stokes, 1992) and either increased (Dean et al., 2005a), reduced (Jaakkola et al., 1991) or did not affect (Stokes, 1992) aerobic stability. Enzyme treatment at ensiling has reduced NDF and ADF concentrations and increased NDF digestibility (Kung et al., 1991; Sheperd and Kung, 1996; Dean et al., 2005a) but did not increase milk production when enzyme-treated and untreated forages were fed (Shepherd and Kung, 1996; Dean et al., 2005b).

Using enzymes as silage additives have had positive effects on silage and ruminal fermentation in many studies but has not consistently improved aerobic stability or animal performance. Future research should examine if combinations of enzymes and spoilage-inhibiting bacteria can improve aerobic stability and animal performance.

Nutrient Additives

Crops with high moisture concentrations are prone to poor clostridial fermentation and loss of nutrients from excessive production of effluent. Effluent seepage into waterways can also cause eutrophication and death of marine species. Therefore, various sources of carbohydrates like cereal straw and sugar-beet pulp have been ensiled with forages as absorbents to increase the DM concentration and reduce the production of effluent (Kung et al., 2003b). Hameleers et al. (1999) studied the effect of ensiling mixtures of molassed sugar-beet pulp and forage corn harvested at four stages of maturity (154 to 235 g kg^{-1} DM). Addition of molassed-sugar beet pulp did not improve the fermentation but decreased effluent production except in the least mature

silage. Ferris and Mayne (1994) reported that ensiling ryegrass with sugar-beet pulp improved silage fermentation, reduced DM losses, and reduced effluent losses from the silo. When the sugar-beet pulp-treated ryegrass silage was fed to dairy cows at three levels, DMI and milk fat and protein yields increased with increasing level of beet pulp inclusion in the diet. Moseley and Ramanathan (1989) ensiled a mixed crop of perennial ryegrass and white clover with nothing (Control), molassed-sugar beet pulp, rolled barley or formic acid and fed them to sheep. Dry matter intake was increased by barley and sugar beet pulp treatments compared to the control and digestibility of DM and OM were greater when treated silages were fed. Rodrigues et al. (2005) studied the effect of ensiling elephantgrass with different levels of citrus pulp. Silage DM concentration, in vitro DMD, and WSC increased linearly with increasing level of citrus pulp but ammonia N and NDF concentrations decreased. These studies reveal that nutrient additives can reduce effluent production and improve nutritive value of silages and enhance animal performance. However, addition of nutrient additives at ensiling is a difficult practice because of the increased labor required and the need for uniform distribution of the adsorbent throughout the silage mass (Kung et al., 2003b).

Summary

Most of the published responses to additive treatment of ensiled forages indicate that they can improve silage fermentation and quality. However, their effects on animal performance have been variable. The use of chemicals as additives for forage conservation and improvement has been reduced due to their corrosiveness. Bacterial inoculants and enzymes are more appealing because they are benign and natural. Some of these have improved silage conservation, nutritive value, and aerobic stability

but others have not. Effects of such biological additives on animal performance have been inconsistent.

The objectives of this series of experiments were as follows:

- To determine the effect of dietary addition of a fibrolytic enzyme preparation on the performance of dairy cows fed low or high concentrate diets.
- To compare simulated preingestive and ruminal effects of a fibrolytic enzyme on various dietary components or diets and to determine which dietary components were most affected by enzyme action.
- To examine the effect of applying bacterial inoculants containing heterofermentative bacteria alone or homofermentative and heterofermentative bacteria on the fermentation, quality, and aerobic stability of corn silage.
- To examine the effect of applying bacterial inoculants containing heterofermentative bacteria alone or homofermentative and heterofermentative bacteria on the performance of lactating dairy cows.

CHAPTER 3
EFFECT OF FIBROLYTIC ENZYME APPLICATION TO DIETS DIFFERING IN CONCENTRATE PROPORTION ON THE PERFORMANCE OF LACTATING DAIRY CATTLE

Introduction

Fibrolytic enzymes have been added to forages and ruminant diets to improve forage quality and animal performance. Enzyme application to forages immediately before in vitro incubation improved digestion of DM and NDF (Feng et al., 1996) suggesting that applying fibrolytic enzymes at or just prior to feeding may enhance digestion of forages by cattle. However, results of fibrolytic enzyme application to ruminant diets have been equivocal. Some studies showed that enzyme supplementation increased milk production by dairy cattle (Rode et al., 1999; Kung et al., 2000b; Yang et al., 2000) but others did not (Beauchemin et al., 2000; Kung et al. 2002; Sutton et al. 2003). Adding fibrolytic enzymes has increased digestibility in some studies (Rode et al., 1999, Yang et al., 2000) but not others (Lewis et al. 1999). These discrepancies may be due to differences in enzyme activity, application rate and composition, stage of lactation of dairy cows, mode and time of enzyme delivery, ruminal activity and stability of direct fed enzymes, enzyme-feed specificity and the portion of the diet to which enzymes are applied (Beauchemin et al., 2004; Adesogan, 2005). A further problem may lie with use of enzymes that are incapable of sufficiently hydrolyzing forage cell walls to give repeatable results across a range of forages or diets. Most previous studies evaluated enzymes containing xylanase and cellulase enzymes, which cannot hydrolyze ester linkages between hydroxycinnamic acids and sugars in plant cell walls. Ferulic acid esterases can hydrolyze such linkages (Williamson et al., 1998; Krueger et al., 2008a), thus releasing ferulic acid and opening

the remainder of the polysaccharides to further hydrolytic attack by other enzymes (Yu et al., 2005). Various studies have demonstrated that esterase enzymes play an important role in ruminal cell wall degradation (Nsereko et al., 2000b; Dhiman et al., 2002; Krueger and Adesogan, 2008) particularly following pretreatment or co incubation with xylanases (Yu et al., 2002; Bartolome et al., 1995, 1997a, b). Most of such studies have been done in vitro. Consequently, little is known about effects of enzymes containing cellulase, xylanase and esterase activities on the performance of dairy cows.

The objective of this study was to determine the effect of dietary addition of a fibrolytic enzyme preparation on the performance of dairy cows fed low or high concentrate diets. The esterase - xylanase enzyme preparation tested had increased the NDF digestibility of alfalfa hay and corn silage by over 25% and it was more effective than using either enzyme alone (Eun and Beauchemin, 2006). We hypothesized that 1) enzyme application to the high concentrate diet would improve milk production, whereas application to the low concentrate diet would improve the efficiency of milk production; 2) enzyme application to the low concentrate diet would result in as much milk production as that from the high concentrate diet that was not treated with the enzyme.

Materials and Methods

Cows, Treatments and Design

Care of animals used in this study followed protocols approved by the University of Florida Institutional Animal Care and Use Committee. Sixty lactating Holstein cows in early lactation (22 $\pm$ 3 DIM) were grouped by milk production and parity (multiparous and primiparous) and randomly assigned to 1 of 4 treatments arranged in a 2 x 2 factorial design. Cows were fed either a high or low concentrate TMR that was treated

with or without a prototype fibrolytic enzyme from Dyadic International Inc., Jupiter, FL. The following treatments were investigated: 1) low concentrate untreated diet (LC; 67:33 roughage to concentrate ratio); 2) LC plus enzyme (LCE); 3) high concentrate untreated diet (HC; 52:48 roughage to concentrate ratio); and 4) HC plus enzyme (HCE). The dietary proportions of concentrate were designed to approximate those typical of diets fed to dairy cows in the US (HC) and Western Europe (LC). Prior to the daily a.m. and p.m. feedings, the enzyme solution was diluted in water (1:3 ratio v/v) and sprayed at a rate of 3.4 mg of enzyme/g DM on the TMR during mixing for 5 min in a 250-kg capacity Calan data ranger (American Calan Inc., Northwood, NH). Separate data rangers were used to mix the enzyme-treated and untreated TMR. The roughage portion of the diets contained approximately 20% alfalfa hay, 72% corn silage, and 8% cottonseed hulls (DM basis). The experimental diets were formulated to be isonitrogenous (Table 3-1) and to meet NRC (2001) guidelines for a dairy cow in early lactation producing 40 kg of milk. The experiment was conducted at the University of Florida Dairy Unit and it lasted for 63 d per cow. Cows were adapted to diets for the first 14 d and the last 49 d of the trial were used for sample collection. Cows were housed in a free-stall, open-sided barn fitted with continuously operated fans and misters for cooling and with Calan gates (American Calan Inc., Northwood, NH) for individual feed intake. Free-stalls were bedded with sand and maintained daily. Sufficient free-stalls were available to provide at least 1 free-stall per cow. Water and diets were available in ad libitum amounts.

Enzyme Activity

Xylanase activity (EC 3.2.1.8) measured using the assay of Bailey et al. (1992) was 3633 U/ml with oat spelt xylan as the substrate (Sigma Chemical Company, St.

Louis, MO, USA). Endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) activities measured with the Wood and Bhat (1988) assays were 880 U/ml and 70 U/ml using 1% (wt/vol) carboxymethylcellulose or microcrystalline cellulose (Avicel) as substrates, respectively (Sigma; Chemical Company, St. Louis, MO, USA). One unit of activity of the respective enzymes was defined as micromoles of xylose or glucose released per min per g Assay conditions were 39^{o}C and pH 6.0 to reflect ruminal conditions. Aryl and carboxyl esterase activities measured using the methods described by Gonzalez et al. (2006) were 0.38 µmol/ min mg^{-1} and 0.28 µmol/min mg^{-1} using *p*-Nitrophenyl and naphthyl esters as substrates.

Sampling and Analysis

Cows were fed individually (0700 and 1200 h) and milked (1100 and 2300 h) twice daily. Milk samples from a.m. and p.m. milkings were collected twice every week and analyzed by Southeast Dairy labs (Belleview, FL) for fat, protein and SCC using a Bentley 2000 Near Infrared Reflectance Spectrophotometer (Bentley Instruments Inc., Chaska, MN). Somatic cell scores were generated as described by Norman et al. (2000) for statistical analysis of SCC. Body weight was measured at the beginning and end of the trial. Body condition score was measured on a 1 to 5 scale (Wildman et al., 1982).

Weight of TMR and orts were recorded daily for each cow. Duplicate samples of corn silage, alfalfa hay, and concentrates were collected weekly and composited monthly. Subsamples from each month were dried at 60ºC for 48 h in a forced air oven, ground to pass the 1-mm screen of a Wiley mill (A. H. Thomas, Philadelphia, PA), and analyzed for DM (105^{o}C for 16 h) and ash (512^{o}C for 8 h). Concentrations of NDF and ADF were measured using the method of Van Soest et al. (1991) in an Ankom 200

Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α-amylase and sulfite were used in the NDF assay. Nitrogen was determined by rapid combustion using a Macro elemental N analyzer (Vario MAX CN, model ID 25.00-5003; Elementar, Hanau, Germany) and CP was calculated as N x 6.25. Blood samples were collected weekly by coccygeal venipuncture into vacutainer tubes containing sodium heparin anticoagulant (Fisher Scientific, Pittsburgh, PA). The blood was centrifuged at 2,500 × *g* for 20 min at 4°C and the plasma was frozen (-20°C). A Technicon Autoanalyzer (Technicon Instruments Corp., Chauncey, NY) was used to measure plasma glucose (Bran and Luebbe Industrial Method 339–19; Gochman and Schmitz, 1972) and BUN (Bran and Luebbe Industrial Method 339–01; Marsh et al., 1965).

In vivo apparent digestibility was calculated using chromic oxide as a marker (Schneider and Flatt, 1975). Chromic oxide powder (Fisher Scientific, Fairlawn, NJ) was weighed (10 g/dose) into gelatin capsules (Jorgensen Lab. Loveland, CO) and dosed twice daily (0700 and 1900h) with a balling gun for 10 consecutive d between d 45 and 60. Fecal samples were collected at 0630 and 1830 h during the last 5 d of dosing. Feces were dried to a constant weight at 55°C in a forced-air oven, ground to pass through a 1-mm screen in a Wiley mill and a composite sample was made from all 10 fecal samples per cow. Chromium concentration in the feces was determined using a Perkin Elmer 5000 atomic absorption spectrometer (Perkin Elmer, Wellesley, MA), according to the procedure described by Williams et al. (1962). Apparent digestibility of CP, ADF and NDF were calculated.

In Situ Ruminal Degradability

Four ruminally-cannulated, nonlactating Holstein cows were used to determine dietary treatment effects on ruminal fermentation and in situ ruminal DM degradation.

This aspect of the experiment had a 4 x 4 Latin square design with four, 18-d periods. The first 14 d of each period were used to adapt cows to a new diet. On d 15, ruminal fluid samples were taken at 0, 2, 4, 6, 8 and 10 h after cows were fed in the morning. Approximately 100 ml of ruminal fluid was immediately filtered through 2 layers of cheesecloth, and pH was measured with an electrode (Accumet pH meter, model HP-71, Fisher Scientific, Pittsburgh, PA). Subsequently, the ruminal fluid was acidified with 2 ml of 50% H_2SO_4 per 100 ml of ruminal fluid and centrifuged at 11,500 × *g* for 20 min. The supernatant was frozen (-20^{o}C) and subsequently analyzed for concentrations of lactate and VFA using the method of Muck and Dickerson (1988) and a High Performance Liquid Chromatograph system (Hitachi®, FL 7485, Tokyo, Japan) coupled to a UV Detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, NJ) set at 210 nm. The column was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) column with 0.015M sulfuric acid mobile phase and a flow rate of 0.7 ml/min at 45°C. Ammonia-N was determined with a Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA) and an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification.

The ruminal degradation kinetics of the experimental diets was measured *in situ* on d 16 to18. The TMR samples were dried (60ºC for 48 h), ground to pass a 4-mm screen with a Wiley mill and weighed (5 g as is) into preweighed polyester bags (10 × 20 cm, pore size 50 µm, Bar Diamond Inc., Parma, ID). Samples were incubated for 0, 4, 8, 16, 24, and 48 h in quadruplicate in each of 2 ruminally fistulated cows fed that same diet in each period. All bags were inserted in the rumen simultaneously. After each incubation period, bags were removed and rinsed with cool water and frozen (-

20°C). Subsequently, all bags were thawed, washed using a rinse cycle without soap in a Kenmore Series 70 washing machine, dried for 48 h at 60ºC and weighed. The exponential model of McDonald (1981) was fitted to the DM degradation data with the NONLIN procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) to generate DM degradation parameters. The model is of the following form:

$$P = A + B\ (1 - e^{-c\ (t-lag)})$$

where P is the DM degradation (%) at time t, A is the Y axis intercept representing the wash fraction (%), B is the potentially degradable fraction (%), and c is the rate of degradation of the B fraction (%/h).

Statistical Analysis

A completely randomized design with a 2 (control vs. enzyme) x 2 (LC vs. HC) factorial arrangement of treatments was used to analyze the data. The MIXED procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) and a model containing treatment, week (repeated measure), parity, all interactions of these terms, and cow nested in treatment x pairty as the random effect was used to analyze the data from measurements that were repeated weekly. Milk production during the first 21 d of lactation was used as a covariate for analyzing milk production data. A similar model excluding the week effect and its interactions was used to analyze digestibility coefficients. The model for analyzing ruminal fermentation data included treatment, period, time (repeated measure), treatment x time and cow (random effect). Least square means are reported and results are presented on a DM basis. Contrast statements were used to determine the effects of enzyme application (control vs. enzyme), concentrate level (LC vs. HC), the interaction (enzyme treatment vs. concentrate level), and to compare the LCE and HC diets. The slice command of SAS

was used to detect differences among means at specific time points and the PDIFF statement of SAS also was used to compare enzyme effects within each concentrate level. Treatment significance was declared at $P \leq 0.05$ and tendencies were declared at $P > 0.05 < 0.10$.

Results and Discussion

Enzyme application did not affect ($P = 0.14$) DMI but tended to reduce intake of CP ($P = 0.06$; 4.5 vs. 4.2 kg/d), NDF ($P = 0.07$; 7.9 vs. 7.4 kg/d), and ADF ($P = 0.07$; 5.3 vs. 4.9 kg/d). Enzyme application increased ($P \leq 0.02$) digestibility of DM (69.8 vs. 72.6%), CP (69.2 vs. 73.3%), ADF (50.4 vs. 54.8%), and NDF (53.7 vs. 55.4%) (Table 3-2). Therefore, adding the enzyme increased nutrient digestion and release. Krueger et al. (2008b) reported that fibrolytic enzyme application to bermudagrass hay increased DMI and DM and NDF digestibility by beef steers. Yang et al. (1999) also reported that supplementation with a xylanase cellulase enzyme increased NDF digestibility of an alfalfa - barley silage-based TMR by dairy cows. They attributed the response to increased nutrient release and solubility of DM and NDF, which likely increased glycocalyx production, and thereby enhanced adhesion of fibrolytic bacteria to substrates. The enzyme-mediated digestibility increases in this study may have occurred via increased microbial colonization of feed particles or by direct cell wall hydrolysis (Cheng et al., 1995; Yang et al., 1999).

Increasing the concentrate amount increased DMI (21.5 vs. 24.8 kg/d, $P = 0.003$; Figure 3-1) and CP intake (4.1 vs. 4.6 kg/d, $P = 0.001$) and tended to increase ADF intake ($P = 0.09$; 4.9 vs. 5.2 kg/d). Likewise, increasing the concentrate amount increased digestibility of DM (69.9 vs. 72.6%; $P = 0.02$) and CP (70.0 vs. 72.6%, $P = 0.01$) and tended ($P = 0.06$) to increase ADF digestibility (50.1 vs. 54.4%). Cows fed

HC instead of LCE had greater ($P \leq 0.01$) DMI (25.7 vs. 20.8 kg/d), CP intake (4.8 vs. 3.9 kg/d), and ADF intake (5.4 vs. 4.7 kg/d), and tended ($P = 0.06$) to have greater NDF intake (8.0 vs. 7.3). Increasing the amount of concentrate in diets has caused linear increases in DMI in other studies (Llamas-Lamas and Combs, 1991; Weiss and Shockey, 1991). Eun and Beauchemin (2005) also reported that cows receiving high concentrate diets had greater DMI and greater DM digestibility than those receiving low concentrate diets. Substitution of concentrates for forages generally increases intake and digestibility because concentrates cause less ruminal fill, have a lower lignified polysaccharide concentration, a faster passage rate, and require less rumination than forages.

Enzyme application effects on plasma glucose concentration differed with the amount of concentrates fed (Enzyme x concentrate interaction, $P = 0.006$) but the differences were small. (The PUN concentration was not affected by enzyme application, but feeding more concentrates increased plasma urea N (13.5 vs. 15.0 mg/dl, $P = 0.001$; Table 3-3), reflecting the increased CP digestibility caused by enzyme treatment.

Enzyme application did not statistically increase milk yield though numerical increases occurred at both concentrate amounts (Table 3-4, Figure 3-2). In some studies, dietary addition of fibrolytic enzymes increased milk production (Lewis et al., 1999; Rode et al., 1999; Yang et al., 2000) but no milk response was reported in others (Beauchemin et al., 2000; Elwakeel et al., 2007). Enzyme application effects on milk fat concentration seemed to differ with the amount of concentrates (Enzyme x concentrate interaction, $P = 0.04$) but milk fat concentrations did not differ ($P > 0.05$) at the same

concentrate feeding amount. Enzyme application increased the efficiency of milk production (P = 0.04; 1.44 vs. 1.60 kg milk/kg DMI) because of increased nutrient supply for milk production. That enzyme application increased feed efficiency across concentrate amounts partly confirms our first hypothesis that enzyme application would increase and the efficiency of milk production by cows.

Increasing the dietary concentrate amount increased milk yield (P = 0.02; 32.2 vs. 34.7 kg/d) and milk protein yield (P = 0.02; 0.89 vs. 0.99 kg/d). These responses are attributable to the increase in concentration of dietary NFC, particularly starch as the dietary concentrate level increased. Starch digestion in the rumen increases propionic acid production, which is a major gluconeogenic precursor in ruminants (Chen et al., 1994). Starch digestion also increases uptake of amino acids and other nutrients by the mammary gland, improving milk and milk protein yields (Theurer et al., 1999).

Despite the lower DMI of cows fed the LCE diet relative to the HC diet, milk production from both diets did not differ (P = 0.43). Consequently the efficiency of milk production was greater (P = 0.01; 1.69 vs. 1.42 kg milk/kg DMI) in cows fed the LCE diet than those fed the HC diet. These results confirm our second hypothesis by indicating that enzyme application to the low concentrate diet made it as effective as the untreated high concentrate diet at stimulating milk production. Similarly, Schingoethe et al. (1999) reported that cows fed low-concentrate diets (45% of TMR) treated with a fibrolytic enzyme increased milk production to the same extent as that achieved when cows were fed a high concentrate (55%) diet without the enzyme. Such results imply that by adding such enzymes, higher forage diets can be fed without jeopardizing milk production, and this could lower the cost of the diet and reduce the risk of acidosis.

One concern about fibrolytic enzyme application to diets containing considerable amounts of concentrates is that fermentation of starch in such diets may indirectly depress ruminal pH and predispose cows to ruminal acidosis (Eun and Beauchemin, 2005). Hristov et al. (1996a) also noted that applying fibrolytic enzymes to feed may decrease both chewing time and saliva output and thereby increase the risk of acidosis. Enzyme application did not affect (P = 0.92; Table 3-5) the ruminal pH of cows in this study, but caused lower values 4 h after feeding (Figure 3-3). Therefore, cows fed enzyme supplemented, high concentrate diets should be monitored closely for signs of subclinical ruminal acidosis.

Enzyme application effects on ruminal ammonia concentration differed with time (P = 0.02; Figure 3-4). Enzyme application increased total VFA concentration (P = 0.03; 114.5 vs. 125.7 *mM*; Figure 3-5). In contrast, several studies reported that enzyme application had no effect on total VFA concentration (Kung et al., 2002; Sutton et al., 2003; Eun and Beauchemin, 2005). Enzyme application also tended (P = 0.10) to reduce acetate molar proportion (58.9 vs. 56.0; Figure 3-6) but did not affect propionate molar proportion or ammonia-N (P > 0.05; Figure 3-7 and 3-4; respectively). Consequently, enzyme application reduced the acetate to propionate ratio (P = 0.04; 3.09 vs. 2.87; Figure 3-8), implying improved efficiency of energy utilization in the rumen.

Increasing the amount of concentrate supplementation produced the expected pH decrease (P < 0.001; 6.31 vs. 6.06), as did feeding the HC diet instead of the LCE diet (P = 0.006; 6.36 vs. 6.10). None of the average pH values was low enough to indicate acute ruminal acidosis, which is characterized by ruminal pH below 5 (Nagaraja and

Town, 1990). However, ruminal pH of cows fed the HC diet reached the subclinical acidosis threshold (pH <5.8; Ghorbani et al., 2002) 10 h after feeding. Feeding more concentrates reduced (P = 0.004) molar acetate proportion (59.8 vs. 55.1), and increased (P < 0.05) concentration of total VFA (114.3 vs. 125.9 *mM*) and lactate (5.5 vs. 7.0 *mM*) and molar proportion of propionate (18.8 vs. 19.9). Consequently, acetate to propionate ratio was reduced (P = 0.003) by feeding more concentrates, implying increased ruminal energetic efficiency. Eun and Beauchemin (2005) also reported that feeding more concentrates had no effect on molar acetate proportion, increased total VFA concentration and molar propionate proportion; and decreased acetate propionate ratio.

Enzyme application did not affect the ruminal degradation of the diets (Table 3-6). Similarly, Krueger et al. (2008b) reported no effect of enzyme application on the rate or extent of degradation of bermudagrass hay. However, feeding more concentrates increased the immediately soluble dietary fraction (P = 0.03; 43.3 vs. 33.6 %), and tended to decrease the potentially degradable fraction (P = 0.06; 45.0 vs. 53.9 %) likely reflecting differences in particle size distribution and NFC and fiber concentration between low and high concentrate diets.

Conclusions

This study shows that application of the cellulase xylanase esterase enzyme preparation did not affect DMI or milk production but increased nutrient digestibility, total VFA concentration, and the efficiency of milk production. Furthermore, enzyme application to the low concentrate diet resulted in as much milk production and DM digestibility as from cows fed the untreated high concentrate diet. These beneficial

effects of enzyme application were primarily attributable to improved nutrient digestion and improved ruminal energy utilization.

Table 3-1. Ingredient and chemical composition of the untreated experimental diets

	Low concentrate	High concentrate
Ingredient composition, % DM		
Corn silage	49.20	37.00
Alfalfa hay	13.50	10.00
Cottonseed hulls	4.63	5.00
Corn meal	7.38	17.89
Citrus pulp	2.00	5.01
Whole cottonseed	1.81	4.84
SoyPlus[1]	7.90	5.93
Soybean meal	2.49	6.01
Cottonseed meal	7.80	5.10
Mineral mix[2]	3.26	3.25
Roughage : concentrate ratio	67:33	52:48
Chemical composition		
DM, %	64.9	72.2
Crude protein, % DM	18.6	18.5
Neutral detergent fiber, % DM	34.3	31.0
Acid detergent fiber, % DM	22.4	20.9
Hemicellulose[3], % of DM	11.9	10.1
Non-fiber carbohydrates[4], % of DM	36.5	39.7

[1] West Central Soy, Ralston, IA.
[2] Mineral mix contained 26.4% CP, 10.2% Ca, 8.6% Na, 5.1% K, 3.1% Mg, 1.5% S, 0.9% P, 2231 mg/kg of Mn, 1698 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 31 mg/kg of Co, 26 mg/kg of I, 7.9 mg/kg of Se, 147,756 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis).
[3] Calculated as NDF – ADF;
[4] Calculated as NFC = 100 – (CP+ash+fat+NDF); NRC (2001) values used for fat concentrations of ingredients.

Table 3-2. Effect of adding a fibrolytic enzyme[1] (Enz) to diets containing low (33%) or high (48%) amounts of concentrate (conc) on intake and digestibility by dairy cows.

	Low Conc		High Conc		SEM	Contrast *P values*			
Item	No Enz (LC)	Enz (LCE)	No Enz (HC)	Enz (HCE)		Enz	Conc	LCE vs. HC	Enz x Conc
Intakes, kg/d									
DM	22.1	20.8	25.7	23.8	1.0	0.14	0.003	0.001	0.79
CP	4.2	3.9	4.8	4.4	0.16	0.06	0.001	0.003	0.80
NDF	7.8	7.3	8.0	7.4	0.29	0.07	0.43	0.06	0.87
ADF	5.1	4.7	5.4	5.0	0.19	0.07	0.09	0.01	0.84
Digestibility, %									
DM	68.5	71.2	71.1	74.0	1.1	0.02	0.02	0.91	0.90
CP	68.4[a]	71.5[b]	70.0[x]	75.1[y]	1.0	0.002	0.01	0.29	0.31
NDF	52.1	55.2	53.3	57.5	1.7	0.03	0.30	0.43	0.75
ADF	48.6	52.9	52.1	56.6	1.8	0.02	0.06	0.75	0.97

[a. b, x, y] At the same level of concentrate supplementation, means in the same row with different superscripts differed ($P < 0.05$).

