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Effect of Exogenous Amylase and Protease on Ruminal Metabolism, Nutrient Digestibility, Rumen Microbiome, and Lactation Performance of Dairy Cows Fed Freshly Ensiled Corn Silage Based Diets

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EFFECT OF EXOGENOUS AMYLASE AND PROTEASE ON RUMINAL METABOLISM, NUTRIENT DIGESTIBILITY, RUMEN MICROBIOME, AND LACTATION PERFORMANCE OF DAIRY COWS FED FRESHLY ENSILED CORN SILAGE BASED DIETS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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To Bradley,

Love Mom

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ABBREVIATIONS

ADF	Acid Detergent Fiber
ADIA	Acid Detergent Insoluble Ash
AMY	Amylolytic Enzyme Treatment
AMYP	Amylolytic and Proteolytic Enzyme Treatment
BCS	Body Condition Scores
BW	Body Weight
CP	Crude Protein
CO_2	Carbon Dioxide
CON	Control Treatment
CS1	Silage weeks 1 to 3
CS2	Corn Silage weeks 4 to 6
CS3	Corn Silage weeks 7 to 9
DHIA	Dairy Herd Improvement Association
DIM	Days in Milk
DM	Dry Matter
DMD	Dry Matter Digestibility
DMI	Dry Matter Intake
DNA	Deoxyribose Nucleic Acid
ECM	Energy Corrected Milk
EE	Ether Extracts
GC	Gas Chromatograph
HMC	High Moisture Corn

K_2EDTA	Potassium Ethylene Diamine Tetra-Acetic Acid
MUN	Milk Urea Nitrogen
NaFl	Sodium Fluoride
NDF	Neutral Detergent Fiber
NH_3-N	Ammonia Nitrogen
OM	Organic Matter
OMD	Organic Matter Digestibility
OTU	Operational Taxonomic Unit
peNDF	Physically Effective NDF
PSPS	Penn State Particle Separator
PUN	Plasma Urea Nitrogen
RDP	Ruminal Digestible Protein
RUP	Ruminal Undigestible Protein
SCC	Somatic Cell Count
SDSU DRTF	South Dakota State University Dairy Research and Training Facility
SEM	Standard Error of the Mean
SNF	Solids Non-Fat
TMR	Total Mixed Ration
VFA	Volatile Fatty Acids

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ABSTRACT

EFFECT OF EXOGENOUS AMYLASE AND PROTEASE ON RUMINAL METABOLISM, NUTRIENT DIGESTIBILITY, AND LACTATION PERFORMANCE OF DAIRY COWS FED FRESHLY ENSILED CORN SILAGE BASED DIETS

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The objective of this study was to examine the effects of feeding exogenous amylolytic and proteolytic enzymes on lactation performance of dairy cows fed freshly ensiled corn silage-based diets. We hypothesized that the addition of these enzymes would improve nutrient utilization and consequently lactation performance. Thirty-six Holstein cows [15 multiparous and 15 primiparous; DIM = 132 ± 48 and 6 cannulated (3 multiparous and 3 primiparous; DIM = 164 ± 50] were blocked by milk yield, DIM, parity, and body weights and used in a 9 wk randomized complete block design study.. Treatments were a 40% (DM basis) corn silage TMR with 1) no enzymes (CON), 2) amylolytic enzymes (AMY; 10g/hd/d), and 3) amylolytic and proteolytic enzymes (AMYP; 10g/hd/d +15 g/hd/d). Corn silage was ensiled for 48 d before the start of the trial. Cows were fed individually with a Calan Broadbent system to determine daily intakes and milked 2×/d with weights recorded. Feed, milk, rumen fluid, fecal, and blood samples collected every 3 wk. An in situ digestibility experiment was conducted during wk 10 with TMR, corn, and triweekly composites of corn silage. Data were analyzed using MIXED procedure of SAS 9.4 with repeated measures and means compared using Tukey's test. Significance was declared at $P \leq 0.05$. There was a treatment effect for milk fat yield, and percent protein and lactose. A treatment by week interaction was observed

for ECM.A treatment effect was observed for rumen isovalerate percentage and a treatment by week interaction was observed for rumen butyrate percentage. There was a treatment by week interaction for *Prevotella* populations. Apparent total tract DM degradation increased in AMY compared to CON and AMYP diets. Ruminal DM rate of degradation for TMR samples decreased in AMY compared to CON and AMYP diets. Ruminal degradation of corn grain tended to increase in DM and Starch digestibility for AMYP compared to CON and AMY diets. Under the conditions of this study, the addition of exogenous enzymes altered the rumen microbiome and fermentation pathways, but maintained lactation performance compared to CON.

INTRODUCTION

Over the last 20 years, corn silage production has increased by 75% in the U.S. dairy industry (Powell et al., 2016). This growth can be attributed to the benefits of ensiling forages, which minimizes nutrient loss during long-term storage for large amounts of feed. In addition, compared to other forages, corn silage is a more digestible, uniformly high energy feedstuff with less input costs (Roth and Heinrichs, 2001). Research on improving corn silage digestibility has focused on kernel degradation as starch, located mainly in the corn kernel's endosperm, accounts for 65% of the corn silage's energy. Improvements in starch digestibility can impact lactation performance by increased feed efficiency (Ferraretto et al., 2013), milk yield (Poore et al., 1993) and milk protein (Firkins e al., 2006).

Recent work by Hoffmann et al. (2011) observed that starch granules are encased in a protein-starch matrix mainly consisting of prolamin proteins which are resistant to degradation in ruminal fluid (McCalister et al., 1993). However, natural degradation of this matrix occurs during the ensiling process (Lawton, 2002) with maximum starch digestibility achieved in 8 months (Ferraretto, 2015b). Unfortunately, producers typically need to utilize corn silage sooner, and increase concentrate levels in the diet to compensate for low starch availability in the rumen, subsequently increasing feed costs.

Researchers have observed that the addition of exogenous amylolytic enzymes to diets have improved lactation and feedlot performance (Harrison and Tricarico, 2007; Klingerman et al., 2009). Additionally, proteolytic enzymes have been shown to improve total tract digestibility of high concentrate diets in lactating dairy cows (Eun and Beuchemin, 2005), and in vitro starch digestibility of corn silage and high moisture corn (Young et al., 2012; Windle et al., 2014, Kung et al., 2014). Supplementing both

amylolytic and proteolytic enzymes may facilitate the degradation of the protein-starch matrix and allow for the utilization of corn silage earlier in the ensiling process.

This thesis describes the potential use of feeding exogenous amylolytic and proteolytic enzymes to improve starch digestibility of lactating dairy cows fed freshly ensiled corn silage-based diets. Our overall hypothesis was that supplementing freshly ensiled corn silage-based diets with both exogenous amylolytic and proteolytic enzymes would help improve milk production feed efficiency, and milk quality by increasing ruminal starch availability and utilization.

CHAPTER 1: LITERATURE REVIEW

Introduction

In the U.S. dairy industry, typically 50-60% of the diet for lactating cows is forage based, with 40-70% of the forage amount composed of corn silage. Feeding corn silage can be challenging for ration balancing as it is a feedstuff with a forage component from the cornstalk and leaves, along with a concentrate component from the corn kernels. Starch accounts for approximately 65% of the energy for corn silage; however, numerous factors can influence the amount of starch digestibility. As the majority of corn silage starch is located in the corn kernel, research on improving the degree of kernel degradation to increase starch digestibility has gained interest.

Natural microbes and their fermentation end products assist in the hydrolysis and break down of cell walls to improve digestibility of corn silage during the ensiling period. Four months of fermentation is recommended before feeding corn silage to cows to maximize starch digestibility. Feeding corn silage with a short fermentation period is common among dairies with high demands for forages. In order to make up for the decreased availability of starch, additional concentrate must be added to the ration. Improvements in corn silage starch digestibility and utilization in dairy cows may help reduce feed costs and increase lactation performance.

While most of the research on exogenous enzymes focuses on fibrolytic enzymes to improve fiber digestibility, recent experiments suggest benefits of feeding exogenous amylolytic enzymes to improve lactation performance. Proteolytic enzymes also show promise by increasing starch digestibility and soluble protein concentration for in vitro experiments. This literature review will focus on corn silage processing techniques and

its effect on starch digestibility and how the addition of exogenous amylolytic and proteolytic enzymes can influence lactation performance.

Corn Silage in the Dairy Industry

Ensiling forages allows producers to preserve large amounts of feed for long-term storage while minimizing nutrient loss, which makes silages a more efficient forage for feed mixing and handling on farms compared to dry forages. While other forages, such as hay, sorghum, barley, or oats can be fermented or ensiled for feed rations, whole plant corn silage is the most common forage choice. In 2012, over 6 million acres of corn were harvested for whole plant corn silage in the United States, which is a 33% increase from 1982 (USDA-NASS, 2012). In the dairy industry alone, corn silage production accounts for 31% of chopped acres, a 75% increase from 1982 (USDA-NASS, 2012). These increases demonstrate the value this feed ingredient has to producers for incorporation into total mixed rations (TMR) to provide a more uniform, digestible, high energy feed source with less input cost compared to other forages (Roth and Heinrichs, 2001).

Ensiling Process

Whether storing in a silo or vertical bunker, proper ensiling goals are to reduce the amount of oxygen present by filling, packing, and sealing the forage as quickly as possible. After the silage is sealed, aerobic respiration occurs by the plant cells and microbes, which removes the remaining oxygen and produce carbon dioxide (CO_2) and heat. Once acceptable CO_2 concentrations are present, fermentation occurs as anaerobic microbes consume the soluble carbohydrates and produce a variety of end products (Kung et al., 2018). Corn silage harvested with a 30-40% dry matter (DM) will typically have 3-6 % concentration of lactic acid, 1-3 % acetic acid, <0.1% propionic acid, 1-3%

ethanol and 5-7% ammonia (Kung and Shaver, 2001). Not only does lactic acid (pK_a of 3.86) account for the majority of the acids present during fermentation, but it also has the greatest influence on the decrease in pH compared to other major acids (pK_a's > 4.75) (Kung et al., 2018). Decreasing the pH below 4.2 is important for nutrient stability and maintaining forage quality by preventing the growth of, or killing, harmful microbes such as clostridia which has negatively impacted lactation performance (Pahlow et al., 2003; Bates, 2015; Kung and Shaver, 2001).

Evaluation of Corn Silage

Several on-farm evaluation tools are available to estimate the quality of fermentation of corn silage. Silages with temperatures greater than 7 to 10 °C above ambient temperature indicate presence of oxygen and aerobic fermentation. While aerobic fermentation is necessary at the beginning of the ensiling, temperatures should begin to drop after 7 days indicating a transition to anaerobic fermentation. An excellent silage is described as a green to yellowish green color, no water seepage when squeezed in hand and has a light, pleasant odor (Bates, 2015). Silages with visible mold or a rancid (clostridial fermentation), vinegar (high acetic acid), or alcoholic (yeast fermentation) odor are considered problematic and can negatively impact dry matter intake (DMI) (Roth and Heinrichs, 2001, Kung and Shaver, 2001).