[1] Produced by Dyadic International Inc., Jupiter, FL

Table 3-3. Effect of adding a fibrolytic enzyme[1] (Enz) to diets containing low (33%) or high (48%) amounts of concentrate (conc) on body weight, average daily gain (ADG), body condition score (BCS), and plasma metabolites by dairy cows.

	Low Conc		High Conc		SEM	Contrast *P values*			
Item	No Enz (LC)	Enz (LCE)	No Enz (HC)	Enz (HCE)		Enz	Conc	LCE vs. HC	Enz x Conc
Growth performance									
Initial wt, kg	574	568	579	558	16.3	0.41	0.89	0.61	0.63
Final wt, kg	593	593	609	584	14.7	0.39	0.82	0.43	0.39
ADG, kg/d	0.32	0.42	0.57	0.38	0.14	0.89	0.44	0.63	0.51
Initial BCS	2.55	2.43	2.46	2.48	0.11	0.29	0.71	0.61	0.19
Final BCS	2.42	2.39	2.40	2.45	0.03	0.81	0.60	0.84	0.20
Plasma metabolites									
Glucose, mg/dl	69.3[a]	71.3[b]	71.9	70.6	0.6	0.53	0.10	0.46	0.006
Urea N, mg/dl	13.4	13.6	14.5	15.4	0.4	0.13	0.001	0.06	0.31

[1] Produced by Dyadic International Inc., Jupiter, FL

Table 3-4. Effect of adding a fibrolytic enzyme[1] (Enz) to diets containing low (33%) or high (48%) amounts of concentrate (conc) on milk production, composition, and efficiency of feed utilization by dairy cows.

	Low Conc		High Conc		SEM	Contrast *P values*			
	No Enz	Enz	No Enz	Enz		Enz	Conc	LCE vs. HC	Enz x Conc
Item	(LC)	(LCE)	(HC)	(HCE)					
Milk yield, kg/d	31.9	32.5	33.6	35.8	1.1	0.21	0.02	0.43	0.47
FCM 3.5%, kg/d	32.5	33.9	35.9	36.2	1.2	0.49	0.03	0.26	0.64
FCM 4%, kg/d	29.9	31.3	33.1	33.4	1.1	0.49	0.03	0.26	0.64
Milk protein, %	2.79	2.85	2.91	2.89	0.05	0.71	0.15	0.43	0.43
Milk fat, %	3.60	3.80	3.90	3.56	0.13	0.62	0.80	0.59	0.04
Milk protein, kg/d	0.88	0.90	0.97	1.00	0.04	0.59	0.02	0.19	0.96
Milk fat, kg/d	1.14	1.20	1.32	1.25	0.08	0.93	0.13	0.24	0.41
SCC x 1000/ml	412	158	417	465	144	0.88	0.68	0.85	0.47
Feed efficiency (kg milk/kg DMI)	1.46[a]	1.69[b]	1.42	1.51	0.08	0.04	0.16	0.01	0.35

[a, b] At the same level of concentrate supplementation, means in the same row with different superscripts differed ($P < 0.05$).

[1] Produced by Dyadic International Inc., Jupiter, FL

Table 3-5. Effect of adding a fibrolytic enzyme[1] (Enz) to diets containing low (33%) or high (48%) amounts of concentrate (conc) on ruminal fermentation characteristics by dairy cows.

	Low Conc		High Conc		SEM	Contrast P values			
Item	No Enz (LC)	Enz (LCE)	No Enz (HC)	Enz (HCE)		Enz	Conc	LCE vs. HC	Enz x Conc
Ruminal pH	6.26	6.36	6.10	6.01	0.08	0.92	<0.001	0.006	0.14
Ammonia-N, mg/dl	12.4	13.0	12.7	13.0	0.78	0.61	0.87	0.81	0.87
Lactate, *mM*	6.57	4.41	5.14	8.94	0.55	0.15	0.01	0.37	<0.001
Total VFA, *mM*	110.4	118.2	118.6	133.1	4.8	0.03	0.01	0.96	0.49
Individual, molar proportion									
Acetate, (A)	60.1	59.5	57.7	52.4	1.73	0.10	0.004	0.42	0.18
Propionate, (P)	18.6	18.9	19.6	20.2	0.34	0.20	0.005	0.16	0.79
Iso-butyrate	2.70	2.64	2.90	2.49	0.22	0.30	0.92	0.39	0.43
Butyrate	11.7	12.0	12.3	12.7	1.40	0.81	0.67	0.90	0.97
A:P ratio	3.23	3.15	2.94	2.59	0.07	0.04	0.003	0.29	0.37

[1] Produced by Dyadic International Inc., Jupiter, FL

Table 3-6. Effect of adding a fibrolytic enzyme[1] (Enz) to diets containing low (33%) or high (48%) amounts of concentrate (conc) on the in situ ruminal degradability of dry matter by dairy cows.

	Low Conc		High Conc		SEM	Contrast P values			
	No Enz	Enz	No Enz	Enz		Enz	Conc	LCE vs. HC	Enz x Conc
Item	(LC)	(LCE)	(HC)	(HCE)					
A, %	31.6	35.5	44.9	41.7	3.38	0.929	0.03	0.11	0.34
B, %	56.3	51.5	43.3	46.6	3.82	0.85	0.06	0.20	0.33
A+B, %	87.9	87.0	88.2	88.3	1.17	0.77	0.54	0.55	0.66
C, per h	0.05	0.06	0.05	0.05	0.01	0.60	0.31	0.30	0.31
L, h	0.0	0.39	1.16	0.63	0.42	0.88	0.15	0.27	0.33

[1] Produced by Dyadic International Inc., Jupiter, FL; A = washout fraction; B = potentially degradable fraction
A+B = total degradable fraction; C = fractional degradation rate of the B fraction; L = lag phase

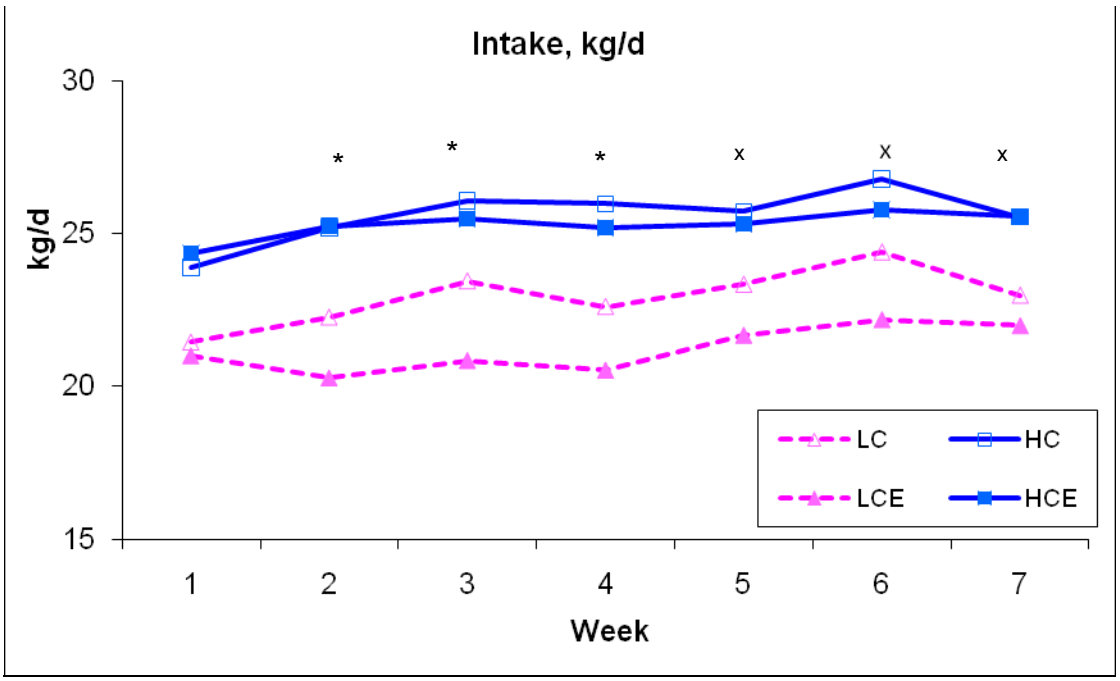

Figure 3-1. Effect of adding a fibrolytic enzyme application to dairy cow diets with low or high levels of concentrates on dry matter intake. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * and x indicate differences (* = $P < 0.05$; x = $P < 0.10$) between treatments at that week. Treatment x week, $P = 0.41$, SEM = 1.23.

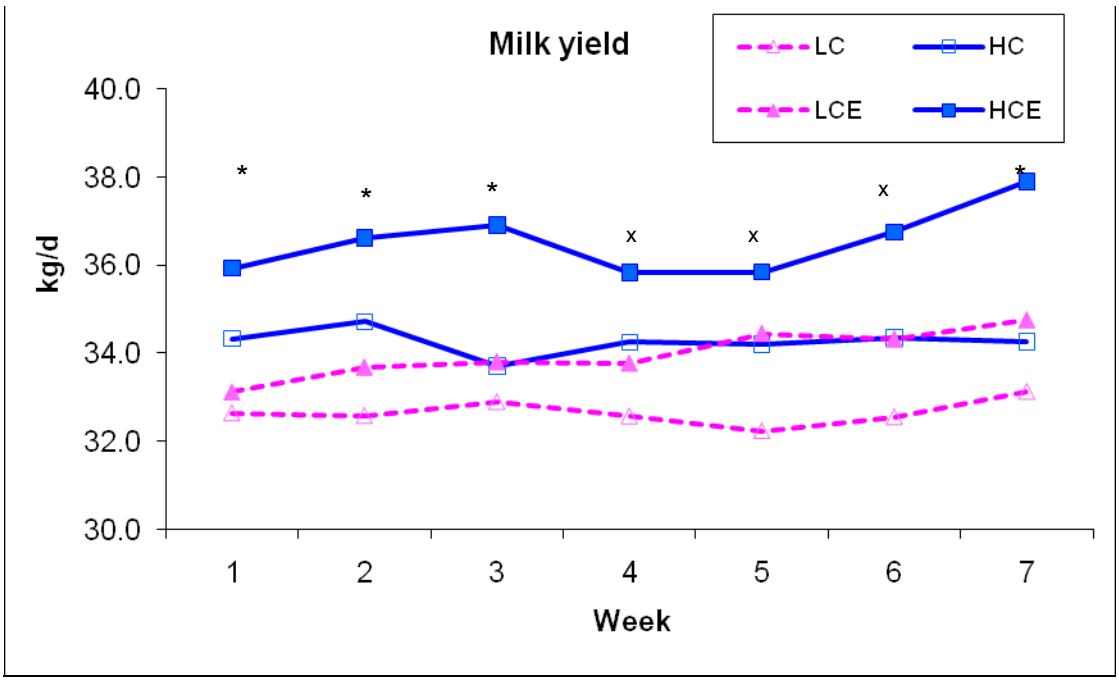

Figure 3-2. Effect of esterase-xylanase enzyme application to dairy cow diets with low or high levels of concentrates on milk yield. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * and ^{x} indicate differences (* = $P < 0.05$; ^{x} = $P < 0.10$) between treatments at that week. Treatment x week, $P = 0.57$, SEM = 1.18.

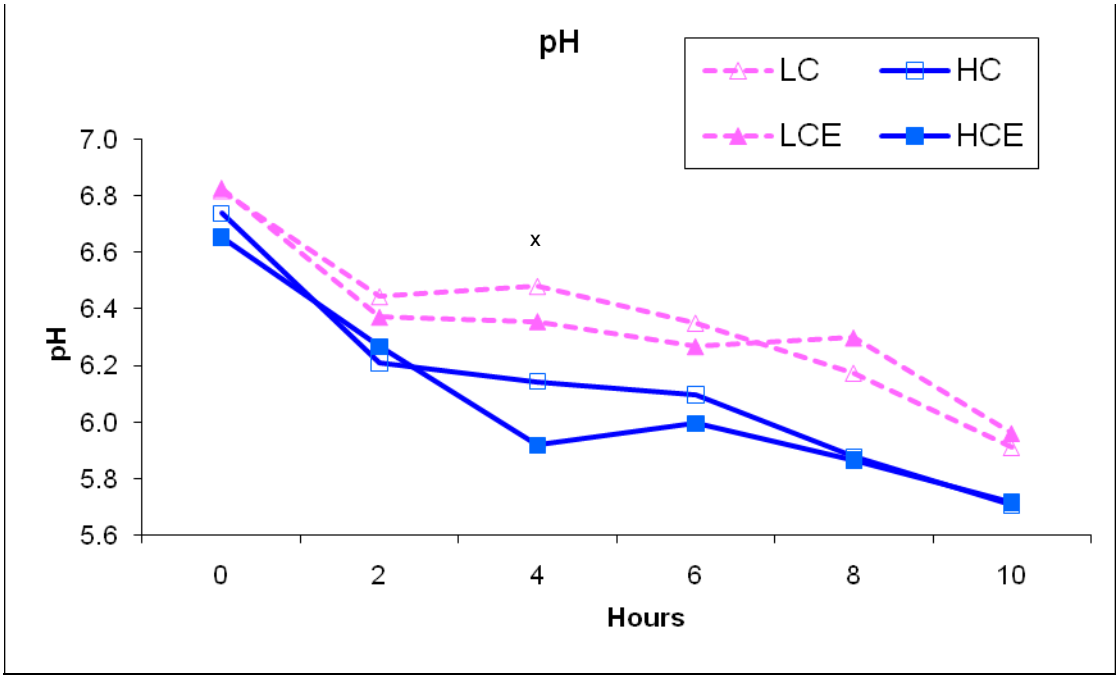

Figure 3-3. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal pH. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. [x] indicates a difference ($P < 0.10$) between treatments at that time. Treatment x time, $P = 1.00$, SEM = 0.16.

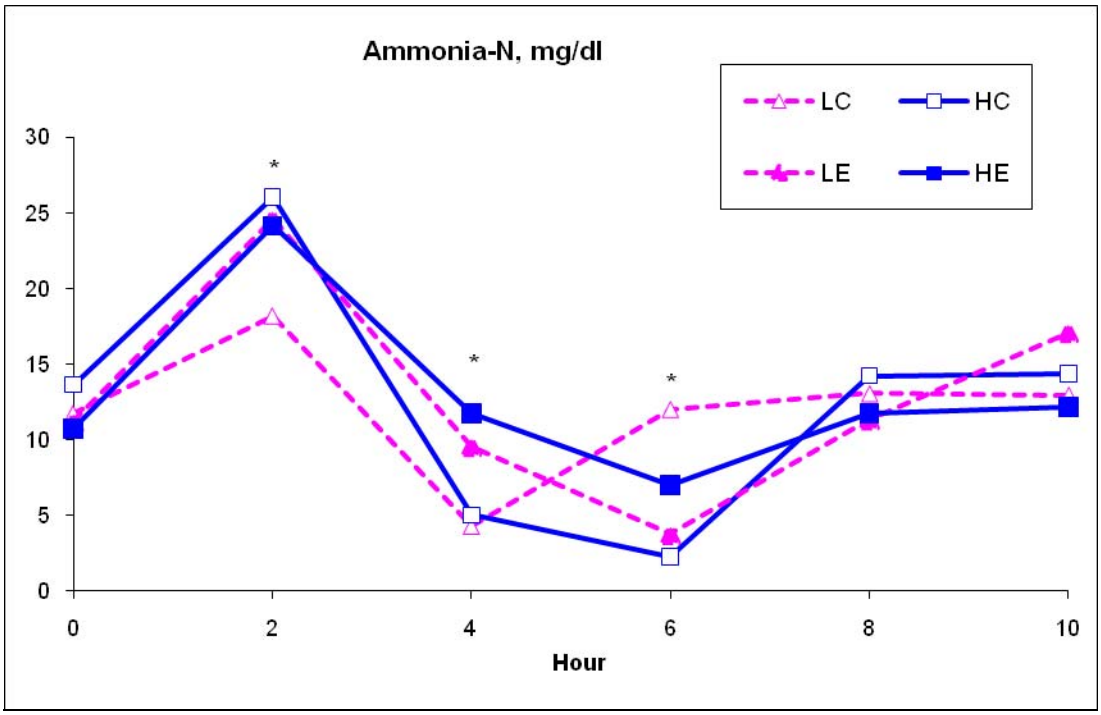

Figure 3-4. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal ammonia nitrogen concentration. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * indicates a difference ($P < 0.05$) between treatments at that time. Treatment x time, $P = 0.002$, SEM = 1.79.

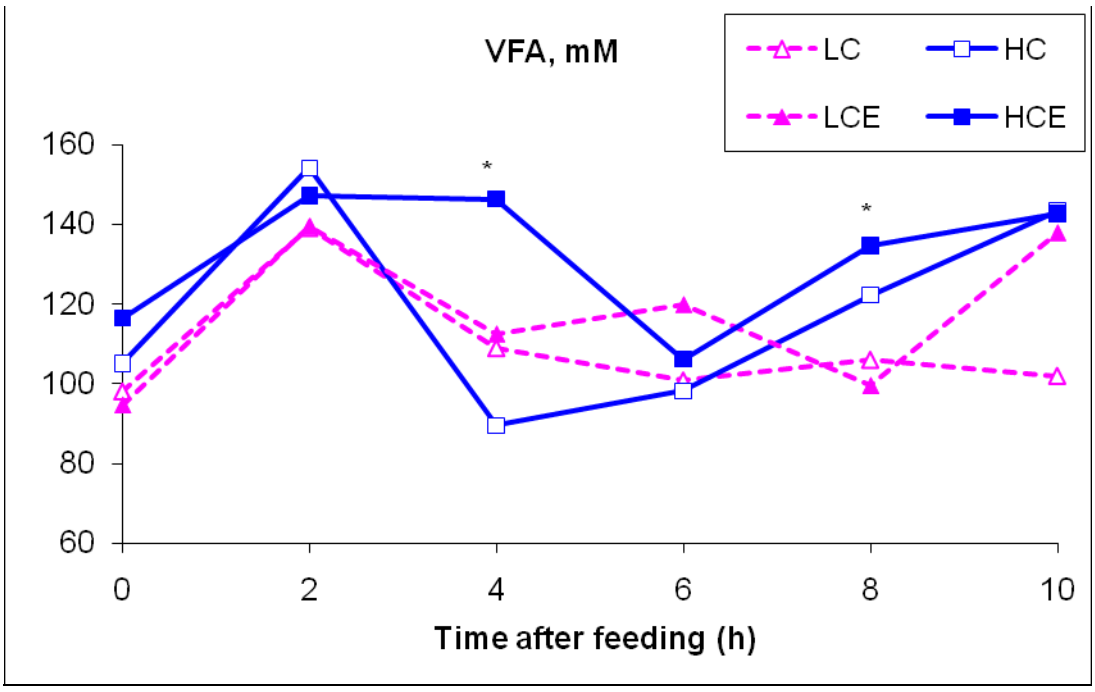

Figure 3-5. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal total VFA concentration. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * indicates a difference ($P < 0.05$) between treatments at that time. Treatment x time, $P = 0.18$, SEM = 6.24.

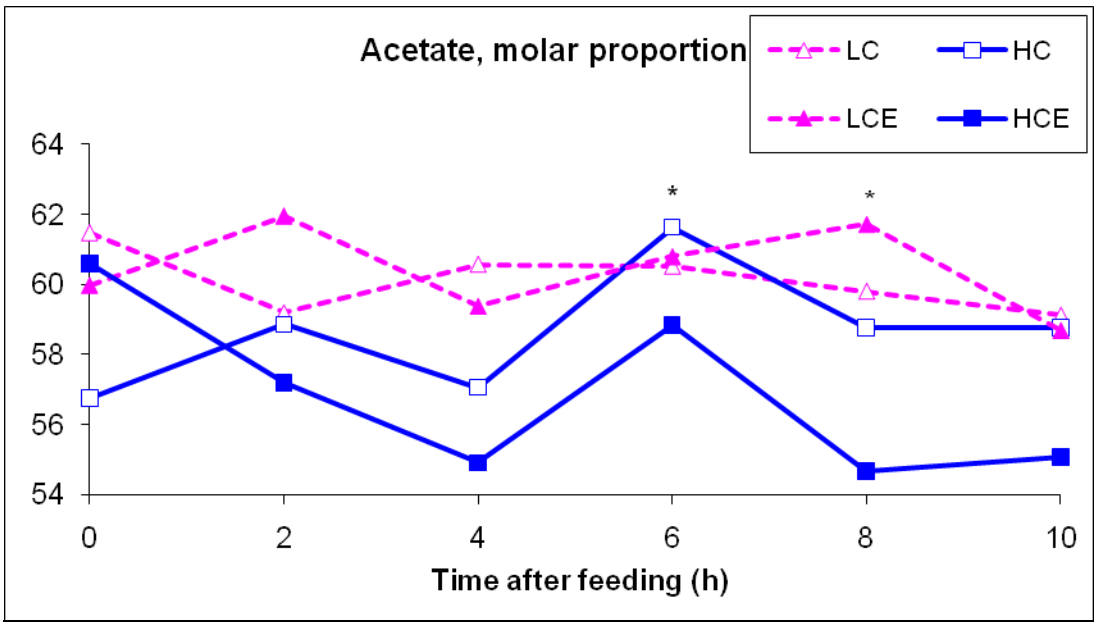

Figure 3-6. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal acetate proportion.LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * indicates a difference ($P < 0.05$) between treatments at that time. Treatment x time, $P = 0.52$, SEM = 4.19.

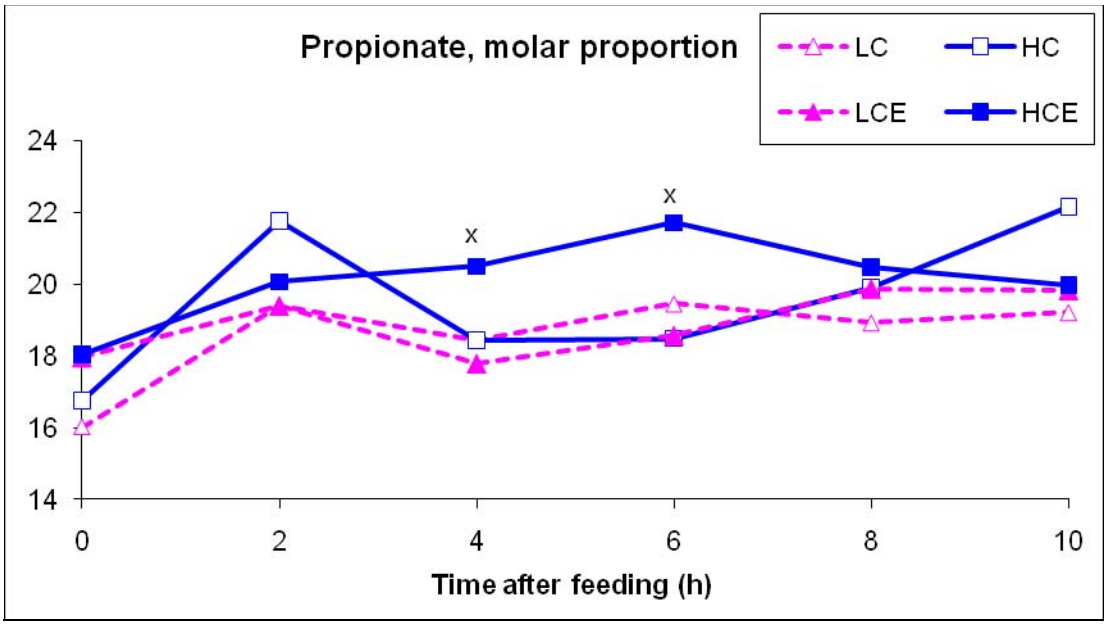

Figure 3-7. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal propionate proportion. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. [x] indicates a difference ($P < 0.10$) between treatments at that time. Treatment x time, $P = 0.30$, SEM = 0.81.

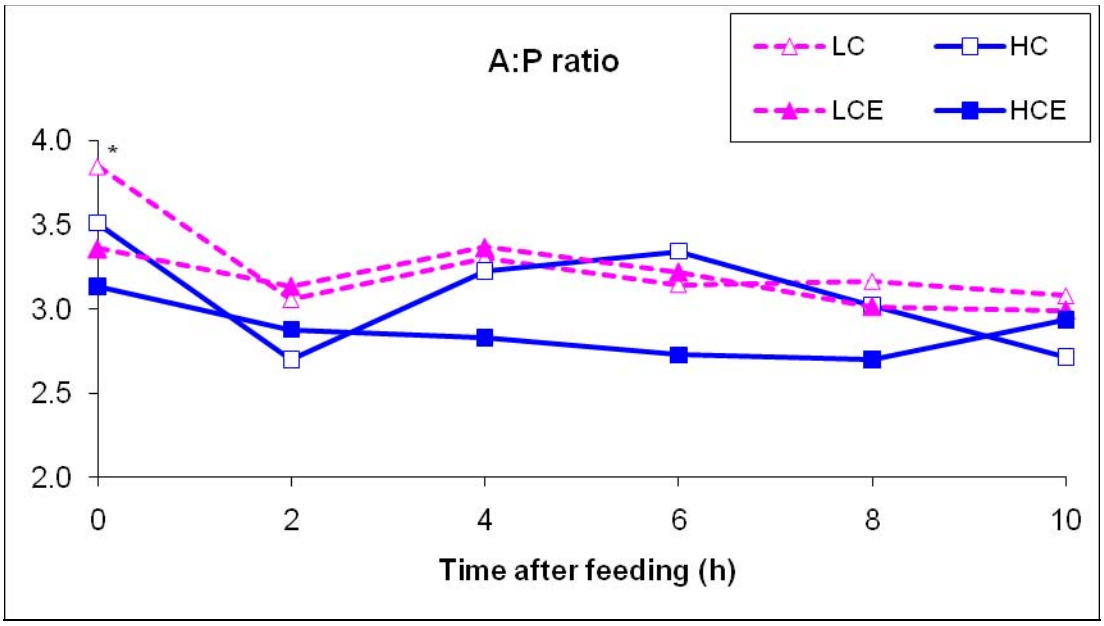

Figure 3-8. Effect of adding a fibrolytic enzyme to dairy cow diets with low or high levels of concentrates on ruminal acetate to propionate ratio. LC = Low concentrate, LCE = Low concentrate + enzyme, HC = High concentrate, HCE = High concentrate + enzyme. * indicates a difference ($P < 0.05$) between treatments at that time. Treatment x time, $P = 0.63$, SEM = 0.17.

CHAPTER 4
EFFECTS OF FIBROLYTIC ENZYME APPLICATION ON THE DIGESTIBILITY OF CORN SILAGE, ALFALFA HAY, TWO CONCENTRATES, AND COMPLETE DIETS UNDER SIMULATED RUMINAL AND PRERUMINAL CONDITIONS

Introduction

Exogenous fibrolytic enzyme products usually are applied to the diet before feeding because a preingestive enzyme-feed interaction is necessary for any significant beneficial effects on ruminal digestion to be realized (Lewis et al., 1996; McAllister et al., 1999). The close enzyme - feed association may enable partial hydrolysis of NDF and ADF (Krause et al., 1998) that causes a release of reducing sugars (Hristov et al., 1996a; Krueger and Adesogan, 2008). The hydrolysis also may modify plant cell wall structure (Feng et al., 1996) and thereby increase fiber digestion. Another reason for applying enzymes to feed prior to ingestion is to enhance binding of the enzyme to the feed, thereby increasing the resistance of the enzymes to ruminal proteolysis (Fontes et al., 1995).

Despite the evidence for preingestive enzyme action, most attention has focused on ruminal enzyme effects. For instance, Beauchemin et al. (2003) stated that most of the improvements in forage quality resulting from exogenous fibrolytic enzyme application are attributable to ruminal effects. Yang et al. (1999) reported that most of the effect of exogenous enzymes in diets of lactating dairy cows occurred in the rumen. Yet infusion of exogenous enzymes into the rumen is not considered an effective method of enzyme application (Lewis et al., 1996; Treacher et al., 1997; Hristov et al., 2000; Sutton et al., 2003) because the proper binding of enzymes to feeds that optimizes efficacy occurs in the preingestive phase. Enzyme application to forage several months prior to feeding increased in vivo digestibility of DM and NDF in beef

steers, whereas enzyme application at feeding had no effect (Krueger et al., 2008b), suggesting that an important preingestive effect occurred in the former treatment. Studies directly comparing preingestive versus ruminal enzyme action are needed to quantify the relative importance of these sites of enzyme action and to inform guidelines about the timing of enzyme application to feeds.

Eun and Beauchemin (2007) reported that application of a certain mixture of developmental fibrolytic enzymes (FF and FT, Dyadic International, Jupiter, FL) improved the in vitro NDF digestibility of both forages by over 20%. Arriola et al. (2007; Chapter 3) reported that adding the same enzyme mixture to diets of lactating dairy cows did not affect DMI but tended to increase milk production and therefore increased the efficiency of milk production. These effects were attributed to improved nutrient digestion and ruminal energy utilization. However, the dietary component most affected by the enzyme was not known. Studies aimed at determining the ideal dietary component to which enzymes should be added have not produced consistent results. Milk production by dairy cows has been improved by adding enzymes to the forage (Lewis et al., 1999; Kung et al., 2000b) or concentrate (Rode et al., 1999; Yang et al., 2000) portions of diets or to the TMR (Beauchemin et al., 1999) in some studies but not others (Phipps et al., 2000; Sutton et al., 2003; Elwakeel et al., 2007). Most of such studies have not involved concurrent enzyme addition to different dietary fractions therefore little is known about the target dietary fraction to which enzymes should be added.

The objectives of this study were to compare simulated preingestive and ruminal effects of a fibrolytic enzyme on various the forages, concentrates, and TMR used by

Arriola et al. (2007) and to determine which dietary components were most affected by enzyme action. The hypotheses were that the effect of the enzyme in the simulated rumen would exceed the simulated preingestive effect and the enzyme would exert the greatest effect on the forage component of the diet.

Materials and Methods

Dietary Substrates

Samples of the alfalfa hay (AH), corn silage (CS), low corn (20%; LC) and high corn (34%; HC) - concentrates, and low - (33%) and high -(48%) concentrate TMR (TMRL and TMRH) from the study of Arriola et al. (2007) were used as substrates in this study. The substrates were dried in a forced-air oven at 60ºC for 48 h, ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) and stored in air-tight plastic bags.

Enzyme Activity

The enzyme mixture was the same developmental enzyme from Dyadic International, Inc. (Jupiter, FL) used by Arriola et al. (2007; Chapter 3). Xylanase activity (EC 3.2.1.8) measured using the assay of Bailey et al. (1992) was 3633 U/ml with oat spelt xylan as the substrate (Sigma Chemical Company, St. Louis, MO, USA). Endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) activities measured with the Wood and Bhat (1988) assays were 880 U/ml and 70 U/ml using 1 % (wt/vol) of carboxymethylcellulose or 1% (wt/vol) solution of microcrystalline cellulose (Avicel, Sigmacell 50; Sigma; Chemical Company, St. Louis, MO, USA) as substrates, respectively. One unit of activity of the respective enzymes is defined as micromoles of xylose or glucose released per min. per g. Assay conditions were 39ºC and pH 6.0. Aryl and carboxyl esterase activities measured using the Gonzalez et al. (2006) assay were

0.38 μmol min^{-1} mg^{-1} and 0.28 μmol min^{-1} mg^{-1} using α-naphthyl and p-nitrophenyl acetate esters as substrates, respectively.

In vitro Fermentation and Degradability

Four experiments were conducted to test the experimental hypotheses. In Experiment 1, the objective was to determine effects of enzyme addition on chemical composition. Exactly 6.8 mg of the fibrolytic enzyme was diluted in 4 ml of citrate phosphate buffer (pH 6.0) and applied to 2 g of each substrate in beakers in triplicate at 23ºC. In addition, beakers containing each substrate and the buffer alone were also prepared and regarded as respective controls. After 8 h, beakers were dried overnight at 60ºC in a forced draft oven. Concentrations of NDF and ADF were analyzed sequentially using the method of Van Soest et al. (1991) in an ANKOM® Fiber Analyzer (ANKOM Technologies, Macedon, NY, USA). Heat stable α-amylase and sodium sulfite were used in the NDF analysis. Hemicellulose was calculated by difference from NDF and ADF. Water-soluble carbohydrate concentration was determined after water extraction, acid hydrolysis, and a colorimetric reaction with potassium ferricyanide (Hall et al., 1999).

In Experiment 2, the objective was to determine effects of enzyme addition on substrate disappearance in a buffer. Exactly 3.4 mg of the fibrolytic enzyme was diluted in 2 ml of citrate phosphate buffer (pH 6.0) and applied to 1 g of each substrate within 250 ml culture bottles in triplicate. After 24 h of incubation at 23ºC in the buffer alone or the buffer - enzyme solution, contents of culture bottles were filtered through a Whatman No. 541 filter paper (#09851D, Fisher Scientific, Pittsburgh, PA, USA), and residues were dried at 60ºC overnight to determine DM disappearance in the buffer in each of 2 runs.

In Experiment 3 the objective was to compare the DM and NDF digestion of enzyme-treated substrates under simulated preingestive and ruminal conditions. Substrates were incubated for 24 h in the buffered - enzyme solution as in Experiment 2 in each of 3 runs. Subsequently, 40 ml of either distilled water (W) or buffered-ruminal fluid (RF) was added to each culture bottle, and the suspensions were incubated at 39ºC for 24 h. Incubation in W was used to simulate preingestive effects of the enzyme, whereas incubation in RF simulated ruminal effects. The RF was collected by aspiration from a non-lactating non-pregnant Holstein cow, filtered through two layers of cheesecloth, immediately transported in a pre-warmed thermos flask to the laboratory, and mixed (1:2 ratio) under a CO_2 stream with an anaerobic culture medium of Tilley and Terry (1963). Ruminal fluid donor cows were fed bermudagrass hay ad libitum supplemented with 750 g of soybean meal daily. The culture medium had been warmed to 39ºC to avoid exposing microorganisms to cold shock. After the 24 h incubation in RF or W, the contents of the culture bottles were filtered through a Whatman No. 541 filter paper and residues were dried at 60ºC overnight to determine DM digestibility. The DM and NDF concentrations of substrates and digestion residues were measured as described previously and DM (DMD) and NDF digestibility (NDFD) were calculated. Even though substrate disappearance in the buffer or W did not involve enzymic or microbial digestion, DMD and NDFD will be used to describe solubility in these media for simplicity.

In Experiment 4 the objective was to determine effects of enzyme addition on DM and NDF digestibility under simulated preingestive and ruminal conditions. Substrates were prepared and incubated in the buffered enzyme solution or in the buffer alone for

24 h. Subsequently, 40 ml of either W or RF was added to each culture bottle, and the suspensions were incubated at 39ºC for 24 h. Dry matter and NDF digestibility were determined as in Experiment 3. A 5 ml aliquot of the RF filtrate was frozen (-20^{o}C) for volatile fatty acid (VFA), pH and NH_3-N analysis. Filtrate samples containing ruminal fluid were analyzed for VFA using a Gas Chromatography system (Perkin Elmer Autosystem XL, Waltham, MA) containing a Supelco (Sigma Aldrich, St. Louis, MO) packed column with the following specifications: 2 m x 2 mm Tightspec ID, 4% Carbowax 20M on 80/120 B-DA. The pH of the filtrate was measured with a pH meter (Accumet, model HP-71, Fisher Scientific, Pittsburgh, PA) and ammonia-N was determined using an adaptation for the Technicon auto analyzer (Technicon, Tarrytown, NY) of the Noel and Hambleton (1976) procedure. The adaptation involved colorimetric quantification of N concentration.

Statistical Analysis

Data from experiments 1 and 2 were analyzed as completely randomized designs with 2 (control versus enzyme) x 6 (substrates) factorial arrangement of treatments. Data from experiments 3 and 4 were analyzed with completely randomized designs with 2 (water versus ruminal fluid) x 6 (substrates) and 2 (control versus enzyme) x 2 (water versus ruminal fluid) x 6 (substrates) factorial treatment arrangements, respectively. The GLIMMIX procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) was used to analyze the data. The model used to analyze individual treatment effects included substrate, enzyme treatment, and the interaction (Experiments 1 and 2), substrate and medium, and the interaction (Experiment 3), and substrate, medium, enzyme treatment and all interactions (Experiment 4). Replicate was the random term in each experiment.

The Tukey test was used to compare least square means and significance was declared at $P < 0.05$.

Results and Discussion

Experiments 1 and 2

The ingredient composition of the concentrates and TMR and the chemical composition of each substrate are shown in Table 4-1. In general, concentrations of NDF, ADF, and hemicellulose were greater in forages than the TMR and least in concentrates, whereas, WSC concentration was greatest in AH followed by concentrates and least in CS (Table 4-2). Across all substrates, enzyme treatment increased WSC concentration by 9% and decreased concentrations of NDF, ADF, and hemicellulose by 3.9, 4.1, and 4.3%, respectively. The release of reducing sugars from hydrolyzed cell wall polysaccharides agrees with reported effects of fibrolytic enzymes on various feeds and forages (Hristov et al., 1996a; Krause et al., 1998; Krueger and Adesogan, 2008). Greater release of sugars from cell walls could stimulate bacterial glycocalyx production, and thereby increase adhesion of bacteria to substrates (Yang et al., 1999). However, the lack of a substrate x enzyme interaction for most of the chemical measures was surprising given that enzyme-feed specificity is often considered an important determinant of enzyme action (Beauchemin et al., 2004).

Application of the enzyme had substrate-dependent effects on DM disappearance in the buffer (substrate x enzyme interaction, $P < 0.001$; Table 4-3). Enzyme application increased the DMD of HC by 17% but did not affect those of other substrates. Therefore, HC seemed more susceptible to preingestive enzymatic hydrolysis in the buffer than other substrates perhaps because it contained the least NDF concentration.

Bowman et al. (2002) and Yang et al. (2000) also reported that enzyme application to the concentrate portion of the diet of lactating dairy cows improved in vivo DMD.

Experiment 3

Incubation of enzyme-treated substrates in RF resulted in greater ($P < 0.001$) in vitro DMD than incubation in W except for AH, which had similar DMD in both media (substrate x medium interaction; $P < 0.001$; Table 4-4). On average, DMD in RF was 76% greater than DMD in W, confirming that for all feeds except AH, ruminal digestion was more extensive than preingestive disappearance. The similar DMD result for AH in RF and W indicates that preingestive solubility of AH was as extensive as ruminal digestion of the substrate and suggests that digestion of AH in RF was due primarily to solubility rather than enzymatic or microbial hydrolysis. This may be because of the high concentration of water-soluble fractions in alfalfa hay (up to 48.5% of DM; Stefanon et al., 1996).

On average, enzyme-treated substrates incubated in RF had greater ($P < 0.001$) NDFD than those incubated in W (Table 4-4) but the magnitude of the increase was least for AH and greatest for the concentrates (substrate x medium interaction, $P < 0.001$). This highlights the greater insolubility of the fiber fractions in the concentrates relative to those in other substrates and illustrates the importance of microbial digestion of such fractions.

Since untreated controls were not included in this experiment, it is not clear if the greater DMD and NDFD in RF versus W reflect the intrinsic digestibility of the substrates or synergy between the exogenous enzyme and ruminal microbes in RF

(Morgavi et al., 2000). This aspect was further explored in Experiment 4, which compared untreated and enzyme-treated substrates in both media.

Experiment 4

Incubation in RF resulted in greater DMD and NDFD than incubation in W but the magnitude of the responses differed with substrate type (substrate x medium interaction; $P < 0.001$ and $= 0.003$, respectively; Table 4-5 and 4-6). Averaged across control and enzyme treatment, increases in DMD of 26, 222, 101, 126; 144, and 152% occurred when AH, CS, LC, HC, TMRL, and TMRH were incubated in RF instead of W, respectively. Therefore, incubation medium had the greatest effect on the DMD of CS and the least on AH. This agrees with the lack of an incubation medium effect on DMD of AH in Experiment 3 and confirms that preingestive hydrolysis had a greater impact on AH than on other substrates.

Enzyme effects on DMD and NDFD depended on the incubation medium ($P =$ 0.002 and <0.001, respectively) suggesting that preingestive and ruminal effects of the enzyme differed. Averaged across substrates, enzyme addition increased DMD in W (26.3 vs. 23.9%) by 10% but had no effect on DMD in RF (53.5 vs. 54.2%). Enzyme treatment increased NDFD in W (14.9 vs. 8.1%) by about 84% but did not affect NDFD in RF (36.4 vs. 36.3%). These results suggest that applying the enzyme contributed more to preingestive hydrolysis of the substrates than to their digestion in ruminal fluid and indicates that the greater DMD and NDFD in RF versus W in Experiment 3 reflects the intrinsic digestibility of the substrates rather than synergy between the exogenous enzyme and ruminal microbes in RF (Morgavi et al., 2000). This preingestive enzyme effect on DMD and NDFD explains some of the enzyme-mediated increases in in vivo

apparent DMD, NDFD, and efficiency of milk production in the previous study (Arriola et al., 2007). That enzymic hydrolysis increased the DMD of only HC after 24 h of incubation in the buffer (Table 4-3) but increased those of all substrates after 24 h of incubation in the buffer followed by 24 h of incubation in W (Table 4-5), suggests that the initial 24 h incubation in the buffer was too short to demonstrate preingestive effects of the enzyme on all substrates.

Reasons why enzyme effects on DMD and NDFD in RF were not apparent are unknown. Many other studies evaluating enzyme effects on DMD of dairy cattle feeds have used 24 h ruminal fluid incubation durations to demonstrate positive enzyme effects (Eun and Beauchemin, 2007; Eun et al., 2007; Krueger and Adesogan, 2008). Nevertheless, greater enzyme effects on DMD and NDFD may have occurred if substrates were incubated for 30 (Oba and Allen, 1999) or 48 h (NRC, 2001) instead of 24 h to reflect ruminal NDF retention times in dairy cattle. Factors other than the duration of ruminal digestion also may be involved. For instance, fibrolytic enzyme application decreased ruminal NDFD of a TMR fed to dairy cows but increased postruminal NDFD for unknown reasons (Sutton et al., 2003).

Ruminal pH and concentrations of total VFA and ammonia-N differed ($P < 0.001$) with substrate type (Table 4-7). Ruminal pH was greatest for AH and least for CS, possibly reflecting the differences in the starch concentrations of the substrates, as well as their ammonia-N concentration and buffering capacity. Total VFA concentrations were less in forages than TMR and butyrate molar proportion was greatest for CS and least for AH. Ammonia-N concentration was least for CS and greatest for LC, partly due to their different CP concentrations.

Enzyme addition increased (P = 0.002) total VFA concentration (194 vs. 176 Mm) suggesting that the enzyme increased supply of energy yielding substrates from fermentation of the dietary substrates. Enzyme treatment also decreased (P < 0.01) ruminal ammonia-N concentration (56 vs. 54 mg/L), which may reflect greater utilization of ammonia-N for microbial growth due to increased availability of WSC during ruminal fermentation. Effects of enzyme addition on the molar proportions of acetate and propionate tended to differ with the substrate. Compared to Control, enzyme-treated AH had greater molar proportion of acetate (P = 0.08) and lower proportion of propionate (P = 0.07) resulting in a greater (P = 0.05) acetate to propionate ratio, but such differences did not occur in other substrates. Eun and Beauchemin (2007) reported that adding the same enzyme to alfalfa hay increased total VFA concentration, propionate proportion, and NDFD, but did not affect acetate to propionate ratio. Whereas adding the enzyme to corn silage decreased acetate and propionate proportions and the acetate to propionate ratio but did not affect total VFA concentration. Differences between their results and those in this study may be due to differences between the forages and ruminal fluid activity as well as procedural differences for estimating these parameters.

Beneficial enzyme effects on concentrations of cell walls and reducing sugars, digestibility of DM or NDF in W or RF, or ruminal fluid VFA or ammonia-N concentrations did not (P > 0.05) depend on the substrate. This indicates that none of the substrates was preferentially hydrolyzed by the enzyme in RF or W and contradicts the hypothesis that enzyme effects on the forages would be greater. Therefore, it is unlikely that beneficial enzyme effects on DMD, NDFD, and efficiency of milk production in the study of Arriola et al. (2007) were due to preferential hydrolysis of the concentrate

or either of the forages in the diets. Rather, the enzyme probably affected the entire TMR. Effects of enzyme addition to specific portions of diets have been contradictory. Yang et al. (2000) compared treating either the TMR or concentrate with an enzyme and reported that improvements in DMD but not NDFD tended to be greater when the concentrate was treated and only concentrate treatment improved milk yield. In contrast, Phipps et al. (2000) reported no differences between milk yield of cows fed enzyme-treated concentrates or TMR or the untreated TMR. Sutton et al. (2003) also reported no differences in DMI or milk yield of cows fed diets in which an enzyme was infused ruminally or added to the TMR or concentrate. These contradictions and the results of this study do not refute the existence of enzyme-feed specificity (Beauchemin et al., 2004) and its' importance in determining enzyme effects. Rather, because the substrates, diets, and ingredients evaluated in this and other studies are comprised of various types and proportions of cell walls and other chemical components, they are probably not homogenous enough to reflect enzyme-feed specificity. Therefore, continued research on the best portion of the diet to which enzymes should be added may not identify an ideal ingredient target for all diets.

Conclusion

Enzyme treatment decreased ruminal ammonia-N concentration and increased hydrolysis of cell walls, release of reducing sugars, digestibility of DM and NDF, and total VFA concentration regardless of substrate type. Therefore, this study provided no convincing evidence that the enzyme preferentially hydrolyzed specific dietary substrates. Substrate digestion in RF was consistently greater than that in W. However, enzyme effects on DMD and NDFD were consistently greater in W than in RF, indicating that preingestive effects of the enzyme were greater than ruminal effects

under the conditions of the study. Preingestive effects were also greater for AH than other substrates, likely reflecting the high concentration of water-soluble fractions in AH. These experiments involved 24 h incubations in RF or W, therefore future research should investigate whether similar results are obtained with longer incubation periods.

Table 4-1. Ingredient composition of concentrate and TMR substrates and chemical composition of all substrates

Item	LC	HC	TMRL	TMRH
Ingredient, % DM				
Corn silage	0	0	49.2	37.0
Alfalfa hay	0	0	13.5	10.0
Cottonseed hulls	12.4	9.4	4.6	5.0
Corn meal	19.8	33.7	7.4	17.9
Citrus pulp	5.4	9.4	2.0	5.0
Whole cottonseed	4.9	9.1	1.8	4.8
SoyPlus[1]	21.2	11.2	7.9	5.9
Soybean meal	6.7	11.3	2.5	6.0
Cottonseed meal	20.9	9.6	7.8	5.1
Mineral mix[2]	8.7	6.1	3.3	3.3

Chemical composition	AH	CS	LC	HC	TMRL	TMRH
DM, %	94.1	35.0	93.9	94.1	64.4	72.2
Ash, % DM	10.7	3.5	10.4	8.1	7.1	6.7
CP, % DM	18.7	9.0	31.1	25.1	18.6	18.5
NDF, % DM	43.6	44.7	27.9	25.4	38.1	33.3
ADF, % DM	23.2	18.4	13.8	12.9	17.9	15.7
Hemicellulose, % DM	20.4	26.2	14.1	12.5	20.2	17.6

[1] West Central Soy, Ralston, IA.
[2] Mineral mix contained 26.4% CP, 10.2% Ca, 8.6% Na, 5.1% K, 3.1% Mg, 1.5% S, 0.9% P, 2231 mg/kg of Mn, 1698 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 31 mg/kg of Co, 26 mg/kg of I, 7.9 mg/kg of Se, 147,756 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis).
LC = Low concentrate; HC = High concentrate;TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

Table 4-2. Effect of enzyme application to different substrates on concentrations of NDF, ADF, hemicellulose, and WSC (Experiment 1).

Measure	Substrate	Control	Enzyme	Substrate Mean
NDF, % of DM				
	Alfalfa hay	43.6	42.7	43.2[a]
	Corn silage	44.7	43.4	44.0[a]
	Low concentrate	27.9	25.6	26.8[d]
	High concentrate	25.4	23.4	24.4[e]
	TMRL	38.1	36.2	37.1[b]
	TMRH	33.3	33.0	33.1[c]
	Enzyme mean	35.5[x]	34.1[y]	
	Effects	*P values*	SEM	
	Substrate	<0.001	0.31	
	Enzyme	<0.001	0.18	
	Substrate x enzyme	0.22	0.44	
ADF, % of DM				
	Alfalfa hay	23.2	23.6	23.4[a]
	Corn silage	18.4	17.9	18.2[b]
	Low concentrate	13.8	12.6	13.2[e]
	High concentrate	12.9	11.9	12.4[e]
	TMRL	17.9	16.7	17.3[c]
	TMRH	15.7	15.3	15.5[d]
	Enzyme mean	17.0[x]	16.3[y]	
	Effects	*P values*	SEM	
	Substrate effect	<0.001	0.20	
	Enzyme effect	<0.001	0.11	
	Substrate x enzyme effect	0.05	0.28	

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a, b, c, d, e] Means within a column with

[x, y] Means within a row with different superscripts differ, P < 0.001

Table 4-2. Continued

Measure	Substrate	Control	Enzyme	Substrate Mean
Hemicellulose, % of DM				
	Alfalfa hay	20.4	19.2	19.8[b]
	Corn silage	26.2	25.5	25.9[a]
	Low concentrate	14.1	13.1	13.6[d]
	High concentrate	12.5	11.4	12.0[e]
	TMRL	20.2	19.5	19.9[b]
	TMRH	17.6	17.7	17.6[c]
	Enzyme mean	18.5[x]	17.7[y]	
	Effects	*P values*	SEM	
	Substrate	<0.001	0.20	
	Enzyme	<0.001	0.12	
	Substrate x enzyme	0.30	0.29	
WSC, % of DM				
	Alfalfa hay	9.4	9.8	9.6[a]
	Corn silage	2.2	3.7	2.9[d]
	Low concentrate	7.7	8.8	8.3[b]
	High concentrate	8.4	8.0	8.2[b]
	TMRL	6.0	6.6	6.3[c]
	TMRH	6.1	7.1	6.6[c]
	Enzyme mean	6.7[y]	7.3[x]	
	Effects	*P values*	SEM	
	Substrate effect	0.003	0.25	
	Enzyme effect	<0.001	0.15	
	Substrate x enzyme effect	0.16	0.36	

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a, b, c, d, e] Means within a column with

[x, y] Means within a row with different superscripts differ, P < 0.001

Table 4-3. Effect of enzyme application on disappearance of DM from substrates incubated in a buffer for 24 h (Experiment 2).

Substrate	DM disappearance %	
	Control	Enzyme
Alfalfa hay	36.7[a]	37.1[a]
Corn silage	20.8[ef]	19.4[f]
Low concentrate	26.5[b]	27.8[b]
High concentrate	22.0[de]	25.9[bc]
TMRL	23.6[cd]	23.7[cd]
TMRH	22.9[de]	22.8[de]
Enzyme mean	25.4[y]	26.1[x]

Effects	*P values*	SEM
Substrate	<0.001	0.34
Enzyme	0.02	0.20
Substrate x Enzyme	<0.001	0.49

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a, b, c, d, e,] Interaction means with different superscripts differ, P < 0.001

[x, y] "Means within a row with different superscripts differ, P < 0.05

Table 4-4. Effect of incubation medium on the dry matter (DMD) and neutral detergent fiber (NDFD) digestibility of enzyme-treated substrates (Experiment 3).

Substrate	DMD %		NDFD %	
	Water	Ruminal fluid	Water	Ruminal fluid
Alfalfa hay	43.4[b]	43.1[b]	10.9[d]	22.7[b]
Corn silage	22.2[f]	54.4[a]	12.0[cd]	31.2[a]
Low concentrate	33.2[c]	55.4[a]	0.8[e]	19.2[bc]
High concentrate	29.4[cd]	55.8[a]	2.1[e]	22.2[b]
TMRL	26.7[de]	52.9[a]	4.6[ed]	33.0[a]
TMRH	24.9[ef]	55.9[a]	5.1[ed]	34.3[a]
Mean	30.0[y]	52.9[x]	5.9[y]	27.1[x]

Effects	*P values*	SEM	*P values*	SEM
Substrate	<0.001	0.44	<0.001	0.73
Medium	<0.001	0.26	<0.001	0.42
Substrate x medium	<0.001	0.62	<0.001	1.03

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration
[a, b, c, d, e,f] Means within a measure (DMD or NDFD) with different superscripts differ, P < 0.001
[x, y] "Means within a row with different superscripts differ, P < 0.05

Table 4-5. Effect of enzyme application and incubation medium on the dry matter digestibility (DMD) of substrates incubated for 24 h in water or ruminal fluid after incubation for 24 h in a buffer (Experiment 4).