The Penn State Particle Separator (PSPS) is also an on farm tool that consists of a series of different size sieves to sort particles (Heinrichs and Kononoff, 2003). The proportion of a sample's particles on different layers can help estimate the effects it will have on rumination and animal performance. Ideal quality corn silage contains small, uniform, sharp angled particles, with 90% greater than 4 mm (Bates, 2015). Particles

greater than this size are used to estimate physically effective neutral detergent fiber (NDF), which stimulates proper rumination and buffering (Heinrichs and Kononoff, 2013). Recommended amount of corn silage particles are 3-8%, 45-65%, 20-30% and <10% for the 19mm, 8mm, 4mm, and bottom pan, respectively. Corn silage chopped to a theoretical length of cut of 4.2 mm resulted in a reduction of chewing time spent eating by 43 minutes per day for cows compared to a 12 mm theoretical length of cut (Fernandez and Michalet-doreau, 2002). A linear decrease in dry matter intake (DMI) was observed as corn silage particle size was increased from 7.4 to 8.8 mm when fed in a TMR (Kononoff et al., 2003).

Composition of the Corn Kernel

Major sources of dietary starch for lactating dairy cows include cereal grains and corn silage (Martin et al., 2017). The three main components of grain are the pericarp, germ, and endosperm. The pericarp surrounds the germ and endosperm and is resistant to microbial attachment and enzymatic digestion when intact (McAllister et al., 1994). Breakage of the pericarp through kernel processing of whole plant corn silage through a 1- to 3- mm roll gap improved total tract starch digestibility in lactating dairy cows by 5.9 % compared to corn silage processed through a 4- to 8- mm roll gap and 2.8 % compared to unprocessed whole plant corn silage. (Ferraretto and Shaver, 2012a).

The germ contains the embryonic portion of the kernel and the majority of the ash, oil, and amino acid concentration. Surrounding the germ is the endosperm, which accounts for 75-80% of the kernel's weight and contains the starch granules. Starch is a polysaccharide which consists of amylose (alpha 1-4 linkages of glucose) and amylopectin (branched amylose with alpha 1-6 linkages) (Mahanna, 2009a). Starch

granules are encased in storage proteins which include albumin, globulin, glutelin, and prolamin.

Prolamin is also known as zein in corn grain while for other cereal grains it is known as gliadin (wheat), hordein (barley), secalin (rye), and kafirin (sorghum). This storage protein can be further broken down into alpha, beta, gamma, and delta fractions (Hamaker et al., 1995). The alpha and beta fractions penetrate into the endosperm while the gamma and delta fractions form crosslinks around the starch granules. These fractions, along with the other storage proteins, form what is known as the starch-protein matrix (Hoffman et al., 2011).

The amount of prolamin in the starch-protein matrix determines the digestibility of grains in the rumen. McAllister et al. (1993) demonstrated that refined starch granules of various grains digested at a similar rate in the rumen; however, there were differences in digestibility among grains when less processed. Corn grain was found to have less starch digestibility compared to other grains (such as wheat, oats, or barley) which was similarly reported by Ferraretto et al. (2013). This was attributed to the higher prolamin concentration of the corn grain, which accounts for 50 to 60 % of the total protein. In vitro degradation rate of prolamin is 0.026%/h as compared to corn globulin-albumin proteins at 0.06 %/h (Romagnolo et al., 1994). The negative correlation between prolamin concentration and ruminal starch digestibility in cereal grains is due to the fact prolamins contain large amounts of proline, which is a rigid amino acid that is hydrophobic in both water and ruminal fluid. Research from Lawton (2002) demonstrated that prolamins are soluble in acetic and lactic acids, which may account for the increased ruminal starch digestibility in corn silage and high moisture corn compared to dry corn.

Factors Affecting Corn Silage Starch Digestibility

Producing large amounts of high-quality corn silage to meet the nutritional requirements of high producing lactating dairy cows is a challenge for producers. The degree of silage fermentation can be influenced by forage characteristics which includes maturity at harvest, kernel vitreousness, and hybrid type. Management decisions at harvest, such as amount of kernel processing, and chop length can also impact silage quality. (Bates, 2015, Barlow et al., 2012).

Increased harvest maturity can lead to a decrease in packing density allowing for a more porous silage with higher concentrations of oxygen (Bates, 2015). Higher oxygen concentrations increase the growth of molds, and harmful yeasts which compete for the same organic solubles as lactic acid producing bacteria. With a limited production in lactic acid, a sufficient or timely decrease in pH does not occur, which can lead to poor quality silage and decreased animal performance (Ferraretto, 2018, Kung et al., 2018). Allen et al. (2003) reported an increase in DM content and starch concentration with a decrease in crude protein (CP), NDF and ash concentration in late-harvested corn silage. Despite the increase in starch concentration, a meta-analysis by Ferraretto and Shaver (2012b) found a reduction in total tract starch digestibility for corn silage greater than 40% DM. Interestingly they also reported a greater NDF digestibility for those same corn silages: however, others have reported a decline in ruminal in situ and in vitro NDF digestibility (Lewis et al., 2004).

On the opposite side of the spectrum, decreased harvest maturity or low dry matter content, can also create poor quality silage. An increase in moisture content

creates seepage, which allows for the loss of soluble carbohydrates needed for proper anaerobic fermentation and lactic acid production (Bates, 2015).

Corn plant varieties and hybrids with differences in endosperm density can also affect the degree of starch utilization. Flint corn, a more vitreous corn kernel variety, consists of a hard –textured, densely packed crystalline starch, while the floury corn kernel variety is soft textured with a weaker encapsulation of the starch. The more vitreous the corn kernel the more prolamins are present in the protein-starch matrix (Hoffman and Shaver, 2010). It was observed that dry corn with greater vitreousness had decreased in situ starch degradability and decreased in vivo starch digestion in lactating dairy cows (Allen et al., 2009). However, by steam flaking or fermentation, the starch digestibility of either variety is similar for ruminants (Mahanna, 2009b).

Greater improvements in total tract starch digestibility are seen for processed whole plant corn silage or high moisture corn, compared to dry corn (Firkins et al., 2001). Owens and Zinn (2005) reported that ensiling shifted the location of starch digestibility to the rumen rather than the hindgut. They observed a ruminal digestibility for high moisture corn and dry rolled corn of 76% and 49%, respectively, compared to the total tract digestibility of 84% and 80% respectively. Differences in digestibility of dry versus high moisture corn are suggested to be due to the degree of degradation of the starch-protein matrix in grain. Fermentation during ensiling, whether by microbial activity or solubolization with microbial end products, allowed for greater degradation of the corn kernel and increased access to the starch granules within the rumen.

Work by Hoffman et al. (2011) evaluated the fate of prolamin proteins in high moisture corn over an ensiling time of 0, 15, 30, 60, 120, and 240 days with or without

bacterial inoculation. They observed a decrease in pH and an increase in lactate, acetate, ammonia nitrogen (NH_3-N), and soluble CP concentrations throughout the entire ensiling period. Additionally, the prolamin protein subunits were also decreased over ensiling time. While all four subunits were decreased (α, β, δ, γ), the γ-zein subunit was decreased the most over the 240-day ensiling period. As this protein subunit is primarily found in the crosslinks of the protein-starch matrix, degradation of this subunit allows for greater access to the starch granules.

While degradation of the protein subunits was not further increased by bacterial inoculation, NH_3-N concentration was further increased for inoculated high moisture corn versus non-inoculated. Hoffman et al. (2011) suggested that the protein degradation is due to microbial proteolytic activity rather than solubolization in lactic and acetic acid in the fermentation process. In corn silage, NH_3-N is associated with degradation of amino acids by proteolytic bacteria rather than a by-product of acid hydrolysis (Ohshima and McDonald, 1978). Further support for proteolytic activity improving starch digestibility is in vitro analysis of corn silage overtime with exogenous proteolytic enzymes by Young et al. (2012). They observed an increase in NH_3-N and soluble protein concentrations with an increase in starch digestibility from 45 days to 150 days of ensiling, with further increases for protease supplemented samples compared to non-supplemented.

Other researchers have also shown metabolic processes continue with longer ensiling for whole plant corn silage. Windle et al. (2014) and Der Bedrosian et al. (2012) both reported further declines in pH after 45 days of ensiling, with an increased storage time of up to 150 days and up to 365 days, respectively. Additionally, concentrations of

lactic acid, acetic acid, and NH_3-N were increased as ensiling period was prolonged in these studies.

While starch concentrations of corn silage remain constant throughout the ensiling period, in vitro and in situ starch digestibility increased with length of ensiling, regardless of hybrid, processing techniques, and harvest maturity (Der Bedrosian et al., 2012; Windle et al., 2014; Ferraretto et al., 2015b; Ferraretto et al., 2016). A 5 to 10% increase in in vitro starch digestibility was reported for the initial 45 days of ensiling (Pahlow et al., 2003). Recent research reported that there is a similar increase in digestibility from 45 days to 120 days of ensiling and that maximal in vitro starch digestibility may not be reached until 9 months of fermentation (Ferraretto et al., 2015a). Positive correlations between in vitro starch digestibility and concentrations of NH_3-N and soluble CP in corn silage were observed, indicating proteolytic activity can improve starch digestibility (Ferraretto et. al, 2015a).

Starch Digestion in Ruminants and the Effects on Lactation Performance

Starch polysaccharides are degraded to glucose by a variety of enzymes produced by the nasal labial glands, the rumen microbes, or the pancreas and small intestine. Amylase is secreted by nasal glands and found in large amounts in the saliva, while alpha-amylase is secreted by the pancreas, and isomaltase, maltase-glucoamylase, trehalase, and lactase are secreted by the intestinal mucosa. Alpha-amylase, beta-amylase, R-enzyme, pullulanase, iso-amylase or alpha-limit dextrinase are produced by the rumen microorganisms (Cerrilla and Martinez, 2003). The site of the starch digestion throughout the digestive tract of the ruminant can impact animal performance.

Microbes metabolize polysaccharides to volatile fatty acids (VFA) with a greater shift towards the production of propionate from diets with greater starch concentrations. These VFA are then transported to the liver where gluconeogenesis occurs, with 27-59% of the glucose derived from propionate (Cerrilla and Martinex, 2003). If the amount of propionate reaching the liver is greater than its ability to transform to glucose, propionate is oxidized, which yields sufficient ATP to signal satiety signals by the vagus nerve and decreases DMI in mid to late lactation dairy cows (Allen et al., 2003).

Glucose is the primary source of energy for the nervous system and for the production of structural polysaccharides, glycoproteins, and glycolipids of cell membranes, cartilage, and mucopolysaccharides in ruminants. The mammary gland is also a major utilizer of glucose, as lactating ruminants can shift 60 to 80% of the glucose production towards lactose and milk synthesis (Cerrilla and Martinez, 2003). Glucose is a precursor for lactose synthesis which is positively correlated with milk yield (Akers, 2002).