	Medium					
	Water			Ruminal fluid		
Substrate	Control	Enzyme	Mean	Control	Enzyme	Mean
Alfalfa hay	34.2	38.4	36.3[d]	44.9	46.5	45.8[c]
Corn silage	15.6	17.8	16.7[g]	54.0	53.6	53.8[b]
Low concentrate	26.5	27.4	27.0[e]	53.3	55.3	54.3[b]
High concentrate	23.3	26.5	25.2[e]	56.4	57.5	56.9[ab]
TMRL	21.9	23.7	22.8[f]	55.7	55.7	55.7[ab]
TMRH	21.7	23.3	22.5[f]	56.7	56.8	56.8[a]
Mean	23.9[y]	26.3[x]		53.5	54.2	

Effects	*P values*	SEM
Substrate	<0.001	0.30
Enzyme	<0.001	0.17
Medium	<0.001	0.17
Substrate x medium	<0.001	0.43
Enzyme x medium	0.002	0.25
Substrate x enzyme	0.10	0.43
Substrate x enzyme x medium	0.28	0.60

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a ,b, c, d, e, f, g] Substrate x medium means with different superscripts differ, P < 0.001

[x, y] Enzyme x medium means with different superscripts differ, P < 0.01

Table 4-6. Effect of enzyme application and incubation medium on neutral detergent fiber digestibility (NDFD) of substrates of substrates incubated for 24 h in water or ruminal fluid after incubation for 24 h in a buffer (Experiment 4)

Substrate	NDFD					
	Water			Ruminal fluid		
	Control	Enzyme	Mean	Control	Enzyme	Mean
Alfalfa hay	7.2	12.1	9.6[d]	27.4	29.4	28.4[bc]
Corn silage	4.4	12.8	8.6[d]	31.9	34.2	33.1[b]
Low concentrate	0.0	0.0	0.0[e]	22.7	24.3	23.5[c]
High concentrate	0.0	7.1	1.3[e]	29.8	30.3	30.0[b]
TMRL	21.2	25.6	23.4[c]	50.6	46.8	48.7[a]
TMRH	28.1	33.5	30.8[b]	55.8	52.6	54.2[a]
Mean	8.1[y]	14.9[x]		36.4	36.3	

Effects	*P values*	SEM
Substrate	<0.001	0.88
Enzyme	<0.001	0.51
Medium	<0.001	0.51
Substrate x medium	0.003	1.25
Enzyme x medium	<0.001	0.73
Substrate x enzyme	0.16	1.25
Substrate x enzyme x medium	0.61	1.76

TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a,b,c,d,e] Substrate x medium means with different superscripts differ, P < 0.001

[x, y] Enzyme x medium means with different superscripts differ, P < 0.01

Table 4-7. Effect of enzyme (Enz) application on pH, VFA and ammonia-N concentrations of substrates incubated for 24 h in ruminal fluid after incubation for 24 h in a buffer (Experiment 4)

Substrate		pH	Total VFA, mM	Acetate (A), mol/100 mol	Propionate (P), mol/100 mol	Butyrate, mol/100 mol	A:P	Ammonia-N, mg/L
AH	Control	7.17	135	57.6	32.0	8.9	1.93	58.3
	Enz	7.16	165	67.3	24.4	7.1	2.76	60.4
	Mean	7.17[a]	150[d]	62.4	28.2	8.0[c]	2.3	59.3[b]
CS	Control	6.01	160	57.4	28.4	13.2	2.03	30.1
	Enz	5.91	194	57.3	28.4	13.2	2.02	26.3
	Mean	5.96[d]	177[c]	57.3	28.4	13.2[a]	2.02	28.2[d]
LC	Control	7.15	178	60.4	28.2	10.3	2.15	76.0
	Enz	6.91	180	59.3	29.0	10.6	2.05	75.9
	Mean	7.03[ab]	179[bc]	59.8	28.6	10.5[b]	2.10	75.9[a]
HC	Control	6.71	184	58.2	30.4	10.4	1.92	65.9
	Enz	6.72	190	57.6	30.4	11.0	1.90	60.8
	Mean	6.72[bc]	187[abc]	57.9	30.4	10.7[b]	1.91	63.3[b]
TMRL	Control	6.44	205	60.3	28.1	10.6	2.15	53.1
	Enz	6.51	216	60.5	27.7	10.8	2.19	49.4
	Mean	6.47[c]	211[a]	60.4	27.9	10.7[b]	2.17	51.3[c]
TMRH	Control	6.43	192	59.4	28.4	11.2	2.10	52.6
	Enz	6.42	221	59.8	28.5	10.8	2.10	48.3
	Mean	6.43[c]	207[ab]	59.6	28.4	11.0[b]	2.10	50.4[c]
SEM[1]		0.10	9.18	1.89	1.44	0.42	0.15	1.55
Effect					*P* values			
Substrate		<0.001	<0.001	0.13	0.58	<0.001	0.16	<0.001
Enzyme		0.45	0.002[2]	0.20	0.16	0.45	0.18	0.01[2]
Substrate x enzyme		0.73	0.23	0.08	0.07	0.11	0.05	0.18

AH = Alfalfa hay; CS = Corn silage; LC = Low energy concentrate; HC = High energy concentrate; TMRL = Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a, b, c, d] Means in the same column with different superscripts differ, $P < 0.001$

[1] Substrate x enzyme SEM.

[2] Main enzyme effect means for total VFA were 176 versus 194 mM, SEM = 3.48 and were 56 versus 54 mg/L, SEM = 0.63 for ammonia-N.

CHAPTER 5
EFFECT OF APPLYING BACTERIAL INOCULANTS ON THE FERMENTATION AND QUALITY OF CORN SILAGE

Introduction

Inoculants containing selected strains of lactic acid bacteria (LAB) have been developed to reduce the influence of epiphytic LAB on the outcome of ensiling forages. The principal function of homofermentative inoculants is to ensure a rapid and efficient fermentation of water-soluble carbohydrates (WSC) into lactic acid, a rapid decrease in pH, and improved silage conservation with minimal fermentation losses (Weinberg et al., 1993a). However, such inoculants have not increased aerobic stability in many studies (Muck and Kung, 2007) and some have decreased aerobic stability (Moon et al. 1980) by enhancing the growth of spoilage yeasts. Inoculating forages at harvest with *Lactobacillus buchneri* improves the aerobic stability of silages (Muck, 1996; Taylor et al., 2002) most likely because this organism converts lactic acid to acetic acid under anaerobic conditions (Oude Elferink et al., 2001). Recently, *L. buchneri* has been combined with homolactic bacteria to improve the aerobic stability and fermentation of silages (Kung et al., 2003a). Driehuis et al. (2001) reported that *L. buchneri* alone or in combination with *P. pentosaceus* and *L. plantarum* improved aerobic stability of grass silage, reduced yeast and mold counts, and had lower DM loss compared to the untreated silage. Other studies reported improved aerobic stability when combinations of homolactic and heterolactic inoculants were applied to sorghum (Filya, 2003) and corn (Huisden et al., 2009). However, Adesogan et al. (2004) reported that treating bermudagrass with *P. pentosaceus* and *L. buchneri* improved the fermentation but did not improve aerobic stability because their control silage was more stable due to a butyric fermentation. Kleinschmit and Kung (2006b) reported that an inoculant

containing *Pedioccocus pentosaceus* and *L. buchneri* reduced yeast counts and improved aerobic stability in a manner that varied with the ensiling duration. Kang et al. (2009) demonstrated that effects of inoculants containing *Lactobacillus casei* and *L. buchneri* on aerobic stability of corn silage varied with the corn hybrid tested. Therefore, more research on effects of combinations of *L. buchneri* and homolactic bacteria is needed.

Propionic acid bacteria can ferment glucose and lactate to acetate and propionic acid (Moon, 1983) and a few studies have shown that such inoculants improved aerobic stability of silage (Dawson et al., 1998; Filya et al., 2004). Effects of combinations of propionibacteria and homolactic bacteria on silage fermentation and aerobic stability are not well known. The objective of this study was to examine the effect of applying bacterial inoculants containing heterofermentative bacteria alone or homofermentative and heterofermentative bacteria on the fermentation, quality, and aerobic stability of corn silage.

Material and Methods

Forage and Treatments

A corn hybrid Vigoro 61R36 (Royster Clark, Inc., Greeley, CO) was grown at the Dairy Research Unit, University of Florida and harvested at 35% DM with a Claas Jaguar 850 (Claas of America, Columbus, IN) forage harvester. Forages were chopped to a theoretical length of cut of 1.9 cm and treated with 1) deionized water (CON); 2) Biotal Plus II (B2), containing 1 x 10^5 cfu/g of *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*, applied at 21.9 mg/kg of fresh forage; 3) Buchneri 40788 (BUC), containing 4 x 10^5 cfu/g of *Lactobacillus buchneri* applied at 8 mg/kg of fresh forage; 4) a combination inoculant, Buchneri 500 (B500), containing 4 x 10^5 cfu/g

of *P. pentosaceus* 12455 and *L. buchneri* 40788 applied at 8 mg/kg of fresh forage. Inoculants were dissolved in 950 mL of deionized water and sprayed uniformly onto the forages under constant mixing. All inoculants were supplied by Lallemand Animal Nutrition, Milwaukee, WI. Four replicates of each treatment were weighed (10 kg) into polyethylene bags, within 20-L mini silos, sealed, and stored for 575 d at ambient temperature (25ºC) in a covered barn. Dry matter recovery was calculated from the initial and final weights and the DM concentrations of the fresh and ensiled forage.

Chemical Analysis

Subsamples of the untreated fresh forage and each treated silage were collected, dried in a forced-air oven at 60ºC for 48 h and ground to pass a 1-mm screen using a Wiley mill (A. H. Thomas, Philadelphia, PA). Concentrations of NDF and ADF were measured using the method of Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Starch was determined using the procedure of Holm et al. (1986). The anthrone reaction assay (Ministry of Agriculture Fisheries and Food, 1986) was used to quantify water-soluble carbohydrates (WSC). Corn silage samples were also analyzed for aerobic stability by placing thermocouple wires at the center of a bag containing 1 kg of silage, within an open-top polystyrene box. The silages were covered with 2 layers of cheesecloth to prevent drying. The thermocouple wires were connected to data loggers (Campbell Scientific Inc., North Logan, UT) that recorded the temperature every 30 min for 14 d. Aerobic stability was denoted by the time (h) before a 2ºC rise in silage temperature above ambient temperature (23ºC). Silage subsamples from each silo were submitted to Dairyland Laboratories, Inc. Arcadia, WI for analysis of yeast and mold counts using YM-11 agar (AOAC, 1995),

VFA by HPLC (Canale et al., 1984), pH using a pH meter (Orion 710+, Thermo Fisher Scientific Inc., Waltham, MA), and ammonia-N by distillation (AOAC, 1995).

Phenol-chloroform Extraction of Total DNA

Silage samples from each treatment were diluted with distilled water (1:2) and macerated in a blender. The DNA was extracted from the water extract using the phenol-chloroform method (Giraffa et al., 2000). A 1.5 ml aliquot of each water extract was centrifuged at 12,500 × *g* for 5 min, the supernatant was discarded and this step was repeated. The pellets were washed twice with TE buffer (10mM Tris-HCl-0.1 mM EDTA, pH 8.0). Washed cell pellets were resuspended in 500 µl of TES buffer (50 mM Tris-HCl-1 mM EDTA-6.7% saccharose, pH 8.0) followed by incubation at 37ºC for 30 min with 200 µl of lysozyme (50 mg/ml). This was followed by a second incubation at 56ºC for 30 min with 15 µl of Proteinase K, and a third incubation at 56˚C overnight with 125 µl of SDS (sodium dodecyl sulfate, 20% wt/vol). DNA was extracted by adding 200 µl of phenol and chloroform, centrifuging at 7,000 × *g* for 5 min at 4ºC, and transferred to a new tube. This step was repeated three times, followed by precipitation with cold (-20˚C) isopropanol (600 µl) and centrifugation at 12,500 × *g* for 30 min at 4ºC. A 1 ml aliquot of ethanol (70%) was added to the pelleted DNA and centrifuged at 12,500 × *g* for 5 min at 4ºC. Purified DNA was dried and resuspended overnight at 4ºC in 150 µl of TE buffer. Ribonuclease HII (1 µl; 5000 U/ml; New England BioLabs, Ipswich, MA) was added to resuspended DNA and after incubation at 37ºC for 1 h, DNA samples were stored at -20ºC until use.

Conventional PCR Conditions

The Polymerase chain reaction was performed to amplify a region of the bacterial 16S rRNA gene of *L. buchneri* with the forward primer LBR2 that corresponds to the

region from base 186 to 215 (5'-GAAACAGGTGCTAATACCGTATAACAACCA-3') and the reverse primer LBR1 that corresponds to the region from base 316 to 345 (5'-CGCCTTGG-TAGGCCGTTACCTTACCAACA-3') (GenoMechanix, LLC. Gainesville, FL). The expected product size was 159 base pairs. A touchdown PCR was done in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Waltham, MA) in order to reduce the incidence of formation of false products, enhance correct annealing, and increase the specificity of the amplification (Kidd and Ruano, 1995). The initial denaturation step was set at 94ºC for 15 min and the annealing temperature was set at 61ºC for the first cycle and reduced by 0.5ºC per cycle for the next nine cycles to reach a final melting temperature (T_m) of 56ºC. The previous melting temperature was used for the remaining 20 cycles. An extension time of 30 s at 72ºC was utilized, with final extension of 7 min and hold at 4ºC. Amplification was done in a standard reaction mixture containing 16.75 µl of H_2O, 2.5 µl of 10x Taq buffer advanced (5 Prime), 1 µl of each primer, 2.5 µl of 2mM dNTP (Thermo Fisher Scientific, Inc), 0.25 µl of Taq Polymerase (5 U/µl; 5 Prime), and 2 µl of the template. Electrophoresis was conducted on 2.0% agarose gel stained with ethidium bromide, and DNA was visualized under UV light and photographed (Sambrook et al., 1989).

Statistical Analysis

The data were analyzed as a completely randomized design using the GLM procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC). The general model was. $Yij = \mu + T_i + eij$, where T = effect of treatment i, and eij = error term. The F-protected least significant difference test was used to compare least square means and significance was declared at $P < 0.05$.

Results and Discussion

The chemical composition of the corn forages was normal for corn silage produced in Florida (Table 5-1; Huisden et al., 2009; Kang et al., 2009). The control silage had the greatest DM concentration but other chemical components did not differ among the forages. Corn forage treated with B2 had a lower DM concentration than B500 and Control silages, whereas CP concentration was greater in CON and B2 silages than in the others (Table 5-2). As expected, WSC concentrations of all silages were reduced during the fermentation. The B500 silage had the greatest ($P < 0.05$) residual WSC concentration (1.49 vs. 1.18 % DM), indicating that plant sugars were less extensively fermented by the bacteria in this inoculant compared to those in others. In contrast, Filya (2003) reported that Control and *L. plantarum*-inoculated corn silages had greater ($P < 0.05$) residual WSC than silages inoculated with *L. buchneri* alone or *L*. *buchneri* and *L. plantarum*. High residual WSC concentrations in silages are desirable because they reflect a more efficient fermentation in the silo and indicate greater availability of energy-yielding substrates for ruminal microbes, but can also predispose to the growth of spoilage yeasts.

All pH values were within the range of 3.8 to 4.1 (Table 5-3), which reflects adequate fermentation for restricting the growth of undesirable microorganisms like clostridia. Dry matter loss was lower in BUC silages compared with Control and B2 silages (5.0 vs. 14.3 %). Control and B2 silages had greater pH and lower lactic acid concentration than BUC and B500 silages. The greater lactate concentration and lower pH of BUC silages explain the lower DM loss from this silage. Propionic acid concentration was greater in Control and B2 silages than BUC and B500 silages. Acetic acid concentration was greatest in the B2 silage (6.46 vs. 4.23 % DM), lowest in

the B500 silage and intermediate in Control and BUC silages. The B500 silage had the greatest lactic: acetic acid ratio (1.54 vs. 0.41).

The fermentation results of the Control, BUC, and B500 silages were unusual. Normally, untreated corn silage undergoes a homolactic fermentation resulting in relatively low pH values (< 4.0) due to high concentrations of lactate and low concentrations of acetate and propionate. In contrast, the Control silage had among the highest pH values, lowest lactate concentrations, and highest acetate and propionate concentrations, suggesting that the fermentation was dominated by heterolactic bacteria. Furthermore, the homolactic bacteria in B2 and B500 did not dominate the fermentation sufficiently to result in greater lactate concentrations and lower DM loss relative to those in the Control silage. Results of inoculation with BUC and B500 were atypical in that application of *Lactobacillus buchneri* to corn forage usually increases the concentration of acetate and reduces the lactate concentration (Ranjit and Kung, 2000; Filya, 2003; Hu et al., 2009). This could be due to the presence of high concentrations of epiphytic *L. buchneri* on the control silages. The conventional PCR analysis was performed to examine this theory. It revealed the presence of similar populations of *L. buchneri* in all silages irrespective of treatment (Figure 5-1) and explains why inoculants containing *L. buchneri* did not have normal effects on the silages. Factors predisposing to high epiphytic populations of *L. buchneri* on corn forages or silages are unknown and warrant further research.

Propionic acid bacteria can ferment glucose and lactate to acetate and propionic acid (Moon, 1983), therefore the presence of *Propionibacteria* in B2 partly explains the greater acetate concentration and numerically greater propionate concentration of B2

silages relative to Control silages. However, inoculation with *Propionibacteria* has produced equivocal results on propionic acid concentration of silages, with few studies showing positive effects (Dawson et al., 1998) and many others showing little to no effect (Higginbotham et al., 1998, Kung and Ranjit, 2001; Pedroso et al. 2010). Consequently, such bacteria have not usually increased aerobic stability (Weinberg et al., 1995; Higginbotham et al., 1998; Pedroso et al., 2010).

Yeast and mold counts were less than the threshold (10^5) typically associated with silage spoilage (Pahlow and Zimmer, 1985; O'Kiely et al., 1987) and did not differ among treatments (Table 5-4). Consequently, all silages were stable for long periods (> 250 h) even though B500 silages were less stable than the others. That application of *L. buchneri* inoculants did not increase the aerobic stability or decrease the yeast counts relative to those of untreated silages contradicts various reports (Kleinschmit and Kung, 2006b; Hu et al., 2009; Huisden et al., 2009; Pedroso et al., 2010). This is likely because of the high population of *L. buchneri* in all silages. The greater deterioration of the B500 silage, which had one of the highest lactate concentrations, reflects the relatively low antifungal property of lactate. In fact, lactate serves as a substrate for several spoilage yeasts such as those of the genera *Candida* and *Pichia* spp. (Pahlow et al., 2003). Acetate, butyrate, and propionate are the main antifungal acids in silages (Moon et al., 1983) and the total concentration of these acids was lower in the B500 silage than in others. Weinberg et al. (1993a) reported that high levels of residual WSC, combined with high lactate concentrations and insufficient concentrations of antifungal VFA in silages inoculated with homofermentative LAB were associated with aerobic spoilage. The greater residual WSC concentration and lower total antifungal acid

concentration may have made the B500 silage more susceptible to deterioration than others in this study.

Conclusions

Application of BUC improved lactate concentration, which made the fermentation more homolactic and contributed to a reduction in DM loss relative to values for Control silages. Control and B2 silages had greater pH values and lower lactate concentrations than BUC and B500 silages. Acetate concentration was greatest in B2 silage, intermediate in Control and BUC silages, and lowest in B500 silage. However, aerobic stability was generally high (> 250 h) and was not improved by inoculant application. A conventional PCR analysis confirmed the presence of similar populations of *L. buchneri* in all treatments and this probably explains the prolonged aerobic stability of all silages and some of the atypical fermentation results.

Table 5-1. Chemical composition of corn forages treated with or without bacterial inoculants before ensiling

Item	CON	B2	BUC	B500	SEM	P values
DM, %	35.4[a]	32.9[b]	31.5[b]	33.1[b]	1.46	0.02
NDF, % DM	42.8	43.0	43.0	41.7	1.07	0.30
ADF, % DM	25.1	25.1	25.3	25.7	0.97	0.81
Starch, % DM	25.5	28.4	22.9	24.8	2.72	0.08
WSC, % DM	16.9	18.5	18.3	17.7	1.35	0.39

Means in the same row with different superscripts differed ($P < 0.05$)
CON = Control, no inoculant; B2 = *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*; BUC = *Lactobacillus buchneri*; B500 = *P. pentosaceaus* and *L. buchneri*.

Table 5-2. Effect of bacterial inoculants on the chemical composition of corn silages

Item	CON	B2	BUC	B500	SEM	P values
DM, %	31.4	29.9	31.2	31.7	0.84	0.09
CP, % DM	10.5[a]	10.3[a]	9.86[b]	9.86[b]	0.31	0.03
NDF, % DM	44.4[a]	43.2[ab]	42.3[b]	42.0[b]	1.06	0.04
ADF, % DM	27.2	27.4	26.2	26.6	0.99	0.42
Starch, % DM	26.1	27.8	28.3	25.2	2.81	0.41
WSC, % DM	1.22[b]	1.14[b]	1.18[b]	1.49[a]	0.12	0.01

Means in the same row with different superscripts differed ($P < 0.05$)
CON = Control, no inoculant; B2 = *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*; BUC = *Lactobacillus buchneri*; B500 = *P. pentosaceaus* and *L. buchneri*.

Table 5-3. Effect of bacterial inoculants on DM losses and fermentation indices of corn silages

Item	CON	B2	BUC	B500	SEM	*P* values
DM loss, %	14.9[a]	13.6[ab]	5.0[c]	8.2[ab]	3.92	0.016
pH	4.10[a]	4.06[a]	3.90[b]	3.80[b]	0.05	<.001
NH_3-N, % CP	8.25	9.68	8.92	8.01	1.15	0.28
Total acids, % DM	7.15[b]	8.88[a]	8.40[a]	7.30[b]	0.58	0.005
Lactic acid, % DM	1.56[b]	1.31[b]	3.34[a]	4.17[a]	0.72	0.001
Acetic acid, % DM	4.85[b]	6.46[a]	4.88[b]	2.97[c]	0.59	<0.001
Propionic acid, % DM	0.72[a]	1.09[a]	0.17[b]	0.08[b]	0.16	<0.001
Butyric acid, % DM	0.01	0.01	0.01	0.07	0.06	0.47
Isobutyric acid, % DM	0.01	0.01	0.01	0.01	0.00	-
Ethanol, % DM	0.01	0.10	0.01	0.01	0.07	0.28
La : Ac ratio	0.33[b]	0.21[b]	0.69[b]	1.54[a]	0.35	0.001

Means in the same row with different superscripts differed ($P < 0.05$)
CON = Control, no inoculant; B2 = *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*; BUC = *Lactobacillus buchneri*; B500 = *P. pentosaceaus* and *L. buchneri*.

Table 5-4. Effect of bacterial inoculants on fungal counts and aerobic stability of corn silages

Item	CON	B2	BUC	B500	SEM	*P* values
Yeasts, log cfu/g	<3.00	<3.00	<3.00	<3.00	0.00	-
Molds, log cfu/g	3.4	3.67	4.15	3.75	0.71	0.54
Aerobic stability, h	390[a]	390[a]	381[a]	276[b]	53	0.07

Means in the same row with different superscripts differed ($P < 0.05$)
CON = Control, no inoculant; B2 = *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*; BUC = *Lactobacillus buchneri*; B500 = *P. pentosaceaus* and *L. buchneri*.

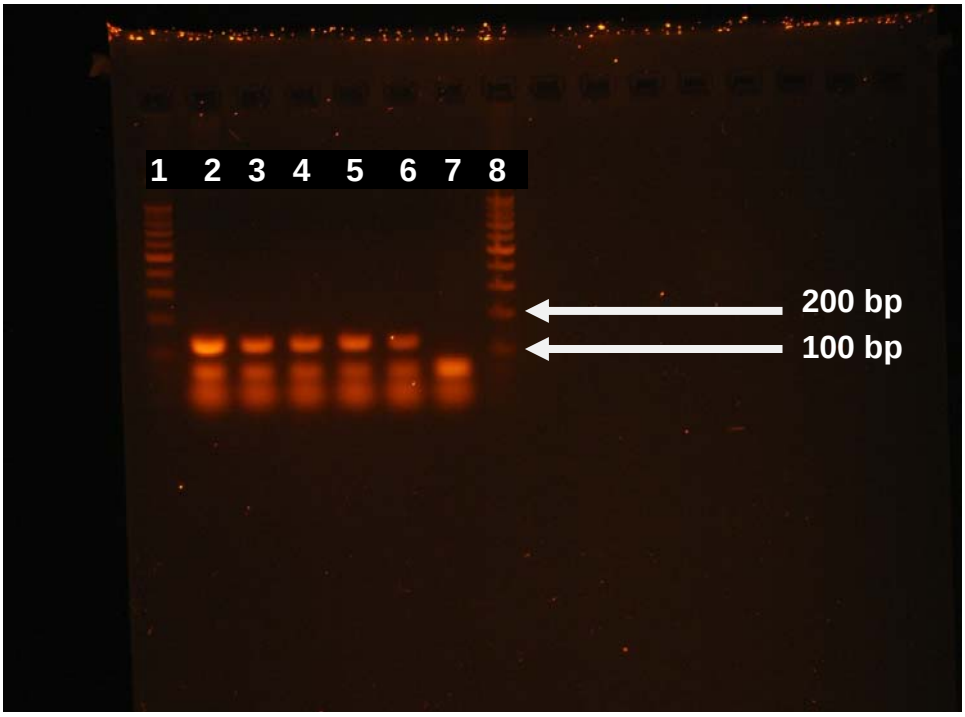

Figure 5-1. Gel electrophoresis analysis after polymerase chain reaction amplification of DNA from silages treated with or without inoculants: lane 1 and 8, DNA ladder/ marker (Promega, Corp.), lane 2, Pure culture of *L*. *buchneri*, lane 3: CON, untreated control, lane 4: B2, *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*, lane 5: BUC*, L. buchneri*, lane 6: B500, *P. pentosaceus* plus *L*. *buchneri,* lane 7: negative control. The expected product size was 159 base pairs (bp).

CHAPTER 6
EFFECT OF APPLYING BACTERIAL INOCULANTS TO CORN SILAGE ON THE PERFORMANCE OF DAIRY CATTLE

Introduction

Ensiling is a preservation method for moist forage crops based on conversion of water-soluble carbohydrates (WSC) into organic acids by lactic acid bacteria (LAB) under anaerobic conditions (McDonald et al., 1991). Homofermentative LAB are used to increase the rate of acidification and minimize DM and nutrient losses during forage fermentation. Bacteria typically used for this purpose include *Lactobacillus plantarum, Enterococcus faecium,* and *Pediococcus spp.* However, such bacteria have not been very successful at enhancing aerobic stability of silage (Muck and Kung, 2007) because the lactic acid they produce may facilitate the growth of spoilage yeasts.

Propionic acid bacteria convert lactate and glucose to antifungal acids like propionic and acetic acids, but they have not improved consistently the aerobic stability of forages (Weinberg et al., 1995; Higginbotham et al., 1996, 1998; Filya et al., 2006) because the growth of propionic acid bacteria is affected adversely by acidic environments (Weinberg et al., 1995; Higginbotham et al., 1996). Application of *Lactobacillus buchneri* inoculants has improved the aerobic stability of corn silage (Kleinschmit et al., 2005; Huisden et al., 2009), alfalfa silage (Kung et al., 2003a), and sugar cane silage (Pedroso et al., 2002). However, *L. buchneri* is heterofermentative, consequently, small losses of DM can occur during the fermentation of forages treated with *L. buchneri* inoculants (Ranjit and Kung, 2000). This led to the recent development of 'dual purpose' inoculants containing a mixture of homofermentative and heterofermentative LAB that reduce DM losses by increasing the acidification rate and increase the aerobic stability, respectively.

Studies on effects of inoculant application to forages on the performance of dairy cows are few and those available have produced equivocal results. Muck (1993) reviewed studies published from 1985 to 1992 and reported that inoculant application increased DMI and weight gain in about 25% of the studies, whereas milk production and feed efficiency were increased in 40 and 45% of studies, respectively. Kung et al. (1993) showed that feeding corn silage inoculated with *L. plantarum* to lactating dairy cows improved 3.5% FCM and DMI. Wohlt (1989) reported that dairy cows fed corn silage inoculated with *L. plantarum* produced 0.7 kg/d more FCM than cows fed the untreated silage. Feeding alfalfa silage inoculated with *L. buchneri* 40788 increased milk production by dairy cows (Kung et al., 2003a), but feeding barley silage inoculated with *L. buchneri* 40788 did not (Taylor et al., 2002). To our knowledge, no studies have evaluated effects of inoculating corn silage with the newer inoculants containing homofermentative and heterofermentative bacteria on the performance of dairy cows. The objective of this study was to examine the effect of applying bacterial inoculants containing heterofermentative bacteria alone or homofermentative and heterofermentative bacteria on the performance of lactating dairy cows.

Material and Methods

Forage and Treatments

A corn hybrid Vigoro 61R36 (Royster Clark, Inc., Greeley, CO) was grown at the Dairy Research Unit, University of Florida, harvested at 35% DM using a Claas Jaguar 850 (Monroe, NC) forage harvester and chopped to achieve a theoretical length of cut of 1.9 cm. Thirty-five tons of forage were ensiled within separate 2.4-m wide bags for 363 d after application of the following treatments: 1) nothing (CON), 2) Biotal Plus II (B2) containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii* (applied

at 1 x 10^5 cfu/g of fresh forage), 3) Buchneri 40788 (BUC) containing *Lactobacillus buchneri* 40788 (applied at 4 x 10^5 cfu/g), and 4) Buchneri 500 (B500) containing *P. pentosaceus* 12455 (1 x 10^5) and *L. buchneri* 40788 (4 x 10^5 cfu/g). Inoculants were dissolved in water (0.95 L/ton) and applied via a sprayer mounted on the chopper. All inoculants were supplied by Lallemand Animal Nutrition, Milwaukee, WI.

Diets, Cows, and Management

The study was conducted from July to October 2007 at the Dairy Research Unit, University of Florida. Each of the four silages was mixed into a TMR consisting of 44% corn silage, 50% concentrate and 6% alfalfa hay (DM basis; Table 6-1). All diets were balanced to meet the nutrient requirement of dairy cows in early lactation (NRC, 2001). Fifty-two lactating Holstein cows were classified according to milk production and parity, and randomly assigned to the four dietary treatments at 22 $\pm$ 3 DIM. Cows were housed in a free-stall, open-sided barn fitted with continuously operated fans and misters and drinking water was constantly available in ad libitum amounts. Free-stalls were bedded with sand and cleaned daily. Sufficient free-stalls were available to provide at least 1 free-stall per cow. Cows were fed at an ad libitum level through individual Calan gates (American Calan Inc., Northwood, NH) twice daily (0800 and 1300 h). The initial 14 d were used for adaptation to diets, and the subsequent 35 d were used to measure DMI and milk production.

Sampling and Analysis

Duplicate samples of each corn silage, alfalfa hay, and concentrate were collected weekly and composited monthly. Subsamples from each month were dried at 60ºC for 48 h in a forced draft oven, ground to pass 1 mm screen in a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) and analyzed for DM (105ºC for 8 h) and ash

(512ºC for 8 h). Concentrations of NDF and ADF were measured using the method of Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat stable α-amylase and sodium sulfite were used in the NDF analysis. Nitrogen was determined by rapid combustion using a Macro elemental N analyzer (Vario MAX CN, model ID 25.00-5003; Elementar, Hanau, Germany) and CP was calculated as N x 6.25. Subsamples of corn silage were submitted to Dairyland Laboratories, Inc. (Arcadia, WI) for analysis of yeast and mold counts using YM-11 agar (AOAC, 1995), VFA by HPLC (Canale et al., 1984), pH using an Orion 710+ electrode (Thermo Fisher Scientific, Waltham, MA), and ammonia-N by distillation (AOAC, 1995). Corn silage also was analyzed for aerobic stability by placing thermocouple wires at the center of a bag containing 1 kg of silage, within an open-top polystyrene box. The thermocouple wires were connected to data loggers (Campbell Scientific Inc., North Logan, UT) that recorded the temperature every 30 min for 12 d. The silages were covered with 2 layers of cheesecloth to prevent drying. Aerobic stability was denoted by the time that elapsed before a 2ºC rise in silage temperature above ambient temperature (23ºC). The quantity of spoiled (visibly heating, moldy, or darker) silage from each bag was weighed daily, composited weekly, and analyzed for DM (48 h at 60ºC).

Body weight and BCS were measured every week at the beginning and end of the trial. Body condition score was measured on a 1 to 5 scale (Wildman et al., 1982) and by the same trained observer. Blood samples were collected weekly by coccygeal venipuncture into vacutainer tubes containing sodium heparin anticoagulant (Fisher Scientific, Pittsburgh, PA). The blood was centrifuged at 2,500 x *g* for 20 min at 4ºC

and the plasma was frozen (-20ºC). A Technicon Autoanalyzer (Technicon Instruments Corp., Chauncey, NY) was used to measure plasma glucose (Bran and Luebbe Industrial Method 339–19; Gochman and Schmitz, 1972) and BUN (Bran and Luebbe Industrial Method 339–01; Marsh et al., 1965).

Cows were milked twice daily (1000 and 2100 h) and milk composition (fat, protein, and SCC) was measured on samples collected from a.m. and p.m. milkings on two consecutive days in each week. Milk samples were analyzed by Southeast Dairy labs (Belleview, FL) using a Bentley 2000 Near Infrared Reflectance Spectrophotometer (Bentley Instruments Inc., Chaska, MN). Somatic cell scores were generated as described by Norman et al. (2000) for statistical analysis of SCC.

In vivo apparent digestibility was estimated by using chromic oxide as a marker. Chromic oxide powder (Fisher Scientific, Fairlawn, NJ) was weighed into gelatin capsules (Jorgensen Lab. Loveland, CO) and dosed twice daily with a balling gun (10 g/dose at 0700 and 1900h) for 10 consecutive d. Fecal samples were collected at 0630 and 1830 h during the last 5 d of dosing. Feces were dried to a constant weight at 55ºC in a forced-air oven, ground to pass through a 1-mm screen in a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) and a composite sample was made from all 10 fecal samples per cow. Chromium concentration in the feces was determined by using a Perkin Elmer 5000 atomic absorption spectrometer (Perkin Elmer, Wellesley, MA). Apparent digestibility of CP, ADF and NDF were calculated by the marker ratio technique (Schneider and Flatt, 1975).

Statistical Analysis

The experiment had a completely randomized design. The data was analyzed with the MIXED procedure of SAS (SAS Institute, Inc. Cary, NC). A model containing

treatment, week (repeated measure), parity and all interactions of these terms was used to analyze all performance measurements except apparent in vivo digestibility, which was analyzed with a similar model excluding the effect of week. Cow nested in treatment x parity was used as the random term. Milk production during the first 21 d of lactation was used as a covariate for analyzing milk production data. Pretrial BW was used as a covariate for analyzing BW and BCS. The covariance structure used was AR(1). The F-protected least significant different test was used to compare least square means. Significance was declared at $P < 0.05$.

Results and Discussion

All silages had normal chemical composition except for relatively low NDF and ADF concentrations in B2 silage (Table 6-2). Chemical composition of silages did not differ ($P > 0.05$) among treatments.

The BUC silage had 51% greater aerobic stability (> 177 h) than other silages. A meta-analysis based on 23 published studies with 43 experiments indicated that applying *L. buchneri* to corn, grass and small-grain silages improved their aerobic stability (Kleinschmit and Kung, 2006a). The improved stability is attributable to inhibition of fungal growth by the acetate produced when *L. buchneri* ferments lactate into acetate and 1, 2 propanediol (Driehuis et al., 1999a) and the propionic acid sometimes formed from fermentation of the 1,2 propanediol by epiphytic *L. diolivorans* (Krooneman et al., 2002). This theory is supported by the numerically greater acetate and propionate concentrations in BUC versus the Control silages. Such differences have been statistically significant in most (Kleinschmit and Kung, 2006a, b; Huisden et al., 2009; Filya et al., 2007) but not all (Mari et al., 2009) other studies.

The B2 inoculant containing homolactic *Pentosaceus pedioccocus* and heterolactic, *Propionibacteria freudenrechii* may have not improved aerobic stability because of the types of bacteria it contained. Effects of homolactic bacteria on aerobic stability have been variable (Weinberg et al., 1993b; Driehuis et al., 2001) because the lactate they produce has weak antifungal properties and it can be used as a growth substrate by certain yeasts. Addition of propionibacteria to forages has resulted in inconsistent effects on concentrations of strong antifungal acids (propionate and acetate) and aerobic stability of silage (Weinberg et al., 1995; Higginbotham et al., 1996, 1998; Filya et al., 2006; Pedroso et al., 2010) because of the slow growth of propionic acid bacteria at acidic pH (Higginbotham et al., 1996; Weinberg et al., 1995). Effects of inoculants containing homolactic bacteria and *L. buchneri* like B500 on aerobic stability have been positive in some studies (Filya, 2003; Huisden et al., 2009) and dependent on ensiling duration or cultivar in others (Kleinschmit and Kung, 2006b; Kang et al., 2009).

Numerically ($P > 0.1$), the quantity of spoiled silage was 22% greater when B2 was applied, and 18 and 13% lower when BUC or B500 were applied, respectively. The lower spoilage losses of the BUC silage agrees with the greater aerobic stability of this silage and supports other studies showing that application of *L. buchneri* increased the aerobic stability of (Driehuis et al., 1999b; Mari et al., 2009) and decreased spoilage losses from (Queiroz et al., 2010) corn silage made in farm-scale silos.

No treatment x time interaction was detected for any of the animal measurements. Inoculant treatment did not affect intake of DM, CP, NDF, or ADF (Table 6-3). One of the early concerns about *L. buchneri* inoculation was that the high acetic acid

concentrations of inoculated silages would reduce feed intake in ruminant livestock. In agreement with previous studies (Driehuis et al., 1999b; Kendall et al., 2002; Taylor et al., 2002; Kung et al., 2003a), this study shows that inoculation with *L. buchneri* resulted in a trend for greater acetate concentration in the silage but this did not decrease DMI.

Inoculant treatment did not affect digestibility of DM or CP, but cows fed B2 had lower NDF (45.3 vs. 52.3 % of DM) and ADF (45.6 vs. 53.8 % of DM) digestibility than cows fed the control diet. Consequently, cows fed B2 had lower digestible NDF (2.5 vs. 3.0 kg/d) and ADF (1.6 vs. 2.0 kg/d) intake than cows fed the control diet. This may have been due to the numerically greater spoilage in the B2 silage. Whitlock et al. (2000) reported that DMI and NDF digestibility markedly decreased as the rate of inclusion of spoiled corn silage increased in a steer ration. Queiroz et al. (2010) also reported spoilage of corn silage resulted in decreased amounts of gross energy, CP, ash, NDF, and ADF in the silage.

Body weight, ADG, BCS, and plasma glucose concentration were not affected by treatment (Table 6-4), whereas PUN concentration was greater in cows fed inoculated silages instead of the Control silage (13.2 vs. 11.4 mg/dl). Inoculant application typically decreases the WSC concentration of silages (McDonald, 1991). Feeding such silages instead of the Control silage may have reduced ruminal ammonia utilization, leading to greater PUN concentrations.

The lower NDF and ADF digestibility of the B2 diet did not adversely affect milk production or composition (Table 6-5), possibly due to differences in NFC concentrations between diets. Milk yield, milk composition, milk component yield, somatic cell counts, and feed efficiency (kg milk/kg DMI) were unaffected by any

treatment. Milk production was increased by *L. buchneri*-treatment in only one (Kung et al., 2003a) of the previous studies that investigated effects of application of *L. buchneri* to silages on milk production by cows (Driehuis et al., 1999b; Kendall et al., 2002; Taylor et al., 2002; Kung et al., 2003a). The improvement in milk production occured when *L. buchneri* was applied to alfalfa at ensiling but application to corn and small-grain cereals in the other studies had no effect. Therefore, the milk production results in this study agree with those in most of the previous studies.

Conclusions

Inoculant application did not affect the fermentation of the corn silage. Inoculation with *L. buchneri* 40788 increased the aerobic stability of the silage and resulted in numerically less spoilage losses than the other treatments. Inoculation with combinations of *L. buchneri 40788* and *L. plantarum* or *Pedioccocus pentosaceus and Propionibacteria freudenrichii* had no effect on aerobic stability. Inoculant application did not affect the feed intake, milk yield or efficiency of feed utilization by dairy cows. These results show that treating corn silage with *L. buchneri* 40788 can improve the aerobic stability of corn silage but none of the inoculants improved the performance of dairy cows.

Table 6-1. Ingredient and chemical composition of the experimental diets.

Item	% of TMR (DM basis)
Ingredient composition	
Corn silage	44.0
Alfalfa hay	6.0
Citrus pulp	4.9
Corn gluten feed	4.6
Soy plus	9.1
Corn meal	17.9
Soybean meal	8.8
Mineral mix[1]	4.7

Chemical composition

	Treatments[2]			
	CON	B2	BUC	B500
DM, %	65.9	65.7	66.2	65.4
Ash, % DM	7.7	7.6	7.7	7.6
CP, % DM	18.2	18.1	18.3	18.3
NDF, % DM	29.9	26.9	28.9	28.6
ADF, % DM	18.7	16.7	17.6	17.8
Hemicellulose, % DM	11.2	10.2	11.2	10.8
NFC, % DM	40.8	43.9	41.8	42.1

[1]Mineral Mix contained 24.0% of CP, 9.5% of Ca, 1.0% of P, 7.0% of K, 2.5% of Mg, 7.9% of Na, 0.5% of S, 1400 mg/kg of Mn, 430 mg/kg of Cu, 1500 mg/kg of Se, 0.05 mg/kg of Cl, 15 mg/kg of I

[2] CON = Control, no inoculant; B2 = Biotal Plus II, containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*; BUC = Buchneri 40788, containing *Lactobacillus buchneri*; B500 = Buchneri B500, containing *P. pentosaceaus* and *L. buchneri*.

Table 6-2. Chemical composition and fermentation indices of corn silages.

Item	CON	B2+	BUC	500	SEM
DM, %	34.0	33.8	34.8	32.9	1.24
CP, % DM	8.8	8.7	9.2	9.1	0.15
NDF, % DM	45.6	38.7	43.0	42.6	0.557
ADF, % DM	28.9	24.3	26.3	26.8	0.43
pH	4.00	3.90	4.02	3.90	0.17
Ammonia-N, % of CP	7.33	8.39	7.61	7.32	0.95
Total acids, % DM	4.34	4.89	5.10	4.63	1.05
Lactic acid, La, % DM	2.27	2.75	2.52	2.47	0.62
Acetic acid, Ac, % DM	1.62	1.86	2.04	1.89	0.57
Propionic acid, % DM	0.43	0.27	0.47	0.25	0.14
Butyric acid, % DM	0.01	0.01	0.01	0.01	0.00
Isobutyric acid, % DM	0.01	0.01	0.07	0.01	0.06
Ethanol, % DM	0.01	0.01	0.01	0.01	0.00
La:Ac ratio	1.49	1.49	1.23	1.36	0.25
Molds, log cfu/g	3.25	3.25	3.58	3.25	0.55
Yeasts, log cfu/g	3.00	3.00	3.00	3.72	0.42
Aerobic stability, h	95.2[a]	85.1[a]	177.8[b]	77.5[a]	23.6
Spoiled corn silage, kg DM/d	60.0	73.3	49.4	52.5	8.2
Spoiled corn silage, % DM	24.9	27.4	20.7	22.2	-

Means in the same row with unlike superscripts differed ($P < 0.05$)

[1] CON = Control, no inoculant; B2 = Biotal Plus II, containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); BUC = Buchneri 40788, containing *Lactobacillus buchneri*; B500 = Buchneri 500, containing *P. pentosaceaus* and *L. buchneri*.

Table 6-3. Effect of applying different inoculants[1] to corn silage at ensiling on feed intake and digestibility in dairy cows.

Item	Control	B2	BUC	B500	SEM
Intake, kg/d					
DM	19.4	20.9	20.3	19.5	0.8
CP	3.5	3.8	3.7	3.6	0.2
NDF	5.8	5.6	5.8	5.6	0.2
ADF	3.6	3.5	3.6	3.5	0.2
Digestibility, %					
DM	73.5	73.6	74.8	73.5	0.8
CP	72.0	72.9	72.3	72.3	0.9
NDF	52.3[a]	45.3[b]	53.9[a]	52.8[a]	2.0
ADF	53.8[a]	45.6[b]	54.2[a]	53.8[a]	2.0
Digestible intake, kg/d					
DM	14.3	15.4	15.2	14.3	0.6
CP	2.5	2.8	2.7	2.6	0.1
NDF	3.0[a]	2.5[b]	3.2[a]	2.9[a]	0.1
ADF	2.0[a]	1.6[b]	1.9[a]	1.9[a]	0.1

Means in the same row with unlike superscripts differed ($P < 0.05$)

[1] CON = Control, no inoculant; B2 = Biotal Plus II, containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); BUC = Buchneri 40788, containing *Lactobacillus buchneri*; B500 = Buchneri 500, containing *P. pentosaceaus* and *L. buchneri*.

Table 6-4. Effect of applying different inoculants[1] to corn silage at ensiling on growth performance and plasma metabolites in dairy cows.

Item	CON	B2+	BUC	500	SEM
Growth performance					
Covariate adjusted	602	631	599	582	16
BW, kg					
ADG, kg/d	0.06	0.19	0.18	0.41	0.15
Covariate adjusted	2.49	2.52	2.47	2.45	0.04
BCS					
Plasma metabolites					
Glucose, mg/dl	67.2	67.5	67.3	66.9	1.1
Urea N, mg/dl	11.4[a]	13.3[b]	13.2[b]	13.0[b]	0.6

Means in the same row with unlike superscripts differed ($P < 0.05$)

[1] CON = Control, no inoculant; B2 = Biotal Plus II, containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); BUC = Buchneri 40788, containing *Lactobacillus buchneri*; B500 = Buchneri 500, containing *P. pentosaceaus* and *L. buchneri*.

Table 6-5. Effect of applying different inoculants[1] to corn silage at ensiling on milk yield, milk composition, and efficiency of feed utilization.

Item	CON	B2	BUC	B500	SEM
Milk yield, kg/d	32.4	33.3	33.3	30.3	1.3
3.5% FCM, kg/d	29.9	31.8	31.8	29.8	1.6
Milk fat, %	3.26	3.11	3.06	3.28	0.15
Milk protein, %	2.79	2.81	2.73	2.81	0.06
Milk fat, kg/d	1.01	1.06	1.05	1.01	0.07
Milk protein, kg/d	1.27	1.28	1.24	1.26	0.03
SCC, x 1000/ml	1247	589	1524	1005	463
Feed efficiency (kg milk/kg DMI)	1.56	1.54	1.57	1.55	0.07

Means in the same row with unlike superscripts differed ($P < 0.05$)

[1] CON = Control, no inoculant; B2 = Biotal Plus II, containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); BUC = Buchneri 40788, containing *Lactobacillus buchneri*; B500 = Buchneri 500, containing *P. pentosaceaus* and *L. buchneri*.

CHAPTER 7
GENERAL SUMMARY AND CONCLUSIONS

A series of experiments was conducted to determine effects of application of biological additives on the quality of dairy cattle feeds and the performance of dairy cows. The objective of Experiment 1 was to determine the effect of dietary addition of a fibrolytic enzyme preparation containing cellulase, xylanase and esterase activities on the performance of dairy cows fed low or high - concentrate diets. Sixty lactating Holstein cows in early lactation (22 $\pm$ 3 days in milk) were assigned to the following treatments: 1) Low concentrate (33%) diet (LC); 2) LC plus enzyme (LCE); 3) High concentrate (48%) diet (HC); 4) HC plus enzyme (HCE). The enzyme was sprayed at a rate of 3.4 mg of enzyme/g of DM on the TMR daily for 63 d. The first 14 d were used for adaptation to diets and the last 49 d for measurements. In addition, four ruminally-fistulated cows were used to determine dietary treatment effects on indices of ruminal fermentation and in situ DM degradation in the rumen. Enzyme application did not affect intake of DM but increased digestibility of DM, CP, ADF, and NDF and increased the efficiency of milk production. Increasing the concentrate level reduced ruminal pH but increased intakes of DM and CP, digestibility of DM and CP, and milk yield and milk protein yield. Cows fed LCE instead of HC had less DMI, similar milk yield and greater efficiency of milk production. Enzyme application did not affect ruminal pH or ruminal degradation of the diets. However, increasing the level of concentrate supplementation decreased the pH, increased the immediately soluble dietary fraction, and tended to decrease the potentially degradable fraction. In conclusion, application of the enzyme increased nutrient digestion and the efficiency of milk production by the cows.

Experiment 2 was designed to determine if the enzyme used in Experiment 1 primarily exerted its hydrolytic effect prior to ingestion or within the rumen. A second objective was to determine if the enzyme was more effective on specific components of the diet. Substrates were incubated in a buffer or a buffer - enzyme solution in triplicate for up to 24 h and chemical composition and DM disappearance were measured. In addition, DMD and NDFD were determined after untreated or enzyme-treated substrates were incubated in W or RF for a further 24 h after the initial incubation in the buffer or buffer-enzyme solution. Application of the enzyme reduced concentrations of NDF and hemicellulose, and increased water-soluble carbohydrate WSC concentration and DM disappearance. Incubation of enzyme-treated substrates in RF resulted in greater DMD than incubation in W except for AH, which had similar DMD in both media. Enzyme addition increased DMD and NDFD in W by 10 and 84% respectively, but had no effect on DMD and NDFD in RF; suggesting that preingestive effects of the enzyme were greater than ruminal effects. Enzyme effects on NDF, WSC, and hemicellulose concentration or DMD and NDFD in W or RF did not depend on the substrate. Therefore, this study provided no evidence that the enzyme preferentially hydrolyzed specific substrates and it suggested that preingestive effects of the enzyme were greater than ruminal effects under the conditions of the study.

Experiment 3 determined the effect of bacterial inoculants on the fermentation and quality of corn silages. A corn hybrid Vigoro 61R36 (Royster Clark, Inc.) was grown and harvested at 35% DM. Chopped corn forage was treated with 1) deionized water (CON); 2) Biotal Plus II (B2) inoculant containing *Pediococcus pentosaceus* and *Propionibacteria freudenreichii*); 3) Buchneri 40788 (BUC) inoculant containing

Lactobacillus buchneri; and 4) Buchneri 500 (B500) inoculant containing *P. pentosaceus* and *L. buchneri*. Four replicates of each treatment were weighed into polyethylene bags within 20-L mini silos, which were stored for 575 d at ambient temperature (25ºC) in a covered barn. After silos were opened, aerobic stability, chemical composition, and yeast and mold counts were determined. The DNA from treated and untreated silages was isolated using a lysozyme/sodium dodecyl sulfate lysis and phenol/chloroform extraction method. The DNA was used as a template for a conventional PCR with primers designed on the 16S rRNA gene to detect the presence of *L. buchneri* in silage samples. The WSC concentrations of all silages were reduced during the fermentation. However, B500 had the greatest residual WSC concentration, suggesting that plant sugars were less extensively fermented by the bacteria in this inoculant compared to those in other treatments. Dry matter loss was lower in BUC silages compared with Control and B2 silages. Control and B2 silages had higher pH and propionic acid concentration and lower lactic acid concentrations than other treatments. The greater lactate concentration and lower pH of BUC silages explain the lower DM loss from this silage. Acetate concentration was greatest in B2 silage, intermediate in Control and BUC silages, and lowest in B500 silage. However, aerobic stability was generally high (> 250 h) and was not improved by inoculant application. The PCR analysis confirmed the presence of similar populations of *L. buchneri* in all treatments, perhaps explaining why aerobic stability was high in all silages. The inoculants had differing effects on the fermentation of the silages with BUC producing the most desirable fermentation and least DM losses. However, none of the inoculants

improved aerobic stability, probably because all treatments had high populations of *L. buchneri.*

Experiment 4 determined the effect of applying three different bacterial inoculants to corn silage on the performance of lactating dairy cows. Corn plants were harvested, chopped, and ensiled in 2.4-m wide bag silos after application of the same treatments as in Experiment 1. Each of the 4 silages was mixed into separate TMR consisting of 44% corn silage, 50% concentrate and 6% alfalfa hay (DM basis). Fifty-two lactating Holstein cows in early lactation (22 DIM) were fed for 49 d. Chemical composition and yeast and mold counts of silages did not differ among treatments. Treatment with BUC improved silage aerobic stability by 200% and numerically resulted in the least losses compared with other treatments. Inoculant treatment did not affect DMI or digestibility of DM or CP. However, cows fed B2 had lower NDF and ADF digestibility than cows fed the control diet. Consequently, cows fed B2 had lower digestible NDF and ADF intake than cows fed the control diet. Nevertheless, milk yield, milk composition, and feed efficiency were not affected by treatment. Therefore, the inoculants did not affect the performance of the cows, but application of *L. buchneri* improved the aerobic stability of corn silage.