A meta-analysis by Ferraretto et al. (2013) of 102 trials investigated the effect of diets with dry corn, high moisture corn (HMC), and steam flaked corn on lactation performance. They observed a trend ($P = 0.12$) for increased ruminal starch digestibility and a significant increase ($P= 0.001$) in total tract starch digestibility for HMC and steam flaked corn compared to dry corn. A decrease of 1.2 kg/d in DMI was observed in HMC compared to dry corn; however, milk yield was similar, which resulted in a greater feed efficiency. For each percentage unit increase in ruminal starch digestibility, milk fat content was reduced and milk protein content was increased, both by 0.02 percentage units. Greater amounts of starch digested in the rumen resulting in greater propionate

concentrations and increased microbial protein production when ruminal digestible protein (RDP) is adequate (Firkins et al., 2006). Milk fat is positively correlated with ruminal acetate: propionate ratio (Allen, 1997). Ruminal starch digestibility was also positively correlated with total tract starch digestibility, with a 0.19 percentage unit increase in total tract starch digestibility for every one percentage increase in ruminal starch digestibility. Poore et al. (1993) found a milk yield increase of 3.4 kg/day and 0.4% decrease in milk fat percent when ruminal starch digestibility increased.

Exogenous Enzymes

Most of the research on exogenous enzymes focuses on improvements of ruminal fiber digestion with fibrolytic enzymes as fiber digestibility is considered to be the most limiting. Limited research exists on exogenous enzymes with amylolytic or proteolytic activity as improvements in ruminal starch digestion might lead to excessive or rapid digestion and create acidotic conditions (Owens et al. 1998) or due to the concern of inefficient N use with excessive protein degradation. However, a positive correlation between ruminal starch digestibility and milk yield indicate benefits of feeding exogenous enzymes that target starch molecules (Ferraretto et al. 2015b). Additionally, increases in grain prices have pushed producers to find ways to reduce feed costs, such as optimizing starch digestibility and decreasing concentrate amount in the ration. (Noziere et al., 2014). Suggested modes of action for proteolytic enzymes could be the breakdown of cell wall proteins which allow for improved fiber digestion (Eun and Beuchemin, 2005), or degradation of the protein-starch matrix in concentrates (Hoffman et al., 2011) which would allow greater accessibility to the starch granules for amylolytic enzymes.

Exogenous amylolytic enzymes are suggested to act similarly to the amylolytic activity of starch digesting ruminal bacteria such as *Ruminobacter amylophilus* and *Streptococcus bovis*. *Aspergillus oryzae* and *Bacillus licheniformis* are the major sources for producing amylase enzymes; however, and other Bacillus have also been used. These enzymes hydrolyze the alpha 1, 4 linkages and release oligosaccharides (maltodextrins) while also separating 1, 6 linkages in the amylopectin (Tricarico et al., 2008). Similar to amylase enzymes, protease enzymes can be produced by a variety of species in the Aspergillus and Trichoderma families. *Aspergillus niger* specifically produces serine and aspartic proteases which cleave peptide bonds and are active in low pH, respectively (Young et al., 2012).

Tricarico et al. (2002) supplemented cows with 0, 12, 24 and 36 g/d of a commercial product with alpha-amylase activity and observed a quadratic response in milk yield, ruminal starch digestibility, and plasma glucose. The greatest response was at 12 g/d, with an increase in milk yield of 1.5 kg/d with no effect on DMI compared to non-supplemented cows. Similar results were observed by Klingerman et al. (2009) with an increase in milk fat and protein yield. When fed at 12g/d to 8,150 cows across 45 commercial dairies in Canada and the United States, Harrison and Tricarico (2007) observed a tendency for milk yield to increase by 1 kg/d with amylolytic enzyme supplementation compared to non-supplemented cows.

Eun and Beuchemin (2005) fed a proteolytic enzyme to lactating dairy cows fed either a high or low forage diet. While the effect of the supplement was a similar trend for both diets, it was more drastic for the low forage diet. Vera et al. (2012) observed a similar trend where supplementing a proteolytic enzyme was more drastic in the finishing

ration (low forage) for beef steers than in the starting ration (high forage). A decrease in DMI was observed while digestibility of DM, CP, and acid detergent fiber (ADF) were increased. This may be due to the decrease in intake which resulted in a decrease in passage rate, which allowed for improvements in digestibility (NRC, 2001). Interestingly, an increase in NDF digestibility was observed for the low forage diet while the high forage diet had greater starch digestibility.

Tricarico et al. (2002) and Hristov et al. (2000) both observed an increase in milk yield and milk fat yield along with an increase in butyrate concentrations for lactating cows fed enzymes with alpha-amylase activity. Tricarico et al. (2005) observed an increase in milk yield but no changes in milk composition, despite a shift towards butyrate synthesis rather than propionate. While some researchers have observed an increase in ruminal starch digestibility with supplementation (Tricarico et al., 2002, Noziere, et al., 2014), Tricarico et al. (2005) did not observe an increase in ruminal starch digestibility for supplemented dairy cows fed a corn-based diet with corn silage. Similar results were reported by Hristov et al. (2008) for dairy cows supplemented with an exogenous amylolytic enzyme with diets containing corn grain, barley grain, and alfalfa.

When lactating dairy cows were supplemented with a proteolytic enzyme milk yield was decreased for both low and high forage diets, with the low forage diet observing an increase in milk fat and decrease in protein yields (Eun and Beuchemin, 2005). Eun and Beuchemin attributed the decreased milk yield to a similar decrease in DMI caused by shifts in energy metabolism due to increased nutrient digestibility which triggered the down regulation of intake. The increased digestibility, despite a decrease in DMI, for cows fed the proteolytic enzyme resulted in similar digestible intakes and a

greater feed efficiency, milk yield/DMI, compared to non-supplemented cows. A decrease in milk protein yield, without a change in microbial protein supply, suggests an imbalance of ruminal digestible protein (RDP) and RUP, possibly due to the degradation of CP with the exogenous enzyme. Ruminal NH_3-N was increased, indicating an ineffective nitrogen usage. Ruminal pH was less for supplemented low forage diets, with a decrease in butyrate concentrations (Eun and Beuchemin, 2005).

A meta-analysis of 20 experiments from Seymour et al., (2005) found a positive correlation between butyrate concentrations and milk production. Ruminal development is stimulated by the production of VFA, primarily butyrate and propionate. Neonatal dairy calves fed an alpha amylase supplemented calf starter had improved ruminal development with increased papillae length and width (Heinrichs et al., 2007). The increased capillary development stimulated by butyrate concentrations could result in improved rumen epithelial blood flow. In a recent study, Storm et al. (2011) reported an increase in rumen epithelial blood flow by 65 ± 19% by increasing butyrate concentration from 4 to 36 mM/L. The experiment also resulted in a positive correlation between rumen epithelial blood flow and the ruminal disappearance of propionate. Butyrate is also a precursor for milk fat synthesis in the mammary gland (Van Soest, 1994).

Improvements in lactation performance due to changes in ruminal fermentation instead of ruminal starch digestibility, suggest exogenous enzymes alter ruminal microbial metabolism or microbial populations. Supplementing amylolytic enzymes to lactating dairy cows fed a corn silage-based diet resulted in an increase of the relative abundance of *F. succinogenes* and *Prevotella spp* to the total bacteria (Noziere, 2014). Improvements in in vitro experiments with a proteolytic enzyme have resulted in

increases in DM and NDF digestibility despite that no cellulolytic or xylanolytic activity were present (Colombatto et al., 2003). When fed to lactating dairy cows on a low forage diet, an increase in endoglucanase, xylanase, and protease activity in ruminal fluid was observed in supplemented cows, even though the supplement had only proteolytic activity (Eun and Beauchemin, 2005).

To understand the effect of exogenous amylolytic enzymes on the microbial population, Tricarico et al. (2008) grew various strains of starch degrading bacteria with or without supplementation. While *Streptococcus Bovis* S1and *Butyrivibrio fibrisolvens* 49 grew rapidly with no improvements with supplementation, *Butyrivibrio fibrisolvens* D1, *Selenomonas ruminantium* GA192, and *Megasphaera elsdenii* T81 only grew on starch mediums if supplemented. Further experimentation with *Butyrivibrio fibrisolvens* D1 on maltodextrin-based mediums, observed an increase in growth with supplementation when maltodextrins had a degree of polymerization greater than 22.1, but supplementation had no effect on growth when degree of polymerization was less than 11.1. By supplementing exogenous amylolytic enzymes, larger polysaccharides were more accessibly metabolized by the bacteria.

A theory proposed for exogenous enzymes is an oligosaccharide cross feeding strategy. Tricarico et al., (2008) hypothesized that these enzymes break down various nutrient components into oligosaccharides. This breakdown not only provides more sites for microbial attachment for further degradation, but the oligosaccharides produced could support microbes that normally do not benefit from the natural degradation of the polymer. These newly benefited microbes might then produce end products not normally

associated with that nutrient's digestion, such as acetate and butyrate from starch digestion.

While current research indicates a benefit of a longer fermentation period of corn silage of at least 4 to 6 months before feeding, producers typically need to utilize these feedstuffs much sooner. This need may be due to a lack of space available to store the correct amount of silage to last 16 to 18 months, or improper management of oxygen and temperature that decreased the predicted amount of useable silage from the previous year (Young et al., 2012). Proteolytic enzymes may facilitate the breakdown of the protein-starch matrix allowing for greater accessibility to the starch granules for degradation of the amylolytic enzymes, or increase the amino acid supply to microbes, stimulating fermentation. Adding exogenous amylolytic and proteolytic enzymes to corn silage-based diets may increase starch digestibility earlier in the ensiling period and may lead to improved lactation performance. The number of studies on the use of proteolytic exogenous enzymes in vivo and paired with amylolytic enzymes on lactation performance are limited. In addition, the understanding of the mode of action these enzymes have on the rumen microbiome is limited.

Therefore, the objective of this study was to determine the effect of both exogenous amylolytic and proteolytic enzymes in diets formulated with freshly fermented corn silage on lactation performance, nutrient digestibility, and rumen fermentation of high producing dairy cows. The hypothesis is that adding both amylolytic and proteolytic enzymes to freshly fermented corn silage would improve the availability of starch directly through the degradation of dietary starch and indirectly via the degradation of prolamin proteins encapsulating starches. Improvements in starch digestibility due to

exogenous enzymes, would allow dairy producers to optimize feed efficiency, ultimately increasing net revenue and decreasing their environmental impact.

CHAPTER 2: EFFECT OF EXOGENOUS AMYLASE AND PROTEASE ON RUMINAL METABOLISM, NUTRIENT DIGESTIBILITY, AND LACTATION PERFORMANCE OF DAIRY COWS FED FRESHLY ENSILED CORN SILAGE BASED DIETS

INTRODUCTION

As corn silage accounts for approximately 25-45% of the typical dairy ration, corn silage digestibility is a concern for dairy producers (Klopfenstein, 2013; Shaver, 2011). Starch accounts for approximately 65% of the energy content of corn silage; however, numerous factors, such as harvest maturity (Bates, 2015), theoretical length of cut (Kononoff et al., 2003) and hybrid variety (Hoffman and Shaver, 2010), can influence corn silage quality and the degree of starch digestibility. Increases in ruminal and total tract starch digestibility have been positively correlated to milk yield (Ferraretto et al., 2013).

The length of ensiling time, or how long the corn silage is allowed to ferment in a bunker or silo before being fed, can greatly impact starch digestibility. Ferraretto et al. (2015a) observed a rapid increase in starch digestibility over the first 4 months, with maximum starch digestibility not reached until 8 months of fermentation. While it is recommended to wait a minimum of four months until feeding corn silage, producers may not be able to wait this long and must add additional concentrate to the diet to make up for the lost starch digestibility; therefore increasing feed costs. As the majority of corn silage starch is located in the corn kernel, research in improving degree of kernel degradation to increase starch digestibility has gained interest

Starch granules in corn are encapsulated in a protein-starch matrix that consists of cross linked proteins of albumins, globulins, glutelins and prolamins, also known as zein.

These prolamins compromise up to 50-60% of the corn protein, with proline as the predominant amino acid. Proline is a very rigid amino acid and is insoluble in water or rumen fluid. Poor breakdown of the prolamin in this protein matrix means less access to the starch granules by ruminal microbes. Research from Hoffman et al. (2011) observed an increase in accessibility to starch granules due to breakdown of the protein starch matrix after fermenting HMC for 240 days.

Through fermentation, the protein-starch matrix can be broken down naturally; although, research with supplementing diets with exogenous amylolytic or proteolytic enzymes shows promise. Previous studies by Harrison and Tricarico (2007), and Klingerman et al. (2009), observed an improvement in animal productivity in both a dairy and feedlot setting with the addition of an exogenous amylolytic enzyme to the diet. Additionally, feeding exogenous proteases to lactating cows resulted in improved total tract digestibility in high concentrate diets (Eun and Beuchemin, 2005). More recently, the addition of proteases to high moisture corn and corn silage was shown to increase starch digestibility, soluble protein, and ammonia concentration, along with a decrease in prolamin concentrations in in vitro studies (Young et al., 2012; Windle et al., 2014; Kung et al., 2014).

While both amylase and proteases individually have shown improvements, these two enzymes fed simultaneously may have greater impacts on digestibility on lactation performance for dairy cows fed freshly ensiled corn silage. Therefore the objective of this study is to determine the effects of feeding exogenous proteolytic and amylolytic enzymes with inadequate fermented corn silage based diets to lactating dairy cows on rumen environment, nutrient digestibility, and lactation performance. Our hypothesis is

that the addition of both exogenous amylolytic and proteolytic enzymes will help improve feed efficiency, milk production, and milk composition by increasing ruminal starch digestibility.

MATERIAL AND METHODS

All procedures and animal use were approved prior to the start of the trial by the South Dakota Institutional Animal Care and Use Committee (Committee Approval Number 17-088A)

Experimental Design

Thirty-six lactating Holstein cows [15 multiparous and 15 primiparous; days in milk (DIM) = 132±48) and 6 cannulated (3 multiparous and 3 primiparous; DIM = 164±50)] were used in a randomized complete block design feeding study with three treatment diets. Cows were blocked in groups of three based on milk yield, DIM, parity, and body weight. Cows were randomly assigned to treatment after assignment to block. A covariate period of two weeks prior to the 9 wk feeding period was utilized for adjustment to the pen and Calan gate feeding system and for establishing a baseline.

Treatment diets were a 40% (DM basis) corn silage TMR with 1) no enzymes (CON), 2) amylolytic enzymes (AMY; 10g/hd/d), and 3) amylolytic and proteolytic enzymes (AMYP; 10g/hd/d +15 g/hd/d). The amylolytic enzyme (*Aspergillus oryzae,* Amaize®, Alltech, Inc.) is a fungal alpha amylase extract containing 600 dextrinizing units of α-amylase activity. Dextrinizing units are defined as grams of soluble starch dextrinized per h, according to the standard procedure for determination of fungal α-amylase activity described in the Food Chemicals Codex (1996). The proteolytic enzyme (Allzyme Vegpro^{TM}, Alltech, Inc.) is an enzyme preparation based on *Aspergillus niger* and *Trichoderma longibrachiatum* fermentation extracts containing

protease, cellulase at 7500 HUT/g and 44 CMCU/g, respectively. Diets were formulated according to the NRC (2001) to meet the recommended values for a mature lactating Holstein cows at a BW of 680 kg, at 100 DIM, with a target milk yield of 40.8 kg/d with a 3.5% milk fat and a predicted dry matter intake (DMI) of 25.8 kg/d. At the start of the study, corn silage had been ensiled for 48 days. The amount of individual ingredients in each ration offered was adjusted weekly using DM analysis of feedstuffs.

Animal Care and Feeding

The farm study was conducted, and all cows were housed, at the South Dakota State University Dairy Research and Training Facility (SDSU DRTF) in Brookings, South Dakota. Cows were observed daily for health problems and were treated according to standard SDSU DRTF management practices. The experiment occurred from October 2017 to January 2018. Cows were housed in a group pen in a free-stall barn with rubber mattresses and water was provided ad libitum. The pen was scraped and cleaned during each milking period, according to SDSU DRTF management practices. Cows were fed using the Calan Broadbent gates and box system (American Calan Inc., Northwood, NH) to monitor and determine daily individual intakes. Diets were fed as a TMR and were fed once daily at 0830 h using a Calan Data Ranger (American Calan Inc., Northwood, NH) at a 10% refusal rate. Refusal of feeds (orts) were collected daily for each cow. Dietary ingredient composition is shown in Table 1. A forage mix of corn silage, alfalfa hay, and grass hay were combined in a vertical mixer wagon (Patz 1200 Series Trailer TMR Vertical Mixer, Patz Corporation, WI) and then transferred to the Calan Data Ranger via conveyor belt. A grain mix, mixed at the South Dakota State University Feed Mill, was added to the Calan Data Ranger after the premixed forages. Treatment mixes contained:

1) ground corn for the CON diet, 2) ground corn with 10g/hd/d of amylolytic enzymes for the AMY diet, and 3) ground corn with 10g/hd/d of amylolytic and 15 g/hd/d of proteolytic enzymes for the AMYP diet. Treatment mixes were mixed in a cement mixer at the SDSU DRTF. A basal diet was formulated (Table 2) with the forage and grain mixes, and specific treatment mixes were individually weighed, added to the basal diet, and mixed by the Calan Data Ranger.

Sample Collections

Feed intakes and orts for individual cows were recorded once daily at 0700 h. Dry matter concentration of corn silage, alfalfa hay, grass hay, grain mix, and ground corn was determined weekly by drying samples for 24h at 105°C. Diets were then adjusted to maintain a constant forage to concentrate ratio throughout the experiment. Samples of corn silage, alfalfa hay, grass hay, treatment TMR, grain mix, and ground corn were collected weekly on two consecutive days and were stored at -20°C until further analysis. At the end of the study, samples were composited by 3 wk periods for nutritional analyses. Additional samples of corn silage and treatment TMR were collected every 3 wk to determine particle size using the Penn State Particle Separator (Jones and Heinrichs, 2013) and the Z-box (Grant and Cotanch, 2005).

Orts and fecal grab samples were collected over 3 days every three wk for analysis of total tract digestibility of nutrients. Fecal grab sampling was scheduled to represent every 3 h over the 24-h period relative to time of feeding. Samples were frozen at -20°C until further analysis. Acid detergent insoluble ash (ADIA) was used as an internal digestibility marker.

Body weight (BW) and body condition score (BCS) were taken on two consecutive days every three wk at 3 h post feeding. Four trained individuals assessed the BCS of cows based on the scale described by Wildman et al. (1982), where 1= emaciated and 5=obese.

Cows were milked twice per day in a double-8 parallel parlor at 0530 and 1750 h, and daily milk were electronically recorded (ALPRO^{TM}, DeLaval, Sweden). Milk from individual cows were collected every three wk over two consecutive days at each milking for component analysis by Dairy Herd Improvement Association (DHIA; MQT Lab Services, Kansas City, MO).

Blood samples were collected over two consecutive days every three wk at 3 h post feeding via venipuncture of the coccygeal artery into vacutainer tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ) containing sodium fluoride (NaFl) for glucose analysis (Cat.#:367729) or potassium ethylene diamine tetra-acetic acid (K_2EDTA) for all other analyses (Cat.# 366643). Immediately after blood collection, samples were placed in ice and then brought to the laboratory within 3 h for processing and storage. Blood collection tubes were centrifuged at 1000 x g for 20 minutes at 4°C (Centrifuge: CR412 Jouan, Inc., Winchester, VA.). Plasma (K_2EDTA tubes) or serum (NaFl tubes) was then transferred using a plastic pipette into polystyrene storage tubes and frozen at -20°C until further analysis.

Rumen fluid was sampled from the cannulated cows before feeding (0 h) and 2, 4, 6, 8, 10, 12, 16 and 24 h post feeding via syringe and filtered probe. The probe was inserted into four different sections of the rumen and 15 mL were collected at each site for a total of 60 mL collected for a more accurate representation of the entire rumen. The

probe was rinsed with water between cows. In addition, initial ruminal fluid drawn through the syringe was discarded in order to remove any contamination in probe between cows. The pH of the sample was analyzed and recorded immediately (Waterproof pH Testr 30, Oakton Instruments, Vernon Hills, IL). A 10 mL aliquot was mixed with 2 mL of 25% (w/v) meta-phosphoric acid for later determination of VFA concentrations, and a 10 mL aliquot was mixed with 200 µL of 50% (v/v) sulfuric acid to determine rumen ammonia nitrogen (NH_3-N). The two samples from all time points were frozen at -20°C until further analysis.

Samples of whole rumen contents, which included both the liquid and solid fractions were collected from the 6 cannulated cows 4 h post feeding during wk 4 and 8 for microbial analysis. A 100 mL sample of whole rumen content was collected from four different locations, and an aliquot of approximately 50 mL from each collection was poured into a 250 mL conical tube (CAT# 05-538-05 Thermo Fisher Scientific). Samples were frozen at -20°C until further analysis.

Lab Analysis

Tri-weekly composites of the forages (corn silage, alfalfa hay, grass hay), concentrates (grain mix, ground corn), and TMR were dried for 48 hr at 55 °C in a forced Despatch oven (style V-23, Despatch Oven Co., Minneapolis, MN) and were ground to a 2 mm particle size using a Wiley Mill (Model 3; Arthur H. Thomas Co., Philadelphia, PA). Further grinding to a 1 mm and 0.5 mm particle size was done using an ultracentrifuge mill (Brinkman Instruments Co. Westbury, NY). In order to express nutrient concentrations on DM basis, a 1 g aliquot of sample was dried for 4 h in a 105 °C oven (AOAC 17th ed., method 935.29). The ash content was analyzed by incinerating

1 g of sample for 8 hr at 450°C in a muffle furnace (AOAC 17th ed., method 942.05). Organic matter (OM) was then calculated as OM= (100-% Ash). Samples were analyzed for nitrogen content via Dumas combustion analysis (AOAC 2002, method 968.06), using a Rapid N Cube (Elementar Analysensysteme, GmbH, Hanau, Germany). CP concentration was calculated as N x 6.25. Neutral detergent fiber (NDF; Van Soest et al., 1991) and acid detergent fiber (ADF; Roberston and Van Soest, 1981) were analyzed sequentially using the Ankom 200 fiber analysis system (Ankom Technology Corp., Fairport, NY) for all samples. Heat stable α amylase and sodium sulfite were used for NDF analysis. Starch concentration (Hall et. al., 2015) was conducted on all samples using the glucose oxidase-peroxidase (GOPOD Kit; #K-GLUC, Vinotec, Napa, CA). Ether extracts (EE) were analyzed using petroleum ether (AOAC 17th ed., method 920.39) in an Ankom XT10 fat analysis system (Ankom Technology Corp., Fairport, NY). Samples were sent to a commercial lab (Dairyland Laboratories Inc., Arcadia, WI) for mineral analysis of Ca, P, Mg, K, Na (method 985.01), S (method 923.01), and Cl (method 915.01) (AOAC, 1998).