Experiment 1 demonstrated that fibrolytic enzymes containing cellulase, xylanase and esterase improved the nutrient digestion and efficiency of feed utilization by dairy cows in early lactation. That feeding the low concentrate enzyme-treated diet resulted in greater efficiency of milk production than the untreated high concentrate diet implies that the enzyme could be strategically used to reduce the level of concentrates in the diets of dairy cows and thereby reduce the risk of acidosis and associated metabolic

diseases without jeopardizing milk production. Future studies should examine the efficacy of the enzyme at other stages of lactation and determine the relative importance of the esterase, xylanase, and cellulase enzymes in the mixture. Such studies are needed to ascertain why this enzyme was effective at improving milk production because enzymes used in many similar studies have been ineffective.

Experiment 2 demonstrated that application of the enzyme increased cell wall hydrolysis, release of reducing sugars, and DMD and NDFD regardless of substrate type. This implies that there was no benefit to adding the enzyme to specific components of the diet instead of the TMR. Application of the enzyme to the TMR is attractive from the viewpoint of maximizing the distribution of the enzyme in the diet but this method could pose logistical difficulties for some producers.

That enzyme application to substrates incubated in water increased their DMD and NDFD, whereas application to substrates incubated in ruminal fluid only affected concentrations of fermentation products, suggests that the preingestive effect of the enzyme was important. Consequently, a preingestive enzyme – substrate interaction period should be ensured when fibrolytic enzymes are added to diets of dairy cows. However, the ideal duration of the period needs to be verified by research. Future research should also use longer incubation periods (30 and 48 h) to compare the preingestive and ruminal effects of the enzyme on the substrates.

Application of bacterial inoculants resulted in contrasting effects on the fermentation and aerobic stability of corn silage in the mini-silo (Experiment 3) and farm-scale silo experiments (Experiment 4) likely because of differences in the epiphytic bacterial populations of the untreated corn silages. In Experiment 3, the presence of *L.*

buchneri in Control silages probably prevented typical effects of inoculation with *L. buchneri* or *Propionibacteria* on aerobic stability. In Experiment 4, *L. buchneri* application produced the normal improvement in aerobic stabililty and resulted in numerically less spoilage losses than other treatments but feeding inoculated silages did not affect the performance of dairy cows. Factors that predispose to high epiphytic populations of *L. buchneri* on corn silages need to be determined because such silages may be inherently aerobically stable. Inoculants that are more potent and consistently effective at improving animal performance are needed. Therefore, in addition to screening inoculant bacteria for their fermentation enhancing and fungicidal properties, they should be selected based on their potential to improve nutrient digestion and utilization by dairy cows.

APPENDIX A
METHOD FOR MEASURING HEMICELLULASE ACTIVITY

Xylanase Assay

Procedure adapted from Bailey et al. (1992)

Xylose Standard

0.1 g of xylose per 100 ml distilled water (stock solution)

Make a serial dilution to obtain 0, 0.2, 0.4, 0.6, and 0.8 mg Xylose

a) 0 = 0 ml stock solution with 2 ml of distilled water
b) 0.2 = 0.2 ml stock solution with 1.8 ml of distilled water
c) 0.4 = 0.4 ml stock solution with 1.6 ml of distilled water
d) 0.6 = 0.6 ml stock solution with 1.4 ml of distilled water
e) 0.8 = 0.8 ml stock solution with 1.2 ml of distilled water

Reagents

Citrate phosphate buffer 0.1 M (pH 6.0):

0.3761 g of Citric acid
0.9114 g of Na_2HPO_4
Bring to 100 ml final volume with distilled water.

DNS reagent 1 L volume (store in a dark bottle for no more than 2 weeks)

10 g 3,5 dinitrosalacylic acid
10 g NaOH
200 g Sodium Potassium Tartrate
0.5 g Sodium Sulfite
2 g Phenol
Bring to 1 L final volume with distilled water

Substrate: 1% xylan from oat spelts.

1 g of xylan is homogenized in approximately 60 ml of distilled water at 60ºC
Heat and boil using a heating stirrer.
Allow to cool overnight with continuous stirring
Next day, bring to 100 ml final volume with distilled water

Blank preparation

Substrate blank: 0.1ml of substrate + 0.9 ml of buffer + 0.1 ml of water

Enzyme blank: 0.9 ml of buffer + 0.1 ml of enzyme (diluted in buffer) + 1.0 ml of distilled water

Assay

** using 15 ml tubes

1) Add 1.0 ml of substrate
2) Add 0.9 ml of buffer
3) Incubate at 39ºC for 10 min
4) Add 0.1 ml of enzyme
5) Incubate at 39ºC for 5 min
6) At this point, standard tubes and blanks should be included for the next steps
7) Add 3 ml of DNS reagent to terminate reaction
8) Boil in water bath for 5 min
9) Allow to cool in water
10) Read at 540 nm
11) Plot xylose standard
12) Plot samples against standard

The following equation is produced:

Xylose equivalents (mg) = $a + b \times x$

Where x is the absorbance obtained after correction for the enzyme and the substrate blanks. Xylose equivalents are plotted on the Y-axis and the absorbance values are plotted on the X-axis. The units of activity are then expressed as µmol xylose equivalents min^{-1} ml^{-1} of enzyme product.

**All assays should be carried out at least in triplicate.

APPENDIX B
METHODS FOR MEASURING CELLULASE ACTIVITY

Endoglucanase Assay

Procedure adapted from Wood and Bhat (1988).

Glucose Standard

0.1 g of glucose per 100 ml distilled water (stock solution)

Make a serial dilution to obtain 0, 0.2, 0.4, 0.6, and 0.8 mg Glucose

a) 0 = 0 ml stock solution with 2 ml of distilled water
b) 0.2 = 0.2 ml stock solution with 1.8 ml of distilled water
c) 0.4 = 0.4 ml stock solution with 1.6 ml of distilled water
d) 0.6 = 0.6 ml stock solution with 1.4 ml of distilled water
e) 0.8 = 0.8 ml stock solution with 1.2 ml of distilled water

Reagents

Citrate phosphate buffer 0.1 M (pH 6.0)

DNS reagent 1 L volume (store in a dark bottle for no more than 2 weeks)

Substrate: 1% (wt/vol) carboxymethylcellulose (CMC).

1 g of CMC is suspended in 100 ml of distilled water
Stir under gentle heat until completely dissolved.
Add sodium azide (0.02% wt/vol) to prevent microbial growth
Stored at 4ºC

Blank preparation

Substrate blank: 0.1ml of substrate + 0.9 ml of buffer + 0.1 ml of water

Enzyme blank: 0.9 ml of buffer + 0.1 ml of enzyme (diluted in buffer) + 1.0 ml of distilled water

Assay

** using 15 ml tubes

1) Add 1.0 ml of substrate

2) Add 0.9 ml of buffer
3) Incubate at 39ºC for 10 min
4) Add 0.1 ml of enzyme
5) Incubate at 39ºC for 5 min
6) At this point, standard tubes and blanks should be included for the next steps
7) Add 3 ml of DNS reagent to terminate reaction
8) Boil in water bath for 5 min
9) Allow to cool in water
10) Read at 540 nm
11) Plot glucose standard
12) Plot samples against standard

The following equation is produced:

Glucose equivalents (mg) = a + b × x

Where x is the absorbance obtained after correction for the enzyme and the substrate blanks, and glucose equivalents are placed on the Y-axis. The absorbance is plotted on the X-axis to allow a direct inclusion of its value into a spreadsheet. The units of activity are then expressed as μmol glucose equivalents min^{-1} ml^{-1} of enzyme product.

**All assays should be carried out at least in triplicate.

Exoglucanase Assay

Procedure adapted from Wood and Bhat (1988).

Glucose Standard

0.1 g of glucose per 100 ml distilled water (stock solution)

Make a serial dilution to obtain 0, 0.2, 0.4, 0.6, and 0.8 mg Glucose

a) 0 = 0 ml stock solution with 2 ml of distilled water
b) 0.2 = 0.2 ml stock solution with 1.8 ml of distilled water
c) 0.4 = 0.4 ml stock solution with 1.6 ml of distilled water
d) 0.6 = 0.6 ml stock solution with 1.4 ml of distilled water
e) 0.8 = 0.8 ml stock solution with 1.2 ml of distilled water

Reagents

Citrate phosphate buffer 0.1 M (pH 6.0)

DNS reagent 1 L volume (store in a dark bottle for no more than 2 weeks)

Substrate: 1% (wt/vol) microcrystalline cellulose (Avicel)

1 g of Avicel is suspended in 100 ml of distilled water
Stir continuously while pipeting (substrate is not soluble in water).

Blank preparation

Substrate blank: 0.1ml of substrate + 0.9 ml of buffer + 0.1 ml of water

Enzyme blank: 0.9 ml of buffer + 0.1 ml of enzyme (diluted in buffer) + 1.0 ml of distilled water

Assay

** using 15 ml tubes

1) Add 1.0 ml of substrate
2) Add 0.9 ml of buffer
3) Incubate at 39ºC for 10 min
4) Add 0.1 ml of enzyme
5) Incubate at 39ºC for 120 min
6) The tubes are then boiled for 10 min to terminate the reaction.

7) After cooling, the tubes are centrifuged at 1000 x g for 10 min
8) A 1.0 ml of sample is withdrawn from each tube and 1.0 ml of distilled water is added
9) At this point, standard tubes and blanks should be included for the next steps
10) Add 3 ml of DNS reagent
11) Boil in water bath for 5 min
12) Allow to cool in water
13) Read at 540 nm
14) Plot glucose standard
15) Plot samples against standard

The following equation is produced:

Glucose equivalents (mg) = a + b × x

Where x is the absorbance obtained after correction for the enzyme and the substrate blanks, and glucose equivalents are placed on the Y-axis. The absorbance is plotted on the X-axis to allow a direct inclusion of its value into a spreadsheet. The units of activity are then expressed as μmol glucose equivalents min^{-1} ml^{-1} of enzyme product.

**All assays should be carried out at least in triplicate.

LIST OF REFERENCES

Adesogan, A. T., N. Krueger, M. B. Salawu, D. B. Dean, and C. R. Staples. 2004. The influence of treatment with dual purpose bacterial inoculants or soluble carbohydrates on the fermentation and aerobic stability of bermudagrass. J. Dairy Sci. 87:3407-3416.

Adesogan, A. T. 2005. Improving forage quality and animal performance with fibrolytic enzymes. Pages 91-109 in Proc. 16th Florida Ruminant Nutrition Symposium. University of Florida, Gainesville, FL.

Adesogan, A. T. 2008. Recent advances in bacterial silage inoculant technology. Pages 81-96 in Proc. 19th Florida Ruminant Nutrition Symposium. University of Florida, Gainesville, FL.

Adesogan, A. T., O. C. M. Queiroz, and M. F. S. Queiroz. 2009. Effects of homolactic inoculants on silage nutrient digestibility. Pages 8-23 in Proc. Tri-State Dairy Nutrition Pre-Conference Symposium. Milwaukee, WI.

Agosin, E. and E. Odier. 1985. Solid-state fermentation, lignin degradation and resulting digestibility of wheat straw fermented by selected white-rot fungi. Appl. Microbiol. Biotechnol. 21:397-403.

Akin, D. E. 1986. Interaction of ruminal bacteria and fungi with southern forages. J. Anim. Sci. 63:962-977.

Akin, D. E. and R. Benner. 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl. Environ. Microbiol. 54:1117-1125.

Akin, D. E.1993. Perspectives of cell wall biodegradation-session synopsis. Pages 73-82 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASA, CSSA, SSSA, Madison, WI.

Albersheim, P., A. G. Darvill., K. R. Davis, J. M. Lau, M. McNeil, J. K. Sharp, and W. S. York. 1984. Why study the structure of biological molecules? Pages 19-51 in Structure, Function and Biosynthesis of Plant Cell Walls. W. M. Dugger and S. Bartnicki-Garcia, ed. Am. Soc. of Plant Physiologists, Rockville, MD.

Albrecht, K. A., W. F. Wedin, and D. R. Buxton. 1987. Cell-wall composition and digestibility of alfalfa stems and leaves. Crop Sci. 27:735-741.

Alii, I. R. Fairbairn, B. E. Baker, L. E. Philip, and H. Garino. 1983. Effects of anhydrous ammonia on fermentation of chopped, high-moisture ear corn. J. Dairy Sci. 66:2343-2348.

AOAC. 1995. Official Methods of Analysis. Vol. I. 16th ed. Association of Official Analytical Chemists, Washington, DC.

Arriola, K. G., A. T. Adesogan, S. C. Kim, T. W. Kang, C. M. Huisden, and C. R. Staples. 2007. Effect of fibrolytic enzyme application to diets differing in concentrate proportion on the performance of lactating dairy cattle. J. Dairy Sci. 90(Suppl. 1):338. (Abstr.).

Ashbell, G., Z. G. Weinberg, Y. Hen, and I. Filya. 2002. The effects of temperature on the aerobic stability of wheat and corn silages. J. Ind. Microbiol. Biotechnol. 28:261-263.

Atalla, R. H. 1990. The structures of cellulose. Pages 89-98 in Material interactions relevant to the pulp, paper, and wood industries: Proc. Materials Research Society Symposium. Caulfield, D. F., J. D. Passaretti, S. F. Sobczynski, ed. San Francisco, CA. Pittsburgh, PA.

Auerbach, H., E. Oldenburg, and F. Weissbach. 1998. Incidence of *Penicillium roqueforti* and roquefortine C in silages. J. Sci. Food Agric. 76:565-572.

Bacon, C. W., and D. M. Hinton. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Can. J. Bot. 74:1195-1202.

Bailey, M. J., P. Biely, and K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23:257–270.

Bal, M. A., J. G. Coors, and R. D. Shaver. 1997. Impact of the maturity of corn for use as silage in the diets of dairy cows on intake, digestion, and milk production. J. Dairy Sci. 80:2497-2503.

Barr, B. K., Y. –L. Hsieh, B. Ganem, and D. B. Wilson. 1996. Identification of two functionally different classes of exocellulases. Biochem. 35:586-592.

Barry, T. N., J. E. Cook, and R. J. Wilkins. 1978. The influence of formic acid and formaldehyde additives and type of harvesting machine on the utilization of nitrogen in Lucerne silages. 1. The voluntary intake and nitrogen retention of young sheep consuming the silages with and without intraperitoneal supplements of DL-methionine. J. Agric. Sci. (Camb.) 91:701-715.

Bass, J. M., J. J. Parkins, and G. Fishwick. 1982. The effect of calcium hydroxide treatment on the digestibility of chopped oat straw supplemented with a solution containing urea, calcium, phosphorus, sodium, trace elements and vitamins. Anim. Feed Sci. Technol. 7:93-100.

Bartolome, B., C. Faulds, M. Tuohy, G. P. Hazlewood, H. J. Gilbert, and G. Williamson. 1995. Influence of different xylanases on the activity of ferulic acid esterase on wheat bran. Biotechnol. Appl. Biochem. 22: 65-73.

Bartolome, B., C. Faulds, P. Kroon, K. Waldron, H. Gilbert, G. Hazlewood, and G. Williamson. 1997a. An *Aspergillus niger* esterase (ferulic acid esterase III) and a recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (Xy1D) release a 5-5' ferulic dehydrodimer (diferulic acid) from barley and wheat cell walls. Appl. Environ. Microbiol. 63: 208-212.

Bartolome, B., C. B. Faulds, and G. Williamson. 1997b. Enzymic release of ferulic acid from barley spent grain. J. Cereal Sci. 25:285-288.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75: 641-644.

Beauchemin, K. A., and L. M. Rode. 1996. Use of feed enzymes in ruminant nutrition. Pages 103-130 in Animal Science Research and Development - Meeting Future Challenges. L. M. Rode, ed. Minister of Supply and Services Canada, Ottawa, ON.

Beauchemin, K. A., W. Z. Yang, and L. M. Rode. 1999. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. J. Dairy Sci. 83:378-390.

Beauchemin, K. A., L. M. Rode, M. Maekawa, D. P. Morgavi, and R. Kampen. 2000. Evaluation of a nonstarch polysaccharidase feed enzyme in dairy cow diets. J. Dairy Sci. 83: 543-553.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81(E. Suppl. 2):E37-E47.

Beauchemin, K. A., D. Colombatto, and D. P. Morgavi. 2004. A rationale for the development of feed enzyme products for ruminants. Can. J. Anim. Sci. 84:23-36.

Bhat, K. M., A. J. Hay, M. Claeyssens, and T. M. Wood. 1990. Study of the mode of action and site specificity of the endo-(1-4)-β-D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-^{3}H labeled, reduced and chromogenic cello-oligosaccharides. Biochem. J. 266:371-378.

Bhat, K. M. and G. P. Hazlewood. 2001. Enzymology and other characteristics of cellulases and xylanases. Pages 11-60 in Enzymes in Farm Animal Nutrition. M. R. Bedford and G. C. Partridge, eds. CABI Publishing, Wallingford, UK.

Bidlack, J., M. Malone, and R. Benson. 1992. Molecular structure and component integration of secondary cell walls in plants. Proc. Okla. Acad. Sci. 72:51-56.

Biely, P., C. R. MacKenzie, J. Puls, and H. Schneider. 1986. Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. Bio/Tech. 4:731-733.

Biely, P., M. Vrsanska, and S. Kucar. 1992. Identification and mode of action of endo-(1-4)-β-xylanases. Pages 81-95 in Xylans and Xylanases. Progress in Biotechnology. Vol. 7. J. Visser, G. Beldan, M. A. Kusters-van Someren, and A. G. J. Voragen, eds. Elsevier, Amsterdam, The Netherlands.

Bolsen, K. K., A. Laytimi, R. Hart, L. Nutzbach, F. Niroomand, and L. Leipold. 1988. Effect of commercial inoculants on fermentation of 1987 crops. Kansas Agric. Exp. Stn. Rep. Prog. 539:137-153.

Bolsen, K. K., C. Lin, B. E. Brent, A. M. Feyerherm, J. E. Urban, and W. R. Aimutis. 1992. Effect of silage additives on the microbial succession and fermentation process of alfalfa and corn silages. J. Dairy Sci. 75:3066-3083.

Bolsen, K. K., D. R. Bonilla, G. L. Huck, M. A. Young, and R. A. Hart-Thakur. 1996. Effect of a propionic acid bacterial inoculant on fermentation and aerobic stability of whole-plant corn silage. Kansas Agric. Exp. Stn. Prog. Rep. 756:77-80.

Borneman, W. S., L. G. Ljungdahl, R. D. Hartley, and D. E. Akin. 1991. Isolation and characterization of *p*-coumaroyl esterase from anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. 57:2337-2344.

Bowman, G. R., K. A. Beauchemin, and J. A. Shelford. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. J. Dairy Sci. 85:3420-3429.

Britt, D. G., and J. T. Huber. 1975. Fungal growth during fermentation and refermentation of nonprotein nitrogen treated corn silage. J. Dairy Sci. 58:1666-1671.

Britt, D. G., J. T. Huber, and A. L. Rogers. 1975. Fungal growth and acid production during fermentation and refermentation of organic acid treated corn silages. J. Dairy Sci. 58:532-539.

Brown, W. F. 1988. Maturity and ammoniation effects on the feeding value of tropical grass hay. J. Anim. Sci. 66:2224-2232.

Brown, W. F., and W. E. Kunkle. 1992. Improving the feeding value of hay by anhydrous ammonia treatment. University of Florida, IFAS Extension. EDIS Reports. http://edis.ifas.ufl.edu/BODY_AA203 Accessed: August 29, 2009.

Burghardi, S. R., R. D. Goodrich, and J. C. Mashe. 1980. Evaluation of corn silage treated with microbial additives. J. Anim. Sci. 50:729-736.

Burritt, E. A., A. S. Bittner, J. C. Street, and M. J. Anderson. 1984. Correlations of phenolic acids and xylose content cell wall with in vitro dry matter digestibility of three maturing grasses. J. Dairy Sci. 67:1209-1213.

Buxton, D. R., and J. S. Hornstein. 1986. Cell wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Sci. 26:180-184.

Byott, G. S. 1976. Air space systems in C_3 and C_4 species. New Phytol. 76:295-299.

Cain, R. B. 1981. Microbial degradation of surfactants and “builder” components. Pages 325-370 in Microbial Degradation of Xenobiotics and Recalcitrant Compounds. Leisinger, T., R. Hutter, A. M. Cook, and J. Nucsch, ed. Academic Press Ltd., London.

Canale, A., M. E. Valente., and A. Ciotti. 1984. Determination of volatile carboxylic acids (C1-C5i) and lactic acid in aqueous acid extracts of silage by high performance liquid chromatography. J. Sci. Food and Agric. 35:1178-1182.

Chaves, C. M., J. E. Moore, H. A. Moye, and W. R. Ocumpaugh. 1982. Separation, identification and quantification of lignin saponification products extracted from digitgrass and their relation to forage quality. J. Anim. Sci. 54:196-203.

Chen, J., M. R. Stokes, and C. R. Wallace. 1994. Effect of enzyme-inoculant systems on preservation and nutritive value of haycrop and corn silage. J. Dairy Sci. 77:501-512.

Chen, K. H., J. T. Huber, C. B. Theurer, R. S. Swingle, J. Simas, S. C. Chan, Z. Wu, and J. L. Sullivan. 1994. Effect of steam-flaking of corn and sorghum grains on performance of lactating cows. J. Dairy Sci. 77:1038–1043.

Cheng, K. J., T. A. McAllister, L. B. Selinger, L. J. Yanke, H. D. Bae, C. W. Forsberg, and J. A. Shelford. 1995. Biotechnology in the feedlot. Proc. West. Sec. Am. Soc. Anim. Sci. Can. Soc. Anim. Sci. Mtg. 46:600-605.

Chesson, A. 1981. Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. J. Sci. Food Agric. 32:745-758.

Chesson, A., A. H. Gordon, and J. A. Lomax. 1983. Substituent groups linked by alkali-labile bonds to arabinose and xylose residues of legume, grass and cereal straw cell walls and their fate during digestion by rumen microorganisms. J. Sci. Food Agric. 34:1330-1340.

Chesson, A. 1994. Manipulation of fibre degradation: An old theme revised. Pages 83-98 in: T. P. Lyons and K. A. Jacques, ed. Biotechnology in the Feed Industry. Proc. Alltech’s 10th Ann. Symp. Nottingham University Press, Loughborough, U.K.

Claus, D., and R. C. W. Berkeley. 1986. Genus *Bacillus*. Pages 1105-1139 in Bergey’s Manual of Systematic Bacteriology. P. H. A. Sneath , N. S. Mair, M. E. Sharpe, and J. G. Holt, ed. Williams and Wilkins, Baltimore, MD.

Cole, R. J., J. W. Kirksey, J. W. Dorner, D. M. Wilson, J. Johnson, D. Bedell, J. P. Springer, K. K. Chexal, J. Clardy, and R. H. Cox. 1977. Mycotoxins produced by *Aspergillus fumigatus* isolated from silage. Ann. Nutr. Aliment. 31:685-691.

Coleman, S. W., J. E. Moore, and J. R. Wilson. 2004. Quality and Utilization. Pages 267-308 in Warm season grasses. L. E. Moser, B. L. Burson and L. E. Sollenberger , eds. ASA, Madison, WI.

Colombatto, D., F. L. Mould, M. K. Bhat, D. P. Morgavi, K. A. Beauchemin, and E. Owen. 2003a. Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms *in vitro*. J. Anim. Sci. 81: 1040–1050.

Colombatto, D., F. L. Mould, M. K. Bhat, and E. Owen. 2003b. Use of fibrolytic enzymes to improve the nutritive value of ruminant diets: A biochemical and in vitro rumen degradation assessment. Anim. Feed Sci. Technol. 107:201-209.

Colombatto, D., D. P. Morgavi, A. F. Furtado, and K. A. Beauchemin. 2003c. Screening of exogenous enzymes for ruminant diets: Relationship between biochemical characteristics and in vitro ruminal degradation. J. Anim. Sci. 81:2628-2638.

Coughlan, M. P., M. G. Tuohy, E. X. F. Filho, J. Puls, M. Clayessens, M. Vrsanská, and M. M. Hughes. 1993. Enzymological aspects of microbial hemicellulases with emphasis on fungal systems. Pages 53-84 in Hemicellulose and Hemicellulases. M. P. Coughlan and G. P. Hazlewood, eds. Portland Press, London.

Danner, H., M. Holzer., E. Mayrhuber, and R. Braun. 2003. Acetic acid increases stability of silage under aerobic conditions. Appl. Environ. Microbiol. 69: 562-567.

Dawson, T. E., S. R. Rust, and M. T. Yokoyama. 1998. Improved fermentation and aerobic stability of ensiled high moisture corn with the use of *Propionibacterium acidipropionici*. J. Dairy Sci. 81:1015-1021.

Dawson, K. A., and J. M. Tricarico. 1999. The use of exogenous fibrolytic enzymes to enhance microbial activities in the rumen and performance of ruminant animals. Pages 303-312 in Proc. 15th Ann. Symp. on Biotech. in the Feed Ind., Loughborough, Leics, UK.

Dean, D. B., A. T. Adesogan, N. Krueger, and R. C. Littell. 2005a. Effect of fibrolytic enzymes on the fermentation characteristics, aerobic stability, and digestibility of bermudagrass silage. J. Dairy Sci. 88:994-1003.

Dean, D. A. Adesogan, C. Staples, K. Arriola, S. Kim, N. Krueger, M. Huisden, S. Chikagwa, and B. Amaral. 2005b. The effect of method of dietary addition of a fibrolytic enzyme on the performance of lactating dairy cows. J. Dairy Sci. 88(Suppl. 1):341. (Abstr.).

Dengler, N. G., and T. Nelson. 1999. Leaf structure and development in C_4 plants. Pages 133-172 in C_4 Plant Biology. R.F. Sage and R. K. Monson, eds. Academic, Press, New York.

DePasquale, D. A., and T. J. Montville. 1990. Mechanism by which ammonium bicarbonate and ammonium sulfate inhibit mycotoxigenic fungi. Appl. Environ. Microbiol. 56:3711-3717.

Dhiman, T. R., M. S. Zaman, R. R. Gimenez, J. L. Walters, and R. J. Treacher. 2002. Performance of dairy cows fed forage treated fibrolytic enzymes prior to feeding. Anim. Feed Sci. Technol.101:115-125.

Driehuis, F., P. G. Van Wikselaar, A. M. van Vuuren, and S. F. Spoelstra. 1997. Effect of bacterial inoculants on rate of fermentation and chemical composition of high dry matter grass silages. J. Agricultural Sci. Cambridge. 128:323-329.

Driehuis, F., S. J. W. H. Oude Elferink, and S. F. Spoelstra. 1999a. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J. Appl. Microbiol. 87:583-594.

Driehuis, F., S. J. W. H. Oude Elferink, and P. G. Van Wikselaar. 1999b. *Lactobacillus buchneri* improves the aerobic stability of laboratory and farm scale whole crop maize but does not affect feed intake and milk production of dairy cows. Pages 106–107 in Proc. 12th Intl. Silage Conference, Uppsala, Sweden. Swedish Univ. Agric. Sci., Uppsala.

Driehuis, F., S. J. W. H. Oude Elferink, and P. G. Van Wikselaar. 2001. Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacilus buchneri*, with or without homofermentative lactic acid bacteria. Grass and Forage Sci. 56:330-343.

D'Mello, J. P. F., C. M. Placinta, and A. M. C. Macdonald. 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim. Feed Sci. Technol. 80:183-205.

Elwakeel, E. A., E. C. Titgemeyer, B. J. Johnson, C. K. Armendariz, and J. E. Shirley. 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. J. Dairy Sci. 90:5226-5236.

Ely, L. O., E. M. Sudweeks, and N. J. Moon. 1981. Inoculation with *Lactobacillus plantarum* of alfalfa, corn, sorghum, and wheat silages. J. Dairy Sci. 64:2378-2387.

Eun, J. –S., and K. A. Beauchemin. 2005. Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. J. Dairy Sci. 88:2140-2153.

Eun, J. –S., and K. A. Beauchemin. 2006. Supplementation with combinations of exogenous enzymes: Effects on in vitro fermentation of alfalfa hay and corn silage Final Report to Dyadic International Inc. Agriculture and Agri-Food Canada, Research Centre Report, Lethbridge, Canada.

Eun, J. –S., and K. A. Beauchemin. 2007. Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. J. Dairy Sci. 90:2839-2851.

Eun, J. –S., K. A. Beauchemin, and H. Schulze. 2007. Use of exogenous fibrolytic enzymes to enhance in vitro fermentation of alfalfa hay and corn silage. J. Dairy Sci. 90:1440-1451.

Fahey, G. C., L. D. Bourquin, E. C. Titgemeyer, and D. G. Atwell. 1993. Post harvest treatment of fibrous feedstuffs to improve their nutritive value. Pages 715-766 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASA, CSSA, SSSA, Madison, WI.

Feng, P., C. W. Hunt, G. T. Pritchard, and W. E. Julien. 1996. Effect of enzyme preparations on in situ and in vitro degradation and in vivo digestive characteristics of mature cool-season grass forage in beef steers. J. Anim. Sci. 74: 1349-1357.

Ferris, C. P., and C. S. Mayne. 1994. The effects of incorporating sugar-beet pulp with herbage at ensiling on silage fermentation, effluent output and in-silo losses. Grass and Forage Sci. 49:216-228.

Filya, I. 2003.The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. J. Dairy Sci. 86:3575-3581.

Filya, I., E. Sucu, and A. Karabulut. 2004. The effect of *Propionibacterium acidipropionici*, with or without *Lactobacillus plantarum*, on the fermentation and aerobic stability of wheat, sorghum and maize silages. J. Appl. Microbiol. 97:818-826.

Filya, I., E. Sucu, A. Karabulut. 2006. The effect of *Propionibacterium acidipropionici* and *Lactobacillus plantarum*, applied at ensiling, on the fermentation and aerobic stability of low dry matter corn and sorghum silages. J. Ind. Microbiol. Biotechnol. 33:353-358.

Filya, I., R. E. Muck, and F. E. Contreras-Govea. 2007. Inoculant effect on alfalfa silage: fermentation products and nutritive value. J. Dairy Sci. 90:5108-5114.

Fontes, C. M. G. A., J. Hall, B. H. Hirst, G. P. Hazelwood, and H. J Gilbert. 1995. The resistance of cellulases and xylanases to proteolysis inactivation. Appl. Microbiol. Biotechnol. 43: 52-57.

Forsberg, C. W., E. Forano, and A. Chesson. 2000. Microbial adherence to the plant cell wall and enzymatic hydrolysis. Pages 79-97 in Ruminant Physiology: Digestion , Metabolism, Growth and Reproduction. P. B. Cronje, ed. CABI Publishing, Wallingford, UK.

Gamble, G. R., A. Sethuraman, D. E. Akin, and K. E. Eriksson. 1994. Biodegradation of lignocelluloses in Bermuda grass by white rot fungi analyzed by solid-state ^{13}C nuclear magnetic resonance. Appl. Environ. Microbiol. 60:3138-3144.

Ghorbani, G. R., D. P. Morgavi, K. A. Beauchemin, and J. A. Z. Leedle. 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim. Sci. 80:1977-1985.

Giraffa, G., L. Rossetti, and E. Neviani. 2000. An evaluation of chelex-based DNA purification protocols for the typing of lactic acid bacteria. J. Microbiol. Methods. 42:175-184

Gochman, N., and J. M. Schmitz. 1972. Application of a new peroxide indicator reaction to the specific, automated determination of glucose with glucose oxidase. Clin. Chem. 18:943-952.

Goeger, D. E., R. T. Riley, J. W. Dorner, and R. J. Cole. 1988. Cyclopiazonic acid inhibition of the Ca2+-transport ATPase in rat skeletal muscle sarcoplasmic areticulum vesicles. Biochem. Pharmacol. 37:978-981.

Goering, H. K., and D. R. Waldo. 1980. Anhydrous ammonia addition to whole corn plant for ensiling. J. Dairy Sci. 63(Suppl. 1):183. (Abstr.).

Gonzalez, C., M. Proudfoot, G. Brown, Y. Korniyenko, H. Mori, A. Savchenko, and A. Yakunin. 2006. Molecular basis of formaldehyde detoxification. Characterization of two S-formyglutathione hydrolases from *Escherichia coli*, FrmB and YeiG. J.Biol. Chem. 281:12514-14522.

Grabber, J. H., J. Ralph, and R. D. Hatfield. 1998. Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. J. Agric. Food Chem. 46:2609-2614.

Haddad, S. G., R. J. Grant, and T. J. Klopfenstein. 1994. Digestibility of alkali-treated wheat straw measured in vitro or in vivo using Holstein heifers. J. Anim. Sci. 72:3258-3265.

Hameleers, A., K. A. Leach, N. W. Offer, and D. J. Roberts. 1999. The effect of incorporating sugar beet pulp with forage maize at ensiling on silage fermentation and effluent output using drum silos. Grass and Forage Sci.54:322-335.

Hanna, W. W., W. G. Monson, and G. W. Burton. 1973. Histological examination of fresh forage leaves after in vitro digestion. Crop Sci. 13:98-102.

Hartley, R. D. 1972. *p*-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility . J. Sci. Food Agric. 23:347-354.

Hatfield, R. D., and C. W. Ford. 1989. Phenolic constituents of plant cell walls and wall biodegradability. Pages 135-145 in Plant Cell Wall Polymers, Biogenesis and Biodegradation. Lewis, N. G., and M. G. Paice, eds. ACS Symposium Series 399, American Chemical Society, Washington, DC.

Hatfield, R. D., J. Ralph, and J. H. Grabber. 1999. Cell wall structural foundations: Molecular basis for improving forage digestibilities. Crop Sci. 39:27-37.

Hatfield, R., and W. Vermerris. 2001. Lignin formation in plants. The dilemma of linkage specificity. Plant Physiology. 126:1351-1357.

Hatfield, R. D., H. J. G. Jung, G. Broderick, and T. C. Jenkins. 2007. Nutritional chemistry of forages. Pages 467-485 in Forages: The Science of Grassland Agriculture. Vol. II. 6th ed. R.F. Barnes, C. J. Nelson, K. J. Moore and M. Collins, eds. Blackwell Publishing. Ames, IA.

Higginbotham G. E., E. J. DePeters, and S. C. Mueller.1996. Effect of propionic acid producing bacteria on corn silage fermentation. Prof. Anim. Sci. 12:176–180.

Higginbotham, G. E., S. C. Mueller, K. K. Bolsen, and E. J. DePeters**.** 1998. Effects of inoculants containing propionic acid bacteria on fermentation and aerobic stability of corn silage. J. Dairy Sci. 81:2185-2192.

Hoffman, P. C., and S. M. Ocker. 1997. Quantification of milk yield losses associated with feeding aerobically unstable high moisture corn. J. Dairy Sci. 80(Suppl. 1):234. (Abstr.).

Holm, J. I., I. Bjorck, A. Drews, and N. G. Asp. 1986. A rapid method for the analysis of starch. Starch/Die Starke. 7:224-226.

Holmes, B. J., and R. E. Muck. 1999. Factors affecting bunker silo densities. Available online: http://www.uwex.edu/ces/crops/uwforage/h&s-fp.htm. Accessed March 19, 2010

Holzer, M., E. Mayrhuber, H. Danner, and R. Braun. 2003. The role of *Lactobacillus buchneri* in forage preservation. TRENDS in Biotechnology. 21:282-287.

Horton, G. M. J., and G. M. Steacy. 1979. Effect of anhydrous ammonia treatment on the intake and digestibility of cereal straws by steers. J. Anim. Sci. 48:1239-1249.

Hristov, A. N., L. M. Rode, K. A. Beauchemin, and R. L. Wuerfel. 1996a. Effect of a commercial enzyme preparation on barley silage in vitro and in sacco dry matter degradability. Proc. West. Section, Am. Soc. Anim. Sci. 47:282-284.

Hristov, A. N., T. A. McAllister, K. –J. Cheng. 1996b. Exogenous enzymes for ruminants. Pages 51-61 in Proc. 17th Western Nutr. Conf. Edmonton, Alberta.

Hristov, A. N., T. A. Mcallister, R. J. Treacher, and K. -J. Cheng. 1997. Stability of exogenous polysaccharides-degrading enzymes in the rumen. In: Proc. West. Sect. Am. Soc. Anim. Sci. 48:145-148.

Hristov, A. N., T. A. McAllister, and K. –J. Cheng. 1998. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzymes on rumen fermentation and nutrient digestibility. J. Anim. Sci. 76:3146-3156.

Hristov, A. N., T. A. McAllister, and K. J. Cheng. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. J. Anim. Sci. 78: 477-487.

Hu, W., R. J. Schmidt, E. E. McDonell, C. M. Klingerman, and L. Kung Jr. 2009. The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus plantarum* MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. J. Dairy Sci. 92:3907-3914.

Huber, J. T., J. Foldager, and N.E. Smith. 1979. Nitrogen distribution in corn silage treated with varying levels of ammonia. J. Anim. Sci. 48:1509-1515.

Huber, J. T., N. E. Smith, and J. Stiles. 1980. Influence of time after ensiling on distribution of nitrogen in corn silage treated with ammonia. J. Anim. Sci. 51:1387-1392.

Huisden, C. M., A. T. Adesogan, S. C. Kim, and T. Ososanya. 2009. Effect of applying molasses or inoculants containing homofermentative or heterofermentative bacteria at two rates on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 92:690-697.

Humphreys, J. M., and C. Chapple. 2002. Rewriting the lignin roadmap. Current Opinion in Plant Biology. 5:224-229.

Iiyama, K., T. B. T. Lam, and B. A. Stone. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochem. 29: 733-737.

Iiyama, K., T. B. T. Lam, and B. A. Stone. 1994. Covalent cross-links in the cell wall. Plant Physiol. 104:315-320.

Jaakkola, S., P. Huhtanen, and K. Hissa. 1991. The effect of cell wall degrading enzymes or formic acid on fermentation quality and on digestion of grass silage by cattle. Grass Forage Sci. 46:75-87.

Jonsson, A., and G. Pahlow. 1984. Systematic classification and biochemical characterization of yeasts growing in grass silage inoculated with *Lactobacillus* cultures. Anim. Res. Dev. 20:7-22.

Jonsson, A., H. Lindberg, S. Sundas, P. Lingvall, and S. Lindgren. 1990. Effect of additives on quality of big-bale silage. Anim. Feed Sci. Technol. 31:139-155.

Jung, H. G., and M. S. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774-2790.

Jung, H. G., and F. M. Engels. 2002. Alfalfa stem tissues: cell wall deposition, composition, and degradability. Crop Sci. 42:524-534.

Kang, T. W., A. T. Adesogan, S. C. Kim, and S. S. Lee. 2009. Effects of an esterase-producing inoculant on fermentation, aerobic stability, and neutral detergent fiber digestibility of corn silage. J. Dairy Sci. 92:732-738.

Kendall, C., D. K. Combs, and P. C. Hoffman. 2002. Performance of dairy cattle fed high moisture shelled corn inoculated with *Lactobacillus buchneri*. J. Dairy Sci. 85(Suppl. 1):385. (Abstr.).

Kennedy, S. J. 1990. Comparison of the fermentation quality and nutritive value of sulphuric and formic acid-treated silages fed to beef cattle. Grass and Forage Sci. 45:17-28.

Khazaal, K. A., E. Owen, A. P. Dodson, J. Palmer, and P. Harvey. 1993. Treatment of barley straw with ligninase: effect on activity and fate of the enzyme shortly after being added to straw. Anim. Feed Sci. Technol. 41:15-21.

Kidd, K. K., and G. Ruano. 1995. Optimizing PCR. Pages 1-22 in PCR, a Practical Approach. M. J. McPherson, B. D. Hames, and G. R. Taylor, ed. Oxford University Press, Oxford, UK.

Kim, S. C., and A. T. Adesogan. 2006. Influence of ensiling temperature, simulated rainfall, and delayed sealing on fermentation characteristics and aerobic stability of corn silage. J. Dairy Sci. 89:3122-3132.

Kirk, T. K., and R. L. Farrell. 1987. Enzymatic “combustion”: The microbial degradation of lignin. Ann. Rev. Microbiol. 41:465-505.

Kleinschmit, D. H., R. J. Schmidt, and L. Kung, Jr. 2005. The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 88:2130-2139.

Kleinschmit, D.H., and L. Kung Jr. 2006a. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89:4005-4013.

Kleinschmit, D. H., and L. Jr. Kung. 2006b. The effects of *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* R1094 on the fermentation of corn silage. J. Dairy Sci. 89:3999-4004.

Klyosov, A. 1990. Trends in biochemistry and enzymology of cellulose degradation. Biochem. 29: 10577-10585.

Knowlton, K. F., J. M. McKinney, and C. Cobb. 2002. Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation Holstein cows. J. Dairy Sci. 85: 3328–3335.

Kopecny, J., M. Marounek, and K. Holub. 1987. Testing the suitability of the addition of *Trichoderma viride* cellulases to feed rations for ruminants. Zivocisna Vyroba. 32:587-590.

Kosugi, A., K. Murashima, and R. H. Doi. 2002. Characterization of noncellulosomal subunits, ArfA and BgaA, from *Clostridium cellulovorans* that cooperate with the cellulosome in plant cell wall degradation. J. Bacteriol. 184:6859-6865.

Koukiekolo, R., H. Cho, A. Kosugi, M. Inui, H. Yukawa, and R. H. Doi. 2005. Degradation of corn fiber by *Clostridium cellulovorans* cellulases and hemicellulases and contribution of scaffolding protein CbpA. Appl. Environ. Microbiol. 71:3504-3511.

Krause, M., K. A. Beauchemin, L. M. Rode, B. I. Farr, and P. Norgaard. 1998. Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. J. Anim. Sci. 76:2912-2920.

Kroon, P. A., C. B. Faulds, and G. Williamson. 1996. Purification and characterization of a novel esterase induced by growth of *Apergillus niger* on sugar-beet pulp. Biotechnol. Appl. Biochem. 23:255-262.

Krooneman, J., F. Faber, A. C. Alderkamp, S. J. H. W. Oude Elferink, F. Driehuis, I. Cleenwerck, J. Swings, J. C. Gottschal, and M. Vancanneyt. 2002. *Lactobacillus diolivorans* sp. Nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Systematic Evol. Microbiol. 52:639-646.

Krueger, N. A., and A. T. Adesogan. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. Anim. Feed Sci. Technol. 145:84-94.

Krueger, N. A., A. T. Adesogan, C. R. Staples, W. K. Krueger, D. B. Dean, R. C. Littell. 2008a. The potential to increase digestibilitly of tropical grasses with a fungal, ferulic acid esterase enzyme preparation. Anim. Feed Sci. Technol. 145:95-108.

Krueger, N. A, A.T. Adesogan, C. R. Staples, W. K. Krueger, S. C. Kim, R. C. Littell, and L. E. Sollenberger. 2008b. Effect of method of applying enzymes or ammonia to bermudagrass hay on feed intake, digestion, and growth of beef steers. J. Anim. Sci. 86:882-889.

Kung, L. Jr., R. S. Tung, K. G. Maciorowski, K. Buffum, and K. Knutsen. 1991. Effects of plant cell-wall degrading enzymes and lactic acid bacteria on silage fermentation and composition. J. Dairy Sci. 74:4284-4296.

Kung, L. Jr., J. H. Chen, E. M. Kreck, and K. Knutsen. 1993. Effect of microbial inoculants on the nutritive value of corn silage for lactating dairy cows. J. Dairy Sci. 76:3763-3770.

Kung, L. Jr., A. C. Sheperd, A. M. Smagala, K. M. Endres, C. A. Bessett, N. K. Ranjit, and J. L. Glancey. 1998. The effect of preservatives based on propionic acid on the fermentation and aerobic stability of corn silage and a total mixed ration. J. Dairy Sci. 81:1322-1330.

Kung, L. Jr., J. R. Robinson, N. K. Ranjit, J. H. Chen, C. M. Golt, and J. D. Pesek. 2000a. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83:1479-1486.

Kung, L. Jr., R. J. Treacher, G. A. Nauman, A. M. Smagala, K. M. Endres, and M. A. Cohen. 2000b. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. J. Dairy Sci. 83:115-122.

Kung, L. Jr. 2001. Direct-fed microbials for dairy cows and enzymes for lactating dairy cows: New theories and applications. Pages 22-42 in Proc. 12th Annual Florida Ruminant Nutr. Symp. University of Florida. Gainesville, FL.

Kung, L. Jr., and N. K. Ranjit. 2001. The effect of *Lactobacillus buchneri* and other additives on the fermentation and aerobic stability of barley silage. J. Dairy Sci. 84:1149-1155.

Kung, L. Jr., M. A. Cohen, L. M. Rode, and R. J. Treacher. 2002. The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ration to lactating dairy cows. J. Dairy Sci. 85:2396-2402.

Kung, L. Jr., C. C. Taylor, M. P. Lynch and J. M. Neylon. 2003a. The effect of treating alfalfa with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for lactating dairy cows. J. Dairy Sci. 86:336-343.

Kung, L. Jr., M. R. Stokes, and C. J. Lin. 2003b. Silage additives. Pages 305-360 in Silage Science and Technology. D. R. Buxton, R. E. Muck, and J. H. Harrison, eds. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. Publications, Madison, WI.

Kung, L. Jr., R. J. Schmidt, T. E. Ebling, and W. Hu. 2007. The effect of *Lactobacillus buchneri* 40788 on the fermentation and aerobic stability of ground and whole high-moisture corn. J. Dairy Sci. 90:2309-2314.

Kung, L. Jr. 2009. Potential factors that may limit the effectiveness of silage additives. Pages 37-45 in Proc 15th Int. Silage Conf. Madison, WI.

Kunkle, W. E., R. Goff, and J. Durrance. 1983. Anhydrous ammonia treatment of low quality hay. Florida Beef Research Reports. http://www.animal.ufl.edu/extension/beef/pubs.html Accessed: September 8, 2009.

Lacey, J. 1989. Pre- and post-harvest ecology of fungi causing spoilage of foods and other stored products. J. Appl. Bacteriol. 67(Suppl.), 11S-25S.

Ladish, M. R., K. W. Lin, M. Voloch, and G. T. Tsao. 1983. Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb. Technol. 5:82-100.

Lam, T. B. T, K. Iiyama, and B. A. Stone. 1990. Primary and secondary walls of grasses and other forage plants: taxonomic and structural consideration. Pages 43-69 in Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants. Akin, D. E., M. G. Ljundahl, R. J. Wilson and P. J. Harris, ed. Elseiver, New York.

Lam, T. B. T, K. Iiyama, and B. A. Stone. 1992. Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes. Phytochem. 31: 1179-1183.

Lancaster, R. J., and L. F. C. Brunswick. 1977. Optimum rate of application of formic acid during ensiling of unwilted lucerne. N.Z. J. Agric. Res. 20:151-156.

Langston, C. W., and C. Bouma. 1960a. A study of the microorganisms of grass silage. I. The cocci. Appl. Microbiol. 8:212-222.

Langston, C. W., and C. Bouma. 1960b. A study of the microorganisms of grass silage. II. The lactobacilli. Appl. Microbiol. 8:223-234.

Leaver, J. D. 1975. The use of propionic acid as an additive for maize silage. J. Br. Grassl. Soc. 30:17-21.

Leibeinsperger, R. Y., and R. E. Pitt. 1988. Modeling the effects of formic acid and molasses on ensilage. J. Dairy Sci. 71:1220-1231.

Lewis, G. E.,C. W. Hunt, W. K. Sanchez, R. J. Treacher, G. T. Pritchard, and P. Feng. 1996. Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. J. Anim. Sci. 74:3020-3028.

Lewis, G. E., W. K. Sanchez, C. W. Hunt, M. A. Guy, G. T. Pritchard, B. I. Swanson, and R. J. Treacher. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. J. Dairy Sci. 82:611-617.

Lindgren, S., K. L. Petterson, A. Jonsson, P. Lingvall, and A. Kaspersson. 1985a. Silage inoculation: Selected strains, temperature, wilting and practical application. Swed. J. Agric. Res. 15:9-18.

Lindgren, S., K. L. Petterson, A. Kaspersson, A. Jonsson, and P. Lingvall. 1985b. Microbial dynamics during aerobic deterioration of silages. J. Sci. Food Agric. 36:765-774.

Llamas-Lamas, G., and D. K. Combs. 1991. Effect of forage to concentrate ratio and intake level on utilization of early vegetative alfalfa silage by dairy cows. J. Dairy Sci. 74:526-536.

Long, S. P. 1999. Environmental responses. Pages 215-249 in C_4 Plant Biology. R. F. Sage and R. K. Monson, ed. Academic Press, New York.

Mahanna, B., and L. E. Chase. 2003. Practical applications and solutions to silage problems. Pages 855—895 in Silage Science and Technology (Agronomy Series No. 42). Buxton D. R., R. E. Muck, and H. J. Harrison eds. American Society of Agronomy, Madison, WI:

Mari, L. J., R. J. Schmidt, L. G. Nussio, C. M Hallada, and L. Kung Jr. 2009. Short communication: An evaluation of the effectiveness of *Lactobacillus buchneri* 40788 to alter fermentation and improve the aerobic stability of corn silage in farm silos. J. Dairy Sci. 92:1174-1176.

Marsh, W. H., B. Fingerhut, and H. Miller. 1965. Automated and manual direct methods for the determination of blood urea. Clinical Chemistry. 11:624-627.

Masai, E., Y. Katayama, S. Nishikawa, M. Yamasaki, N. Morohoshi, and T. Haraguchi. 1989. Detection and localization of a new enzyme catalyzing the β-aryl ether cleavage in the soil bacterium (*Pseudomonas paucimobilis* SYK-6). FEBS Lett. 249:348-352.

McAllister, T. A., S. J. Oosting, J. D. Popps, Z. Mir, L. J. Yanke, A. N. Histrov, R. J. Treacher, and K. J. Cheng. 1999. Effects of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. Can. J. Anim. Sci. 79:353-360.

McAllister, T. A., and A. N. Hristov. 2000. The fundamentals of making good quality silage. Advances in Dairy Technology. 12:381-399.

McAllister, T. A., A. N. Hristov, K. A. Beauchemin, L. M. Rode, and K. -J. Cheng. 2001. Enzymes in Ruminant Diets. Pages 273-298 in Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge, ed. CABI Publishing. Wallingford, UK.

McDonald, I. 1981. A revised model for the estimation of protein degradability in the rumen, J. Agric. Sci. (Cambridge) 96: 251–252.

McDonald, P., A. R. Henderson, and S. J. E. Heron. 1991. The Biochemistry of Silage. 2nd ed. Chalcombe Publications. Marlow, UK.

McDonald, P., A. R. Henderson, and R. Whittenbury. 1996. The effect of temperature on ensilage. J. Sci. Food Agric. 17:476-480.

McSweeney, C. S., A. Dulieu, Y. Katayama, and J. B. Lowry. 1994. Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. Appl.Environ. Microbiol. 60:2985-2989.

Middelhoven, W. J., and A. H. M. van Baalen. 1988. Development of the yeast flora of wholecrop maize during ensiling and subsequent aerobiosis. J. Sci. Food Agric. 42:199-207.

Miller, W. J., and G. D. O'Dell. 1969. Nutritional problems of using maximum forage or maximum concentrates in dairy rations. J. Dairy Sci. 52:1144.

Ministry of Agriculture, Fisheries and Food. 1986. The Analysis of Agricultural Materials. Reference Book 427. HMSO, London.

Moon, N. J., L. O. Ely, and E. M. Sudweeks. 1980. Aerobic deterioration of wheat, lucerne and maize silage prepared with *Lactobacillus acidophilus* and *a Candida sp.* J. Appl. Bacteriol. 49:75-87.

Moon, N. J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. 55:454-460.

Moore, K. J., K. J. Boote, and M. A. Sanderson. 2004. Physiology and developmental morphology. Pages 179-216 in Warm Season (C_4) Grasses. L. E. Moser, B. L. Burson, and L. E. Sollenberger, ed. ASA. CSSA. SSSA. Madison, WI.

Moran, J. P., and T. R. Owen. 1994. The effects of Ecosyl treated silage on milk production by lactating cows. Page 126 in Proc. Natl. Conference on Forage Quality, Evaluation and Utilization. Univ. of Nebraska, Lincoln, NE.

Moran, J. P., and T. R. Owen. 1995. The effects of feeding silage treated with an inoculum of *Lactobacillus plantarum* on beef production from growing and finishing cattle. Ann. Zootech. 44(Suppl.):383.

Morgavi, D. P., K. A., Beauchemin, V. L. Nsereko, L. M. Rode, A. D. Iwaasa, W. Z. Yang, T. A, McAllister, and Y. Wang. 2000. Synergy between ruminal fibrolytic enzymes and enzymes from *Trichoderma Longibrachiatum*. J. Dairy. Sci. 83:1310-1321.

Moseley, G., and V. Ramanathan. 1989. The effect of dry feed additives on the nutritive value of silages. Grass and Forage Sci. 44:391-397.