For apparent total tract digestibility analysis, fecal and ort samples were composited on an as-is basis by volume for each cow. Samples were processed (dried and ground) as described for the monthly feed composites. Fecal and ort samples were also analyzed for DM, Ash, CP, NDF, ADF, and Starch as previously described for feeds. Acid detergent insoluble ash (ADIA) concentration was measured for all feed composites, fecal samples, and orts. The method for ADIA analysis consists of analyzing the sample for ADF (Robertson and Van Soest, 1981) and then determining the ash

concentration using a modified procedure of the AOAC 17th ed., method 935.29. Digestibility calculations were performed according to Merchen (1988).

Milk samples were sent every three weeks to DHIA (MQT Lab Services, Kansas City, MO) for composition analysis. Fat, protein, and lactose were analyzed via mid-infrared spectroscopy (AOAC, 2006, Bently 2000 Infrared Milk Analyzer, Bentley Instruments, Chaska, MN). Milk urea nitrogen (MUN) was determined using a modified Berthelot reaction (ChemSpec 150 Analyzer, Bentley Instruments, Chaska, MN). Somatic cell count (SCC) was analyzed using laser technology (Soma Count 500, Bentley Instruments, Chaska, MN).

Plasma samples were thawed, homogenized, and analyzed for plasma urea nitrogen (PUN), glucose, and cholesterol concentrations using a commercially available enzymatic or colorimetric assay kits on a micro-plate spectrophotometer (Cary 50, Varian Inc., Walnut Creek, CA). Diacetyl monoxide was used to analyze PUN (procedure 0580, Stanbio Laboratory, Boerne, TX). Glucose was determined by the glucose oxidase reaction (Trinder, 1969), using a glucose kit (Catalog #: G7521, Pointe Scientific, Inc., Canton, MI). Cholesterol concentrations were analyzed using a cholesterol esterase reagent (Catalog #: C7510, Pointe Scientific, Inc., Canton, MI). Plasma triglycerides concentrations were analyzed using glycerol phosphate oxidase after hydrolysis by lipoprotein lipase as described by Fossati and Prencipe (1982) that paired the reaction with the classic Trinder (1969) reaction.

Rumen fluid samples preserved with sulfuric acid were thawed and centrifuged at 30,000 x g for 20 minutes at 4°C (Centrifuge: Eppendorf 5403, Eppendorf North America, Hauppauge, NY), and were analyzed for NH_3-N using a colorimetric assay

performed on a micro-plate spectrophotometer (Cary 50, Varian Inc., Walnut Creek, CA), according to Chaney and Marbach (1962). The rumen fluid samples preserved with meta-phosphoric acid were thawed and centrifuged at 30,000 x g for 20 min at 4°C and were analyzed for the following VFA concentration: acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate. Concentrations were measured using an automated gas chromatograph (GC) (model 6890; Hewlett-Packard Co., Palo Alto, CA) using a flame-ionization detector. Volatile fatty acids were separated on a capillary column (15 m x 0.25 mm i.d.; Nukol, 17926-01C; Supelco Inc., Bellefonte, PA) using 2- ethylbutyrate as an internal standard. The split ratio of 100:1 in the injector port was at a temperature of 250°C with flow rate of 1.3 mL/min of helium. The column and detector temperature were maintained at 140°C and 250°C, respectively.

Microbial Deoxyribose Nucleic Acid (DNA) was isolated from whole rumen content samples using the repeated bead beating plus column method (Yu and Morrison, 2004). Briefly, 250 µL of sample were lysed by bead beating with 0.4 g of zirconium beads at 3000 rpm (3 min) in lysis buffer (0.5 M NaCl, 50 mM Tris.HCl, 50 mM EDTA, 4% SDS), followed by heat treatment at 70 ◦C (15 min). Lysate was recovered by centrifugation (14,000× g or greater, 5 min, 4 ◦C). Residual SDS and impurities were removed by ammonium acetate precipitation (10 *M*, 20% volume). Microbial DNA was recovered from the lysate using isopropanol precipitation, then further purified using a commercial kit following the manufacturer's specifications (QIAamp DNA Stool Kit, QIAGEN, Hilden, Germany). The V1–V3 region of bacterial 16S rRNA gene sequences was PCR-amplified using the 27F forward and 519R reverse primer pair. The PCR reactions were performed with the Phusion Taq DNA polymerase (Thermo Scientific)

under the following conditions: hot start (4 min, 98 ◦C), followed by 35 cycles of denaturation (10 s, 98 ◦C), annealing (30 s, 50 ◦C) and extension (30 s, 72 ◦C), then ending with a final extension period (10 min, 72 ◦C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500 bp) were excised for gel purification using the QiaexII Gel extraction kit (QIAGEN). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the MiSeq 2x300 platform (Illumina, San Diego, CA, USA) to generate overlapping paired end reads.

In Situ Digestibility

Following the 9 wk study, the six cannulated cows remained on their respective treatment diets for an in situ digestibility analysis. Five feeds were analyzed: ground corn, a composited corn silage sample from weeks 1 to 3 (CS1), 4 to 6 (CS2), and 7 to 9 (CS3), and the cow's respective treatment TMR. Samples were dried for 48 h at 55°C in a Despatch oven (Style V-23, Despatch Oven Co., Minneapolis, MN) and were ground to a 2 mm particle size using a Wiley Mill (Model 3; Arthur H. Thomas., Philadelphia, PA). Five grams of each feed were weighed in replicate in 10 x 20 cm Dacron bags with a pore size of 50 µm (Ankom Technology, Macedon, NY) and heat sealed using an impulse sealer. Duplicate sample bags were incubated for each feed and cow for the 0, 2, 4, and 8 h time points. Eight bags per feed were incubated for the 16 h time point and three bags per feed were incubated for time points 24, 48 and 72 h. Duplicate blank bags were also used during each time point to correct for microbial attachment and foreign debris. All bags were soaked in approximately 39°C water for 20 minutes prior to incubation in the rumen. The 0 h samples were soaked and rinsed, but were not placed in the rumen. Bags

for all other time points were inserted into a large mesh nylon bag (36 x 42 cm) with a weight to submerge samples below the particulate mat layer of the rumen and in reverse order so they were removed at the same time. Once removed from the rumen, bags were submerged in a 15 L bucket, gently agitated and rinsed in cold water until runoff was clear. Bags were then stored at -20°C until further analysis.

Bags were thawed and submerged in a methylcellulose solution held at 39°C in a shaking water bath and gently agitated for 30 minutes. Bags were then rinsed with cold water and allowed to dry at 23°C for 12 h. Bags were then dried for 48 h at 55 °C in a forced Despatch oven (style V-23, Despatch Oven Co., Minneapolis, MN). Dry matter disappearance at each time point was calculated as the weight difference of the original sample and the residue of the post – ruminal incubation after a correction for DM concentration. Residues after ruminal incubation were then composited by time point for each feed/cow and ground through a coffee grinder until samples were a consistent particle size. Original feed ingredients and residues were then analyzed for nitrogen content via Dumas combustion analysis (AOAC 2002, method 968.06), using a Rapid N Cube (Elementar Analysensysteme, GmbH, Hanau, Germany). Nitrogen content was then multiplied by 6.25 to calculate CP. Neutral detergent fiber (NDF; Van Soest et al., 1991) and acid detergent fiber (ADF; Roberston and Van Soest, 1981) were analyzed sequentially using the Ankom 200 fiber analysis system (Ankom Technology Corp., Fairport, NY) for the original corn silage and TMR ingredients and residues. Heat stable alpha amylase and sodium sulfite were used for the NDF. Starch analysis (Hall et. al., 2015) was conducted on the original feed ingredients (ground to 0.5 mm using an ultracentrifuge mill (Brinkman Instruments Co. Westbury, NY)) and residues for time

points 0, 2, 4, 8, and 16 h using the glucose oxidase-peroxidase method (GOPOD Kit; #K-GLUC, Vinotec, Napa, CA).

Mathematical and Statistical Analysis

All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). All data are presented as least square means with the highest standard error of the mean (SEM) among the values. Significant differences among treatments were declared at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$.

Analyzed feedstuffs were evaluated using PROC MEANS in SAS to calculate the mean and standard error for individual nutrient contents. Lactation performance, blood metabolite data, and total tract digestibility analysis data were analyzed as a randomized complete block design experiment with week as the repeated measure and cow (block) as the subject using the MIXED procedures of SAS (Littell et al., 2006). The model included treatment, week, and interaction of both terms. Energy-corrected milk (ECM) were calculated by using the following equation: ECM = [(0.327 x kg milk) + (12.95 x kg fat) + (7.2 x kg protein)] (Orth, 1992). Feed efficiency was calculated by ECM/DMI. Rumen fermentation data was analyzed as a randomized complete block design experiment with time point (wk) as the repeated measure and cow (block) as the subject using the MIXED procedures of SAS (Littell et al., 2006). The model included treatment, week, time point and the interaction of these terms. Akaike's criterion was used to determine the most suitable covariance structure in repeated measures for each parameter. The structure that provided the smallest value was used for the analysis.

Analysis of Generated 16S Sequences

Unless specified otherwise, the following steps were performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1–V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2 × 300 paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nucleotides, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15. Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity. The OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR (v.1.36.1, University of Michigan, Ann Arbor, MI, USA) open source software package. Secondly, the integrity of the 50 and 30 ends of OTUs was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nucleotide database, as determined by BLASTN (2.5.0), OTUs with more than five nucleotides missing from the 50 or 30 end of their respective alignments were discarded as artifacts. Single read OTUs were subjected to an additional screen, where only sequences that had a maximum of 1% of dissimilar nucleotides compared to a sequence in the NCBI nucleotide database were kept for analysis.

After removal of sequence chimeras and artifacts, the bacterial composition of each sample was determined by calculating the relative abundance of valid OTUs. This was defined as the number of sequence reads assigned to an OTU in a given sample,

divided by the number of total reads in that sample. Rarefaction analysis was performed using MOTHUR (v.1.36.1). Taxonomic assignment of valid OTUs was determined using a combination of RDP Classifier and BLAST. The phylogenetic composition was summarized for phylum, family, and genus level based on sequence abundance (sum of sequences per taxon divided by total sequences). Unclassified taxa within a given taxonomic level were not pooled together, but rather were pooled according to their closest classifiable parent (i.e., unclassified family belonging to Bacteroidetes).

Phylogenetic composition and OTU abundance were analyzed using PROC MIXED of SAS v. 9.4. Relative abundance data were arcsine square root transformed before performing ANOVA. The compliance of the data with the assumptions of ANOVA was tested using PROC UNIVARIATE of SAS. The model included the fixed effects of treatment, wk, and treatment x wk interaction. Phyla, families, and genera that were above a threshold of 1% of total sequence data and only top 35 OTU were considered for the analysis.

In Situ Digestibility Equations

Ruminal degradation constants of CP were analyzed using nonlinear regression modeling (Ørskov and McDonald, 1979; SAS 9.4). The following equation describes the model used to determine the ruminal degradation of CP as a percentage at time t (Y).

$$Y = A + B\ [1 - e^{\ -Kd\ (t)}]$$

The rapidly degradable CP fraction that disappears at 0 h after rinsing is represented by A; B represents the potentially degradable CP fraction; and Kd is the rate of degradation of the B fraction and t represents time of incubation (h). The C fraction represents the indigestible fraction as 100 – (A + B). Rumen undegradable crude protein or RUP of the

samples was calculated as 100 – RDP%. The RDP fraction is calculated using the following equation

$$RDP = A + B\ [\frac{K_d}{K_d+K_p}]$$

The particulate passage rate (%/h) is the variable Kp and is calculated according to the NRC (2001) equation for concentrates.

$$Kp = 2.904 + 1.375 \times X1 - 0.020 \times X2$$

In this equation X1 represents the DMI, as a % of BW; X2 is the concentrate amount, as a % of the diet DM. The passage rate for this particular study was found to average 6.0%/h among the three cows. Fraction A, B, C, RDP, and RUP were analyzed using MIXED procedure of SAS 9.4. Mean comparisons were performed using Tukey's test with $P < 0.05$ designated as significant and a tendency was declared at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Six cows were dropped throughout the study, one for Calan gate training issues, one for lameness, one for acute acidosis, and three for displaced abomasum.

Feed Analysis

The analyzed nutrient composition of the individual ingredients and of the treatment diets is presented in Table 3. Corn silage, Alfalfa Hay, Grass Hay NDF, ADF, CP, and EE values are less than similar feedstuffs reported in the NRC (2001) while NDF and ADF values for corn grain were greater than expected. Variation of quality of

feedstuffs are within acceptable ranges with non-fibrous carbohydrates (NFC) for grass hay, which had the largest variation over the course of the experiment. Similar nutrient concentrations were observed between the analyzed nutrient compositions of the CON, AMY, and AMYP diets (Table 4). Similar DM concentrations were found for actual diets (53%) compared to the formulated basal diet (55%). While analyzed CP, ADF, and EE concentrations are less than the expected values from the calculated ration, the concentrations are above lactating dairy cow requirements (NRC, 2001). Differences in the mineral composition of diets formulated versus analyzed were minimal, except for the concentration of K, however these differences are unlikely to have a significant effect on lactation performance.

Particle size of the CON TMR measured with the Penn State Particle Separator (PSPS) and Z-box demonstrated little variation overall (Table 5). However, percentages of particle sizes analyzed do not follow the PSPS guidelines for TMRs of 2-8%, 30-50%, 10-20%, and 30 to 40% for the upper (>19mm), middle (8-19mm), lower (4-8mm) screens and summation of the sieve screen (1.18-4mm) and bottom pan (<1.18mm), respectively (Jones and Heinrichs, 2013). Mertens (1997) considered particles greater than 1.18 as resistant to escaping the rumen and useful for calculating the physically effective NDF (peNDF), which is dietary fiber that effectively stimulates proper rumination and buffering. Recent studies indicate particles retained on the 4 mm sieve describe the number of resistant particles more accurately for high producing dairy cows (Maulfair et al., 2011). The majority of particles of the experimental rations were retained below the lower sieve, with 32% and 22% on the sieve screen (1.18mm) and bottom pan, respectively, for a total of 54% of the total particles. Similar estimates of peNDF were

found for both the PSPS and Z-box, an additional on-farm tool for estimating peNDF using a 3.18mm screen (Grant and Cotanch, 2005). Nonetheless, both estimates are below the 21% effective dietary effective fiber threshold suggested by Mertens. This suggests the basal diet does not support proper rumination to maintain a pH of 6.0 and may lead to acidotic conditions (Owens, 1998).

Lactation Performance

Lactation production responses to the addition of exogenous enzymes in the diets are presented in Table 6. Milk yield, was similar for all treatments, however there was a wk affect ($P < 0.01$) with a decreasing trend overtime (Figure 1), and this decrease may be attributed to cows progressing later into lactation (Akers, 2002). The DMI (Figure 2) demonstrated a treatment × wk interaction ($P < 0.01$), where AMY had significant increased intake at wk 2 and 6, and decreased intake for wk 7 and 8, compared to CON. The AMYP had decreased intake for wk 3 and 9 compared to CON. The AMY had an increased intake for wk 6 and decreased intake for wk 7 compared to AMYP. We observed a wk affect but no treatment effects on BW and BCS.

Milk yield (wk 3, 6, 9), energy corrected milk (ECM), and component percent and yields are presented in Table 7. There was a treatment by wk interaction ($P = 0.05$) for ECM. The ECM was higher during wk 3 for AMY diet compared to the AMYP diet and for wk 6 compared to both the CON and AMYP diets. No differences were observed among treatments during wk 9. This may be due to the significant increased DMI during wk 6 for cows fed AMY diet compared to CON and AMYP, as DMI and milk yield have been found to be positively correlated (Ferraretto et al., 2013). An increase in milk yield with cows fed an amylolytic supplement at 12 g/d has been reported (Tricarico et al.,

2002, Tricarico et al., 2005, Harrison and Tricarico, 2007), with a decrease in milk yield for cows supplemented with a proteolytic enzyme on both a high and low forage diet (Eun and Beuchemin, 2005). Milk fat yields demonstrated a treatment ($P = 0.03$) and wk effect ($P < 0.01$), with an observed decrease in milk fat yield for AMYP compared to AMY but similar to control. Across treatments, milk fat yields decreased overtime. Decreases in milk fat content for AMYP may indicate improved ruminal starch digestibility. A percentage unit increase in ruminal starch digestibility can decrease milk fat and increase milk protein by 0.02 percentage units (Ferraretto et al., 2013); however, protein yields were similar among treatments. A wk effect was observed for Lactose yield ($P \leq 0.01$) SNF yield ($P = 0.01$) with a decrease in yield over time. There was a tendency for a treatment × wk interaction ($P = 0.06$) for SNF yield. A treatment by wk interaction was observed for MUN ($P = 0.02$) with an increase in concentration for CON at wk 9 compared to AMY and AMYP, possible evidence of improved N utilization for supplemented cows. No differences ($P \geq 0.05$) were observed in somatic cell counts (SCC).

Blood Metabolites

Concentrations of blood metabolites are described in Table 8. A wk effect was observed with cholesterol, with a decrease in concentration during wk 9 compared to wk 6. Tricarico et al, (2002) observed an increase in plasma glucose concentration for cows supplemented an amylolytic enzyme, however we observed no differences ($P \geq 0.05$) for glucose, PUN, and Triglyceride concentrations.

Rumen Fermentation

Rumen fermentation parameters by wk are shown in Table 9. There was a wk effect observed for pH, with an increase in pH at week 6 compared to wk 3. Subacute

ruminal acidosis occurs when ruminal pH drops below 5.8 for a prolonged period, typically 300 minutes. Based on the estimated percent of physical effective NDF in our diets (<21%) ruminal acidosis conditions should have been observed; however, since pH was measured at specific time points rather than continuously with an inserted probe, we cannot determine if subacute ruminal acidosis conditions were created. There was a time point effect for pH (Figure 3) with an observed pH below 6.0 from 4 to 16 h post feeding, with pH below 5.8 from 12 to 16 h post feeding and returning to 6.4 24 h post feeding.

Changes in Rumen fermentation parameters by time point are shown in Table 10. A time effect was also observed for total VFA concentration (Figure 4) with a significant increase at 12 h post feeding. A time effect was observed for NH_3-N concentrations with a significant increase at 2, 12 and 24 h post feeding. A tendency for a time effect was observed for acetate as a proportion of total VFA with a decrease at 12 and 16 h post feeding. A treatment by wk effect was observed for butyrate as a proportion of total VFA, with an increase in concentration in cows fed the AMY diet for wk 3 compared to the CON and AMYP diet. A meta-analysis of 20 experiments from Seymour et al., (2005) found a positive correlation between butyrate concentrations and milk production. Tricarico et al. (2002) and Hristov et al. (2000) both observed an increase in milk yield and milk fat yield along with an increase in butyrate concentrations for lactating cows fed enzymes with alpha amylase activity. Tricarico et al. (2005) observed an increase in milk yield but no changes in milk composition, despite a shift towards butyrate synthesis rather than propionate. In the present experiment, an increase in butyrate concentrations did not result in an increase in milk yield. A time point effect was also observed for the proportion of butyrate with an increase at 12 and 16 h post feeding. A tendency for a wk

effect was also observed for the proportion of butyrate, with concentrations decreasing overtime. Butyrate is also a precursor for milk fat synthesis in the mammary gland (Van Soest, 1994), however butyrate proportions and milk fat yields did not follow similar trends in the present experiment.

Isobutyrate was not detected in any samples. The proportion of isovalerate had a significant treatment by time point interaction with an increase in concentration at 16 h post feeding for cows on the CON diet, and 10 h post feeding for cows on the AMYP diet. A tendency for an increase in isovalerate as a proportion of total VFA for the AMYP diet was observed compared to the CON and AMY diet. Valerate had a significant time point effect, with an increase at 16 h post feeding. A tendency for a wk by time point effect ($P = 0.08$) was observed for valerate, with an increase at 16 h post feeding for wk 3 and 6. No differences ($P \geq 0.05$) were observed for propionate nor acetate: propionate ratio.

Microbial Populations

The study produced a total of 482,844 sequences across treatments and both collection time points. The resulting sequences were then clustered into 26, 298 OTU, in which 19, 269 were non-singleton OTU. Phylogenic taxonomy resulted in classification into 28 phyla, with 2.5 to 14 percent of sequences, depending on diet and time point, that did not classify into any known phyla. Further classification yielded 36 groups at the class level, 52 groups at the order level, 91 groups at the family level, and 4 groups at the genus level. However, when unclassified groups were broken down into their closest classifiable parent, at any given taxa level, the number of groups increased to 44, 66, 126, and 127 for the class, order, family, and genus levels, respectively.

Assigning phylogenic taxonomy of individual OTU resulted in 94, 91, 67, and 34% that were classifiable into phylum, class, order, and family levels, respectively. Due to the large number of classified groups statistical analysis were only completed on abundant groups (>1% of the total OTU across all taxa levels) and the top 35 OTU. This resulted in a reduction of groups into 4, 3, 4, and 2 for the phylum, class, order and family levels (Table 11), which represented 92, 89, 63, and 29% of the total OTU sequences respectively. There was no significant differences among treatments and time points for the phylum groups. There was a treatment effect for the percentage of unclassified bacteria at the order level, with an increase in cows fed the AMYP diet. There were no significant differences for the remaining order groups. *Prevotellaceae* had a significant treatment by wk interaction, with an increase in CON diet, but a decrease in AMY diet overtime. The abundancy of unclassified bacteria at the class level had a tendency for a treatment by wk interaction, with a decrease in CON diet overtime. There were no significant differences for the remaining class groups. There was a significant treatment by wk effect for *Prevotella*, with an increase in the CON diet, but a decrease in the AMY diet. There was also a significant treatment by wk interaction for the unclassified bacteria at the family level, with a decrease in the CON diet in wk 3 but an increase in the CON diet in wk 8 compared to the treatment diets. *Prevotella* are able to utilize a wide variety of polysaccharides, and while not considered a highly cellulolytic bacteria, they are thought to be important contributors to xylan degradation in the rumen and a range of glycosyl hydrolases have been identified (Kruase, 2003). There was no significant differences for the remaining family groups.