Moss, R. A., D. I. Givens, and J. M. Everington. 1990. The effect of sodium hydroxide treatment on the chemical composition, digestibility and digestible energy content of wheat, barley and oat straws. Anim. Feed Sci. Technol. 29:73–87.

Mueller-Harvey, I., R. D. Hartley, P. J. Harris, and E. H. Curzon. 1986. Linkage of pcoumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carb. Res. 148: 71-85.

Muck, R. E. 1988. Factors influencing silage quality and their implications for management. J. Dairy Sci. 71:2992-3002.

Muck, R. E., and J. T. Dickerson. 1988. Storage temperature effects on proteolysis in alfalfa silage. Trans. ASAE 31:1005–1009.

Muck, R. E. 1989. Initial bacterial numbers on Lucerne prior to ensiling. Grass Forage Sci. 44:19-25.

Muck, R. E., and K. K. Bolsen. 1991. Silage preservation and silage additive products. Page 105 in Field Guide for Hay and Silage Management in North America. K. K. Bolsen, J. E. Baylor, and M. E. McCullough, eds. Natl. Feed Ingred. Assoc., West Des Moines, IA.

Muck, R. E. 1993. The role of silage additives in making high quality silage. Pages 106-116 in Silage Production from Seed to Animal. Northeast Reg. Agric. Eng. Serv., Ithaca, NY. NRAES-67.

Muck, R. E. 1996. A lactic acid bacteria strain to improve aerobic stability of silages. Pages 42–43 in Research Summaries. U.S. Dairy Forage Research Center, Madison, WI.

Muck, R. E., and L. Kung, Jr. 1997. Effects of silage additives on ensiling. Pages 187-199 in Silage: Field to Feedbunk. North Am. Conf. Hershey, PA. NRAES-99.

Muck, R. E. 2001. Corn silage inoculants that work. Page 1 in Proc. Forage Teaching and Technol. Conf. University of Wisconsin Extension, Madison, WI.

Muck, R. E., L. E. Moser, and R. E. Pitt. 2003. Postharvest factors affecting ensiling. Pages 251-304 in Silage Science and Technology. D. R. Buxton, R. E. Muck, and J. H. Harrison, ed. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. Publications, Madison, WI.

Muck, R. E. 2004. Effects of corn silage inoculants on aerobic stability. Trans. ASAE. 47 (4):1011-1016.

Muck, R. E., and L. Kung Jr. 2007. Silage production. Pages 617-633 in Forages: The Science of Grassland Agriculture. Vol. II. 6th ed. R. F. Barnes, C. J. Nelson, K. J. Moore, and M. Collins, ed. Blackwell Publishing, Ames, IA.

Mulrooney, C. N., and L. Kung Jr. 2008. Short communication: The effect of water temperature on the viability of silage inoculants. J. Dairy Sci. 91:236-240.

Munkvold, G. 2003. Cultural and generic approaches to managing mycotoxins in maize. Ann. Rev. Phytopath. 41:99-116.

Murashima, K., A. Kosugi, and R. H. Doi. 2003. Synergystic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. J. Bacteriol. 185:1518-1524.

Nagaraja, T. G., and G. Town. 1990. Ciliated protozoa in relation to ruminal acidosis and lactic acid metabolism. Page 194 in Rumen Ecosystem: Microbial Metabolism and Regulation. R. Oneora, H. Minato, and H. Itabashi, ed. Springer-Verlag, New York, NY.

Nagel, S. A., and G. A. Broderick. 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. J. Dairy Sci. 75:140-154.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

Nelson, C. J. 1995. Photosynthesis and carbon metabolism. Pages 31-43 in Forages: An Introduction to Grassland Agriculture. R. F. Barnes, D. A. Miller, and C. J. Nelson, ed. Iowa State Univ. Press. Ames.

Neylon, J. M., and L. Kung Jr. 2003. Effects of cutting height and maturity on the nutritive value of corn silage for lactating cows. J. Dairy Sci. 86:2163-2169.

Nishino, N., H. Wada, M. Yoshida, and H. Shiota. 2004. Microbial counts, fermentation products, and aerobic stability of whole crop corn and a total mixed ration ensiled with and without inoculation of *Lactobacillus casei* or *Lactobacillus buchneri*. J. Dairy Sci. 87:2563-2570.

Nocek, J. E., and S. Tamminga. 1991. Site of digestion of starch in the grastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci. 74:3598-3629.

Noel, R. J., and L. G. Hambleton. 1976. Collaborative study of a semi automated method for determination of crude protein in animal feeds. J. Assoc. Off. Anal. Chem. 59: 134-140.

Norman, H. D., R. H. Miller, J. R. Wright, and G. R. Wiggans. 2000. Herd and state means for somatic cell count from dairy herd improvement. J. Dairy Sci. 83:2782–2788.

Nout, M. J. R., H. M. Bouwmeester, J. Haaksma, and H. van Dijik. 1993. Fungal growth in silages of sugarbeet press pulp and maize. J. Agric. Sci. 121:323-326.

Nsereko, V. L., D. P. Morgavi, K. A. Beauchemin, A. F. Rode, and T. A. McAllister. 2000a. Effects of feeding fungal feed enzyme preparation on the rumen microbial population. Reprod. Nutr. Dev. 40:219-225.

Nsereko, V. L., D. P. Morgavi, L. M. Rode, K. A. Beauchemin, and T. A. McAllister. 2000b. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay fiber by mixed rumen microorganisms in vitro. Anim. Feed Sci. Technol. 88:153-170.

Nsereko, V. L., K. A. Beauchemin, D. P. Morgavi, L. M. Rode, A. F. Furtado, T. A. McAllister, A. D. Iwaasa, W. Z. Yang, and Y. Wang. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. Can. J. Microbiol. 48:14–20.

Nussio, L. G.,J. T. Huber, C. B. Theurer, C. B. Nussio, J. Santos, M. Tarazon, R. O. Lima-Filho, B. Riggs, M. Lamoreaux, and R. J. Treacher. 1997. Influence of cellulase/xylanase complex (C/X) on lactational performance of dairy cows fed alfalfa hay (AH) based diets. J. Dairy Sci. 80 (Suppl.1):220 (Abstr.).

Oba, M., and M. S. Allen. 1999. Effects of brown midrib 3 mutation in corn silage on dry matter intake and productivity of high yielding dairy cows. J. Dairy. Sci. 82:135-142.

Ocumpaugh, W. R., C. Moore, C. Schultz, and D. Martinez. 1984. Steer performance on ammonia treated Coastal bermudagrass hay. Forage Research in Texas, Texas Agric. Exp. Stn. CPR-4253:9-12.

O'Kiely, P., R. E. Muck, and P. L. O'Connor. 1987. Aerobic deterioration of alfalfa and maize silage. Irish Grassland Anim. Prod. Assoc. J. 21:145.

Ostling, C. E., and S. E. Lindgren. 1991. Bacteria in manure and on manured and NPK-fertilized silage crops. J. Sci. Food Agric. 55:579-588.

Oude Elferink, S. J. W. H., F. Driehuis, J. Krooneman, J. C. Gottschal, and S. F. Spoelstra. 1999. *Lactobacillus buchneri* can improve the aerobic stability of silage via a novel fermentation pathway, the anaerobic degradation of lactic acid to acetic acid and 1,2-propanediol. Pages 266-267 in T. Pauly (ed.) Proc. 12th Intl. Silage Conference. Uppsala, Sweden.

Oude Elferink, S. J. W. H., J. Krooneman, J. C. Gottschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl. Environ. Microb. 67:125–132.

Owen, E., T. J. Klopfenstein, and N. A. Urio. 1984. Treatment with other chemicals. Pages 248-275 in Straw and Other Fibrous By-products as Feed. F. Sundstol and E. Owen, ed. Elsevier, Amsterdam.

Pahlow, G., and E. Zimmer. 1985. Effect of a Lactobacillus inoculant on fermentation and aerobic stability of grass silage. Pages 24-31 in Proc. 15th Intl. Grassld. Congress, Kyoto, Japan. Institute of Grassland and Forage Research, FAL, Braunschweig, Fed. Rep. Germany.

Pahlow, G., R. E. Muck, F. Driehuis, S. J. W. H. Oude Elferink, and S. F. Spoelstra. 2003. Microbiology of ensiling. Pages 31-93 in Silage Science and Technology (Agronomy Series No. 42). D. R. Buxton, R. E. Muck, and H. J. Harrison, eds. American Society of Agronomy. Madison, WI.

Patterson, J. A., R. Stock, and T. J. Klopfenstein. 1980. Calcium hydroxide treatment. Nebraska Beef Cattle Report, EC 80:218-221.

Payne, G. A. 1999. Compendium of corn diseases. 3rd ed. White, D.G. ed. APS Press., St. Paul, MN.

Pedroso, A. F., L. G. Nussio, S. F. Panziani, D. R. S. Loures, M. S. Igarasi, L. J. Mari, R. M. Coelho, J. L. Ribeiro, M. Zopollatto, and J. Horii. 2002. Bacterial inoculants and chemical additives to improve fermentation in sugar cane (*Sccharum officinarum*) silage. Proc. 13th Intl. Silage Conference. SAC, Ayr, UK.

Pedroso, A. F., A. T. Adesogan, O. C. M. Queiroz, and S. K. Williams. 2010. Control of *Escherichia coli* O157:H7 in corn silage with or without various inoculants: Efficacy and mode of action. J. Dairy Sci. 93:1098-1104.

Pendleton, B. 2000. The regulatory environment. Pages 49-55 in Direct-fed Microbial, Enzyme and Forage Additive Compendium. S. Muirhead, ed. The Miller Publishing Company, Minnetonka, MN.

Phipps, R. H., J. D. Sutton, D. E. Beever, M. K. Bhat, G. F. Hartnell, J. Vicini, and D. L. Hard. 2000. Effect of cell-wall degrading enzymes and method of application on feed intake and milk production of Holstein-Friesian dairy cows. J. Dairy Sci. 83(Suppl. 1): 236-237 (Abstr.).

Pond, K. R., W. C. Ellis, and D. E. Akin. 1984. Ingestive mastication and fragmentation of forages. J. Anim. Sci. 58:1567-1574.

Queiroz, O. C. M., A. T. Adesogan, K. G. Arriola, and M. F. Queiroz. 2010. Effect of a bacterial inoculant on the quality of and nutrient losses from corn silage produced in farm-scale silos. ADSA-PSA-AMPA-CSAS-ASAS Joint Annual Meeting. Denver, CO. (Abstr.). In Press.

Ralph, J., R. D. Hatfield, S. Quideau, R. F. Helm, J. H. Grabber, and H. –J. G. Jung. 1994. Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. J. Am. Chem. Soc. 116:9448-9456.

Rammer, C., C. Ostling, P. Lingvall, and S. Lindgren. 1994. Ensiling of manured crops - Effects on fermentation. Grass Forage Sci. 49:343-351.

Randby, A. T., I. Selmer-Olsen, and L. Baevre. 1999. Effect of ethanol in feed on milk flavor and chemical composition. J. Dairy Sci. 82:420-428.

Ranjit, N. K., and L. Kung, Jr. 2000. The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 83:526-535.

Rauramaa, A., J. Setala., T. Moisio, T. Heikkila, and M. Lampila. 1987. The effect of inoculants and cellulase on the fermentation and microbiological composition of grass silages. J. Agric. Finl. 59:371-377

Rode, L. M., W. Z. Yang, and K. A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82:2121-2126.

Rodrigues, P. H. M., L. M. O. Borgatti, R. W. Gomes, R. Passini, and M. Meyer. 2005. Effect of increasing levels of citrus pulp on the fermentation quality and nutritive value of elephantgrass silage. R. Bras. Zootec. 34:1138-1145.

Rooke, J. A., F. M. Maya, J. A. Arnold, and D. G. Armstrong. 1988. The chemical composition and nutritive value of grass silage prepared with no additive or with application of additives containing either *Lactobacillus plantarum* or formic acid. Grass Forage Sci. 43:87-95.

Rooke, J. A., and R. D. Hatfield. 2003. Biochemistry of ensiling. Pages 95-140 in Silage Science and Technology. (Agronomy Series No. 42) ed. Buxton D. R., R. E. Muck, and H. J. Harrison. Madison, WI: American Society of Agronomy.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sarkanen, K. V. and C. H. Ludwig. 1971. Definition and nomenclature. Pages 1-18 in Lignins, Occurrence, Formation, Structure and Reactions, K. V. Sarkanen, C. H. Ludwig eds., Wiley Interscience, New York.

Sawai, A., T. Kondo, and S. Ara. 1983. Inhibitory effects of phenolic acid esters on degradability of forage fibers. J. Jpn. Grassl. Sci. 29:175-179.

Scalbert, A., B. Monties, J. Y. Lallemand, E. Guittet, and C. Rolando. 1985. Ether linkages between phenolic acids and lignin fractions from wheat straw. Phytochemistry 24:1359-1362.

Schingoethe, D. J., G. A. Stegeman, and R. J. Treacher. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. J. Dairy Sci. 82:996-1003.

Schlegel, H. G. 1987. General Microbiology. 6th ed. Cambridge University Press, Cambridge, UK.

Schneider, B. H., and W. P. Flatt. 1975. The indicator method. Page 168 in The evaluation of feeds through digestibility experiments. Univ. Georgia Press, Athens.

Scudamore, K. A., and C. T. Livesey. 1998. Occurrence and significance of mycotoxins in forage crops and silage, a review. J. Sci. Food Agric. 77:1-7.

Sheperd, A. C., and L. Kung Jr. 1996. An enzyme additive for corn silage: effects on silage composition and animal performance. J. Dairy Sci. 79: 1760-1766.

Siciliano-Jones. 1999. Strategies for coping with poor forage digestibility in dairy rations: Response to enzyme supplements. Pages 87-96 in Proc. 15th Annual Symp. Biotechnol. in The Feed Industry. Alltech, Loughborough, Leics, UK.

Solaiman, S. G, G. W. Horn, and F. N. Owens. 1979. Ammonium hydroxide treatment on wheat straw. J. Anim. Sci. 49:802-808.

Spoelstra, S. F., L. Tjoonk, and A. Steg. 1985. Admixing cage layer manure improves the aerobic stability of whole crop maize silage. Pages 159-166 in Agricultural Waste Utilization and Management. Proc. Int. Symp. on Agric. Wastes, 5th. ASAE Publ., St. Joseph, MI.

Spoelstra, S. F. 1991. Chemical and biological additives in forage conservation. Pages 48-70 in Conference on Forage Conservation - Towards 2000. G. Pahlow and H. Honig, ed. European Grassland Federation Conference. Braunschweig, Germany.

Stefanon, B., A. N. Pell, and P. Schofield. 1996. Effect of maturity on digestion kinetics of water-soluble and water-insoluble fractions of alfalfa and brome hay. J. Anim. Sci. 74:1104-1115.

Stokes, M. R. 1992. Effects of an enzyme mixture, an inoculant, and their interaction on silage fermentation on dairy production. J. Dairy Sci. 75:764-773.

Sundstol, F. 1988. Straw and other fibrous by-products. Livest. Prod. Sci. 19:137-158.

Sutton, J. D., R. H. Phipps, D. E. Beever, D. J. Humphries, G. F. Hartnell, J. L. Vicini, and D. L. Hard. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-Friesian cows. J. Dairy Sci. 86: 546-556.

Taylor, C. C., J. M. Neylon, J. A. Lazartic, J. A. Mills, R. M. Tetreault, A. J. Whither, R. Charley, and L. Kung, Jr. 2000. *Lactobacillus buchneri* and enzymes improves the aerobic stability of high moisture corn. J. Dairy Sci. 83(Suppl. 1): 111.

Taylor, C. C., N. J. Ranjit, J. A. Mills, J. M. Neylon, and L. Kung, Jr. 2002. The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. J. Dairy Sci. 85:1793–1800.

Taylor, C. C., and L. Kung, Jr. 2002. The effect of *Lactobacillus buchneri* 40788 on the fermentation and aerobic stability of high moisture corn in laboratory silos. J. Dairy Sci. 85:1526-1532.

Theurer, C. B., J. T. Huber, A. Delgado-Elorduy, and R. Wanderley. 1999. Invited Review: Summary of steam-flaking corn or sorghum grain for lactating dairy cows. J. Dairy Sci. 82:1950-1959.

Tien, M., and T. K. Kirk. 1983. Lignin degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science 221:661-663.

Tilley, J. M. A., and R. A. Terry. 1963. A two stage technique for the in vitro digestion of forage crops. J. Br. Grassl. Soc. 18:104-111.

Treacher, R, T. A. McAllister, J. D. Popp, Z. Mir, P. Mir, and K. –J. Cheng. 1997. Effects of exogenous cellulases and xylanases on feed utilization and growth performance of feedlot steers. Can. J. Anim. Sci. 77:541. (Abstr.)

Tricarico, J. M., and K. Dawson. 2001. Contribution of an acetyl esterase containing enzyme preparation to the action of exogenous enzyme supplements for ruminants. J. Dairy Sci. 84(Suppl. 1):283 (Abstr.).

Vagnoni, D. B., W. M. Craig, R. N. Gates, W. E. Wyatt, and L. L. Southern. 1995. Monensin and ammoniation or urea supplementation of bermudagrass hay diets for steers. J. Anim. Sci. 73: 1793-1802.

Van Soest, P. J., R. H., Wine, and L. A. Moore. 1966. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. Pages 438-441 in Proc.10th Int. Grassl. Congress. Finnish Grassl. Assoc., Helsinki, Finland.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3568-3597.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press, Ithaca, NY.

Vicini, J. L., H. G. Bateman, M. K. Bhat, J. H. Clark, R. A. Erdman, R. H. Phipps, M. E. Van Amburgh, G. F. Hartnell, R. L. Hintz, and D. L. Hard. 2003. Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. J. Dairy Sci. 86: 576-585.

Wanapat, M., F. Sundstol, and T. H. Garmo. 1985. A comparison of alkali treatment methods to improve the nutritive value of straw. I. Digestibility and metabolizability. Anim. Feed Sci. Technol. 12:295–309.

Wang, Y., T. A. McAllister, L. M. Rode, K. A. Beauchemin, D. P. Morgavi, V. L. Nsereko, A. D. Iwaasa, and W. Yang. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique. Brit. J. Nutr. 2001. 85:325-332.

Weinberg, Z. G., G. Ashbell, Y. Hen, and A. Azrieli. 1993a. The effect of applying lactic acid bacteria at ensiling on the aerobic stability of silages. J. Appl. Bacteriol. 75:512-518.

Weinberg, Z. G., G. Ashbell, Y. Hen, A. Azrieli, and I. Brukental. 1993b. Ensiling peas, ryegrass and wheat with additives of lactic acid bacteria (LAB) and cell wall degrading enzymes. Grass Forage Sci. 48:70-78.

Weinberg, Z. G., G. Ashbell, K. K. Bolsen, G. Pahlow, Y. Hen, and A. Azrieli. 1995. The effect of a propionic acid bacterial inoculant applied at ensiling, with or without lactic acid bacteria, on the aerobic stability of pearl millet and maize silages. J. Appl. Bacteriol. 78:430-436.

Weinberg, Z. G., and R. E. Muck. 1996. New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiol. Rev. 19:53-68.

Weinberg, Z. G., G. Szakacs, and Y. Hen. 1998. The effect of temperature and *Lactobacillus amylovorus* and *Lactobacillus-plantarum*, applied ensiling, on wheat silage. J. Appl. Microbiol. 84:404-408.

Weinberg, Z. G., G. Szakacs, G. Ashbell, and Y. Hen. 1999. The effect of *Lactobacillus buchneri* and *L. plantarum*, applied at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages. J. Ind. Microbiol. Biotechnol. 23:218-222.

Weinberg, Z. G., G. Szakacs, G. Ashbell, and Y. Hen. 2001. The effect of temperature on the ensiling process of corn and wheat. J. Appl. Microbiol. 90:561-566.

Weiss, W. P., and W. L. Shockey. 1991. Value of orchardgrass and alfalfa silages fed with varying amounts of concentrates to dairy cows. J. Dairy Sci. 74:1933-1943.

White, B. A., R. I. Mackie, and K. C. Doerner. 1993. Enzymatic hydrolysis of forage cell walls. Pages 455-484 in Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. ASA, CSSA, SSSA, Madison, WI.

White, G. F., N. J. Russell, and E. C. Tidswell. 1996. Bacterial scission of ether bonds. Microbiol. Rev. 60:216-232.

Whitlock, L. A., T. J. Wistuba, M. K. Seifers, R. V. Pope and K. K. Bolsen. 2000. Effect of level of surface-spoiled silage on the nutritive value of corn silage diets. J. Dairy Sci. 83(Suppl. 1):110. (Abstr.).

Wildman, E. E., G. M. Jones, P. R. Wagner, and R. L. Boman. 1982. A dairy cow body condition scoring system and its relation to selected production characteristics. J. Dairy Sci. 65:495-501.

Wilkinson, J. M., and R. Gonzalez-Santillana. 1978. Ensiled alkali-treated straw. 1. Effect of level and type of alkali on the composition and digestibility in vitro of ensiled barley straw. Anim. Feed Sci. Technol. 3:117-132.

Wilkinson, J. M., and R. H. Phipps. 1979. Development of plant-components and their effects on the composition of fresh and ensiled forage maize. 2. Effect of genotype, plant-density and date of harvest on the composition of maize silage. J. Agric. Sci. 92:485-491.

Williams, C. H., D. J. David, and O. Iismaa. 1962. The determination of chromic oxide in feces samples by atomic absorption spectrophotometry. J. Agric. Sci. 59:381-385.

Williamson, G., P. A. Kroon, and C. B. Faulds. 1998. Hairy plant polysaccharides: a close shave with microbial esterases. Microbiology. 144:2011-2023.

Wilson, J. R., D. E. Akin, M. N. McLeod, and D. J. Minson. 1989. Particle size reduction of the leaves of a tropical and a temperate grass by cattle. II. Relation of anatomical structure to the process of leaf breakdown through chewing and digestion. Grass and Forage Sci. 44:65-75.

Wilson, J. R., and P. W. Hattersley. 1989. Anatomical characteristics and digestibility of leaves of *Panicum* and other grass genera with C_3 and different types of C4 photosynthetic pathway. Aust. J. Agric. Res. 40: 125-136.

Wohlt, J. E. 1989. Use of a silage inoculant to improve feeding stability and intake of a corn silage-grain diet. J. Dairy Sci. 72:545-551.

Wood, T. M., and M. K. Bhat. 1988. Methods for measuring cellulase activities. Pages 87-112 in Methods in Enzymology. Vol. 160. W.A. Wood and S.T. Kellogg, ed. Academic Press Inc., London, UK.

Wood, T. M., McCrae, S. I. and Bhat, K. M. 1989. The mechanism of fungal cellulase action. Synergism between enzyme components of *Penicillium pinophilum* cellulase in solubilizing hydrogen bond ordered cellulose. Biochem. J. 260: 37-43.

Wood, T. M., C. A. Wilson, and S. I. McRae. 1994. Synergism between components of the cellulose system of the anaerobic rumen fungus *Neocallimastix frontalis* and those of the aerobic fungi *Penicillium pinophilum* and *Trichoderma koningii* in degrading crystalline cellulose. Appl. Microbiol. Biotechnol. 41:257-261.

Woolford, M. K. 1977. Studies on the significance of three Bacillus species to the ensiling process. J. Appl. Bacteriol. 43:447-452.

Woolford, M. K, K. K. Bolsen, and L. A. Peart. 1982. Studies on the aerobic deterioration of whole crop cereal silages. J. Agric. Sci. 98:529-535.

Woolford, M.K. 1984. The Silage Fermentation. Microbiological series, 14. Marcel Dekker, Inc., New York, NY.

Wyatt, W. E., W. M. Craig, R. N. Gates, F. G. Hembry, and D. L. Thompson. 1989. Effects of ammoniated bermudagrass hay and monensin supplementation on heifer post weaning growth. J. Anim. Sci. 67:1698-1706.

Yang, W. Z., K. A. Beauchemin, and L. M. Rode. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. J. Dairy Sci. 82: 391-403.

Yang, W. Z., K. A. Beauchemin, and L. M. Rode. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. J. Dairy Sci. 83:2512-2520.

Yildirim, M. 2001. Purification of buchnericin LB produced by *Lactobacillus buchneri* LB. J. Biol. 25:59-65.

Yildirim, Z., Y. Kemal Avsar, and M. Yildirim. 2002. Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactobacillus buchneri*. Microbiol. Res. 157:103-107.

Yu, P., D. D. Maenz, J. J. McKinnon, V. J. Racz, and D. A. Christensen. 2002. Release of ferulic acid from oat hulls by *Aspergillus* FAE and *Thichoderma* xylanase. J. Agric. Food Chem. 50:1625-1630.

Yu, P., J. J. McKinnon, and D. A. Christensen. 2005. Improving the nutritional value of oat hulls for ruminant animals with pretreatment of a multienzyme cocktail: In vitro studies. J. Anim. Sci. 83:1133-1141.

Zadrazil, F., A. K. Puniya, and K. Singh. 1995. Pilot scale reactor for biological treatment of lignocellulosics for animal feed production. Indian J. Dairy Sci. 48:110-117.

Zheng,W., D. J. Schingoethe, G. A. Stegeman, A. R. Hippen, and R. J. Treacher. 2000. Determination of when during lactation cycle to start feeding a cellulose xylanase enzyme mixture to dairy cows. J. Dairy Sci. 83:2319-2325.

ZoBell, D. R., R. D. Wiedmeier, K. C. Olson, and R. Treacher. 2000. The effect of an exogenous enzyme treatment on production and carcass characteristics of growing and finishing steers. Anim. Feed Sci. Technol. 87: 279-285.

BIOGRAPHICAL SKETCH

Kathy Gisela Arriola, the youngest of four children was born in San Francisco, California but she grew up in Lima, Peru. She earned her B.S. in Animal Science and acquired pre-professional experience in the Investigation Program for swine, beef, and dairy cattle at the Universidad Nacional Agraria La Molina, Peru in 1998. After she graduated, she worked for one year as a management consultant for dairy farmers and then returned to Universidad Nacional Agraria La Molina to write a thesis for which she earned the title of Engineer in Animal Sciences in 2000. In 2004, Kathy was accepted into the M.S. program in the Department of Animal Sciences, University of Florida under the guidance of Dr. Adegbola Adesogan. She received her M.S. degree in ruminant nutrition 2006 and was accepted into the Ph.D. program in the Department of Animal Sciences at the University of Florida under the guidance of Dr. Adegbola Adesogan. While pursuing her graduate degrees, Kathy taught different classes and participated in several research projects. She was also an active member of the Animal Science Graduate Student Association and Gamma Sigma Delta. Kathy would like to be employed as a consultant in the dairy industry in the future.