Only three groups were classified at the genus level which were a *Bifidobacterium*, *Coriobacteriaceae,* and *Corynebacteriaceae* which represented 0.93, 0.12 and 0.01% of the total OTU sequences, respectively. *Bifidobacterium,* is considered to play a role in rumen acidosis, as it has been identified in animals eating high starch diets, which implies a role for these micro-organisms in the fermentation of starch to produce acetic and lactic acid (Stewart et al. 1997). This is in agreement with results from cultured *Bifidobacterium* spp. taken from ruminal cannulated Holstein dairy cows, which, when placed on a starch based medium, produced L-lactate (Hernandez, 2008). *Coriobacteriaceae* are associated with animals with high feed efficiency. Higher concentrations of this microbe were observed in steers with high feed efficiency and were increased when the high feed efficient steers rumen contents were replaced with the rumen contents of a low feed efficient steer (Zhou et al., 2018). The authors suggested that these microbes were challenged by the switch and stimulated in order to maintain the fermentation efficiency. *Coriobacteriaceae* was also positively correlated with molar valerate production, however we did not observe any significant changes in valerate proportions.

Of the top 35 OTUs, which represented 0.18% of the total OTUs, 7 OTUs were found to have a significant effect (Table 12). The OTUs 3, 5 and 9 had a significant treatment by wk interactions and OTU 15 had a tendency for a treatment by wk interaction, where there was an increase in cows fed the CON diet overtime, but a decrease in cows fed the AMY diet overtime. OTU 21 had a tendency for a wk effect, with an increase in abundance overtime. The OTU 22 had a significant treatment by wk interaction with a decrease in abundance for the CON diet overtime. There was a

tendency for a treatment effect for OTU 34 with a decrease in abundance for AMYP diet. All of these OTU were classified as *Prevotella*, except for OTU 34, which was classified as an unclassified *Ruminococcaceae* at the genus level.

Apparent Total Tract Digestibility

Total tract digestibility for DM, OM, CP, NDF, ADF and Starch are presented in Table 13. There was a significant treatment effect observed for dry matter digestibility (DMD), and organic matter digestibility (OMD) with an increase in cows fed the AMY diet compared to CON and AMYP diets. Similar results were reported by Noziere et al. (2014) with an increase in OMD and Starch for amylase supplemented cows compared to non- supplemented cows. Eun and Beauchemin (2005) reported an increase in OMD for protease supplemented cows, but only a significant increase in starch digestibility for protease supplemented cows fed a high forage diet compared to non-supplemented cows and supplemented cows fed a low forage diet. There was a tendency for wk effects for DMD and OMD with an increase in digestibility in wk 6 compared to wk 9. There was a significant wk effect observed for CP digestibility with a decrease in wk 9 compared to wk 3 and 6. There was a significant wk effect for NDF digestibility and ADF digestibility with an increase in wk 6 compared to wk 3 and 9. There was also a tendency for a treatment effect for NDF digestibility, with an increase in cows fed AMY diet compared to CON and AMYP; however, there was no treatment significance for ADF digestibility. While others reported improved fiber digestibility with supplementation with a proteolytic enzyme (Colombatto et al., 2003, Eun and Beauchemin, 2005, Vera et al., 2012), this was not observed in the present study, but rather an improvement for cows

supplemented with an amylolytic enzyme. There was a tendency for a wk effect of starch digestibility, with an increase in wk 6 compared to wk 3.

In Situ Digestibility

We observed a significant treatment effect for ruminal DM K_d for TMR samples (Table 14), with a decrease in AMY compared to CON and AMYP diets. This slower rate of passage for dry matter in the rumen may explain the increased total tract digestibility in cows fed the AMY diet compared to CON and AMYP. Ruminal degradation of corn grain resulted in a tendency for an increase in DM and starch degradation for cows fed AMYP diets compared to CON and AMY diets (Table 15). This may explain the significant decrease in milk fat yield in AMYP diets compared to CON and AMY diets. A meta-analysis by Ferraretto et al., (2013) observed a negative correlation between ruminal starch degradation and milk fat content, with an increase in percentage unit of ruminal starch meaning a decrease in milk fat by 0.02 percentage units. There was a tendency for ruminal NDF and ADF degradation of CS1 to increase with cows fed the CON diet compared to AMY and AMYP diet, and for cows fed the AMY diet compared to AMYP (Table 16). There was a tendency for ruminal DM degradation of CS2 to decrease for cows fed the AMY diet compared to CON and AMYP (Table 17). In addition, there was a tendency for the A fraction of ruminal ADF disappearance of CS2 to increase for cows fed the CON diet compared to AMY and AMYP diets. There was a significant increase in the ruminal DM K_d of CS3 for cows fed the CON diet compared to the treatment diets (Table 18). Eun and Beuchemin (2005) observed an increase in DM, CP and ADF digestibility in proteolytic supplemented cows fed low or high forage diets compared to non-supplemented cows, with greater improvements in the low forage diets.

Research on amylolytic supplemented diets in lactating cows have reported both improved (Tricarico et al., 2002; Noziere, et al., 2014) and unaffected (Tricarico et al., 2005; Hristo et al., 2008) ruminal starch digestibility. In vitro experiments on corn silage with proteolytic enzymes have resulted in increases in DM and NDF digestibility, despite the lack of cellulolytic and xylanolytic activity (Colombatto et al., 2003).

CONCLUSIONS

The addition of feeding exogenous amylolytic and proteolytic enzymes maintained lactation performance compared to non-supplemented cows. Greater responses were observed for cows supplemented with only amylolytic exogenous enzymes compared to cows supplemented with both proteolytic and amylolytic enzymes. However changes in microbial communities and ruminal fermentation, specifically butyrate concentrations, along with improved organic matter, NDF, and starch digestibility, suggest exogenous enzymes can influence fermentation and metabolic pathways. Further research is warranted to better understand the interactions of exogenous amylolytic and proteolytic enzymes within the ruminant.

Figure 1. Milk yield (kg/d) of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

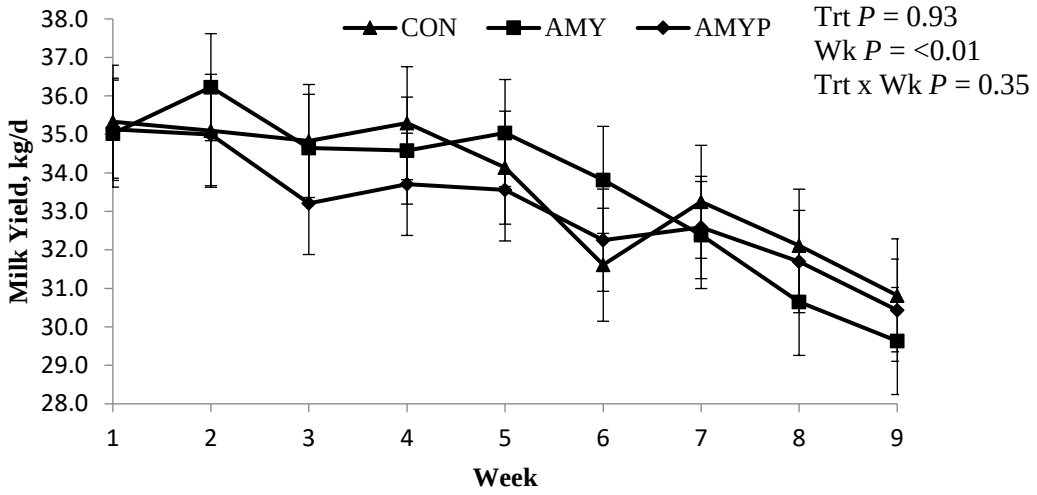

Figure 2. DMI (kg/d) of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

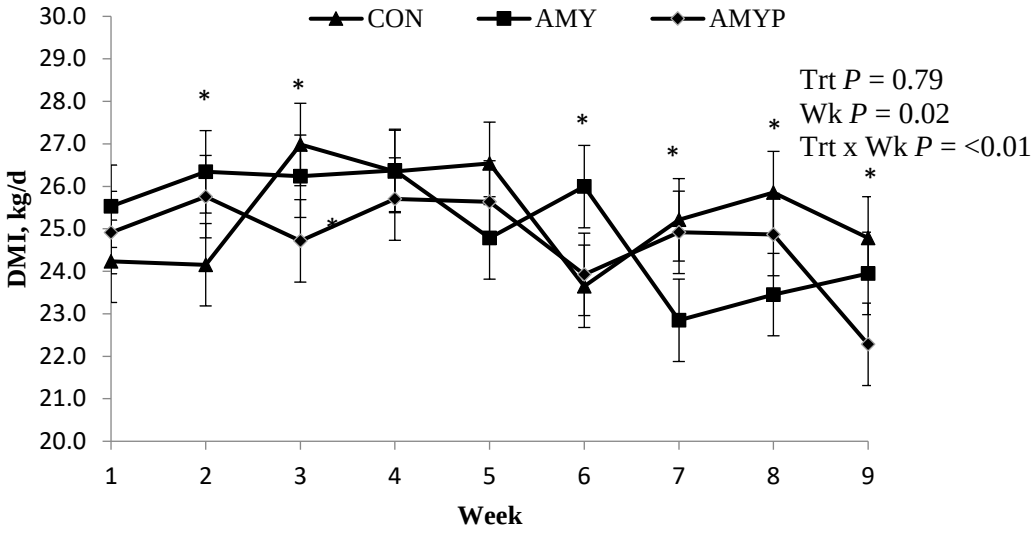

Figure 3. Changes in rumen pH of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets over 24 h.

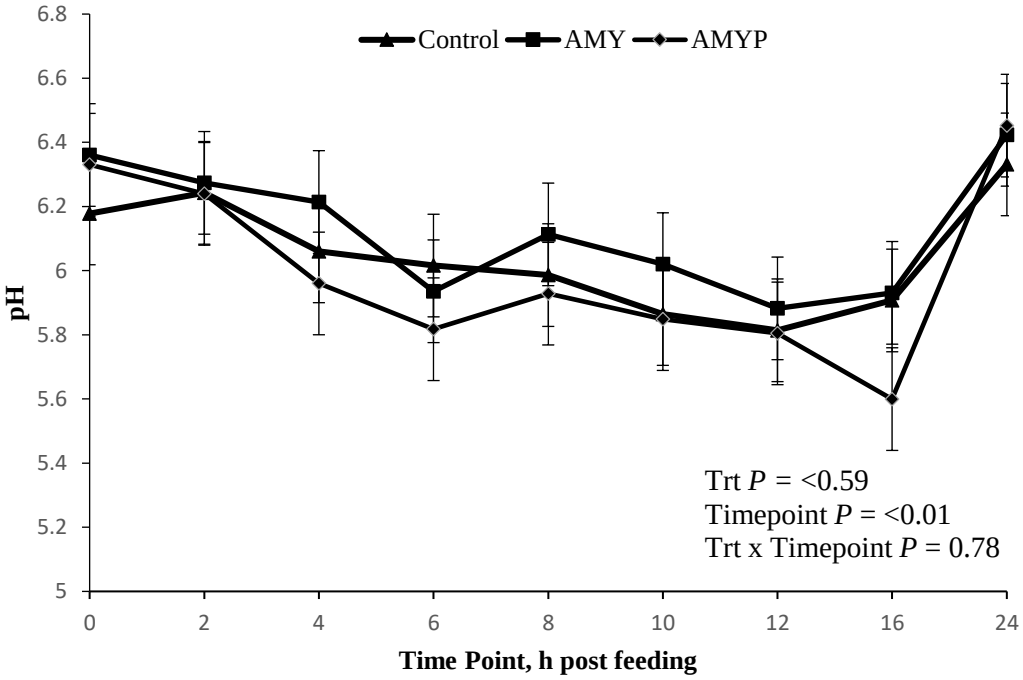

Figure 4. Changes in Total VFA of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets over 24 h.

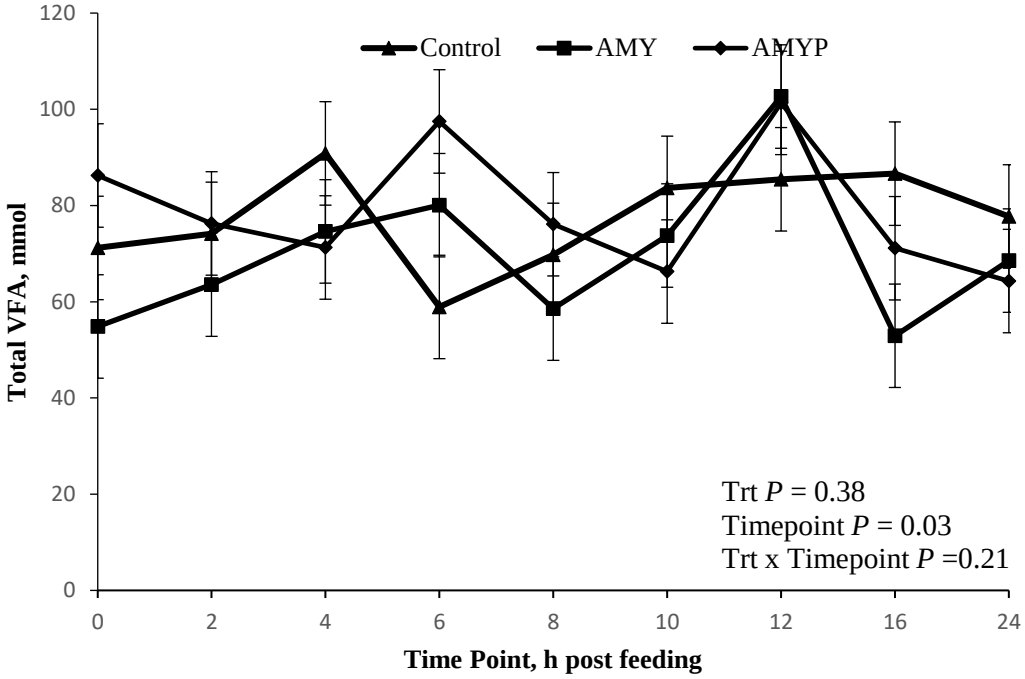

Table 1. Ingredient composition for the control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) treatment diets fed to lactating dairy cows.

	Treament[1]		
Ingredient[2], % DM	CON	AMY	AMYP
Corn Silage	40.00	40.00	40.00
Alfalfa hay	15.00	15.00	15.00
Grass hay	3.25	3.25	3.25
Soybean meal	9.25	9.25	9.25
Soybest L	6.25	6.25	6.25
Soybean hulls, ground	12.50	12.50	12.50
Corn, Ground	11.50	7.50	7.50
AMY Treatment Mix	0.00	4.00	0.00
AMYP Treatment Mix	0.00	0.00	4.00
Calcium carbonate	0.40	0.40	0.40
Sodium bicarbonate	0.15	0.15	0.15
Rumen Protected Fat	0.90	0.90	0.90
Magnesium Oxide	0.02	0.02	0.02
Vitamin E	0.05	0.05	0.05
Salt	0.60	0.60	0.60
Dairy Mineral[4]	0.07	0.07	0.07
Dairy Vitamin Premix[5]	0.06	0.06	0.06

[1] No Enzymes (CON); amylase enzyme (AMY); amylase and protease enzymes (AMYP).
[2] Formulated using Dairy NRC (2001).
[3] Contained: 2.5% C12:0 Myristic, 28.0% C16:0 Palmitic, 45.0% C18:0 Stearic, 8.3% C18:1 Oleic, 1.5% C18:2 Linoleic, and 0.1% C18:3 Linolenic (Energy Booster 100, Milk Specialties Global, Eden Prairie, MN).
[4] Contained: 11.7 % Ca (DM basis), 1.96 % S, 10,527 mg/kg Fe, 63,158 mg/kg Zn, 12,632 mg/kg Cu, 63,158 mg/kg Mn, 325 mg/kg Se, 632 mg/kg Co, and 1,053 mg/kg I (JPW Nutrition, Sioux Falls, SD).
[5] Contained: 25.8 % Ca (DM basis) 1,545 IU/kg Vitamin A, 387 IU/kg Vitamin D, and 4,826 IU/kg Vitamin E (JPW Nutrition, Sioux Falls, SD)

Table 2. Formulated[1] nutrient composition for the basal total mixed[2] ration fed to lactating dairy cows.

Item[3], % DM	Basal TMR
Dry Matter	55.18
CP	17.20
NDF	32.60
ADF	21.10
Ether extract	3.90
NFC[4]	41.80
Ca	0.70
P	0.34
Mg	0.24
K	1.35
S	0.16
Na	0.25
Cl	0.58
NE_L (Mcal/kg)	1.59

[1] Formulated using Dairy NRC (2001).
[2] 10 g/hd/d of amylase enzymes was added to AMY diet and 10 g/hd/d of amylase enzymes and 15 g/hd/d of protease enzymes added to AMYP diet.
[3] %DM, unless otherwise indicated.
[4] NFC = 100 - (%NDF+%CP+%EE+%Ash) (NRC, 2001).

Table 3. Nutrient composition of major ingredients used in the control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Forages[2]						Concentrates[3]			
Item[1], % DM	CS	SE	AH	SE	GH	SE	Corn	SE	GM	SE
DM[4], %	35.24	0.91	84.53	0.92	86.89	0.66	85.22	0.97	88.23	0.59
Ash[4]	4.19	0.07	9.18	0.21	9.18	0.32	1.49	0.05	9.76	1.04
OM[4]	95.81	0.07	90.82	0.21	90.82	0.32	98.51	0.05	90.24	1.04
CP[4]	7.43	0.09	18.47	0.23	8.25	1.23	8.27	0.04	26.38	1.32
NDF[4]	36.18	0.49	38.74	0.10	65.01	1.23	8.01	0.42	23.05	1.03
ADF[4]	19.12	0.15	28.42	0.06	35.50	0.41	2.32	0.06	14.74	1.12
Ether extract[4]	2.24	0.19	1.52	0.05	1.67	0.17	0.50	0.10	3.38	0.15
Starch[5]	32.56	1.11	2.96	-	1.83	-	64.98	-	17.36	-
NFC[4,6]	49.96	0.46	32.09	0.05	15.89	2.56	81.72	0.48	37.43	1.48
Ca[5]	0.22	0.01	1.44	-	0.37	-	0.12	-	1.46	-
P[5]	0.29	0.01	0.27	-	0.16	-	0.28	-	0.54	-
Mg[5]	0.17	0.01	0.32	-	0.21	-	0.1	-	0.30	-
K[5]	1.08	0.03	2.24	-	1.75	-	0.33	-	1.66	-
S[5]	0.09	0.00	0.24	-	0.16	-	0.09	-	0.29	-
Na[5]	0.01	0.00	0.11	-	0.04	-	0.01	-	0.94	-
Cl[5]	0.22	0.01	0.66	-	0.91	-	0.11	-	1.39	-

[1] %DM, unless otherwise indicated.
[2] CS=corn silage, AH=alfalfa hay, GH=grass hay
[3] GM=grain mix
[4] Results from analysis of tri-weekly analysis
[5] CS results from analysis of tri-weekly analysis, all other ingredients results from analysis of 9 week composite
[6] NFC = 100 - (%NDF+%CP+%EE+%Ash) (NRC, 2001).

Table 4. Nutrient composition of the control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment					
Item[1,2], % DM	CON	SE	AMY	SE	AMYP	SE
DM, %	53.64	0.37	53.42	0.06	54.23	0.59
Ash	7.20	0.04	7.01	0.05	7.04	0.06
OM	92.80	0.04	92.99	0.05	92.96	0.06
CP	17.05	0.35	16.33	0.38	16.48	0.42
NDF	31.88	1.03	32.77	1.09	32.20	0.30
ADF	18.93	0.73	19.51	0.24	19.47	0.21
Ether extract	3.49	0.04	3.67	0.16	3.65	0.11
Starch	19.89	0.68	19.59	0.27	20.33	0.31
NFC[3]	40.38	0.81	40.21	0.76	40.63	0.31
Ca	0.76	0.01	0.74	0.03	0.79	0.03
P	0.36	0.00	0.36	0.00	0.36	0.00
Mg	0.26	0.01	0.26	0.00	0.25	0.00
K	1.53	0.01	1.53	0.01	1.55	0.04
S	0.19	0.00	0.20	0.00	0.20	0.00
Na	0.29	0.00	0.28	0.01	0.28	0.02
Cl	0.62	0.01	0.67	0.02	0.63	0.02

[1] %DM, unless otherwise indicated.

[2] Results from analysis of tri-weekly analysis unless otherwise indicated

[3] NFC = 100 - (%NDF+%CP+%EE+%Ash) (NRC, 2001).

Table 5. Particle distribution and physically effective fiber using the Penn State Particle Separator[1] and Z-box[2] of the basal total mixed ration.

Item	Basal TMR	SE
Penn State Particle Separator		
Screen[3]		
Upper (19mm)	1.84	0.00
Middle (8mm)	30.47	0.02
Lower (4mm)	11.74	0.01
Sieve (1.18 mm)	32.03	0.01
Bottom Pan	21.86	0.01
peNDF[4]	14.01	0.75
Z- Box		
3.18 mm	46.34	0.02
peNDF	14.74	0.53

[1] Jones and Heinrichs, 2013.
[2] Grant and Cotanch, 2005.
[3] Average percentage retained on each screen.
[4] peNDF = pef x NDF, where pef= Sum of percent greater than 4mm and NDF=31.8.

Table 6. Milk yield, dry matter intake (DMI), feed efficiency, and body characteristics of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
Milk[1], kg/d	33.6	33.6	33.1	1.20	0.93	<0.01	0.35
0	29.5	28.5	27.9	1.12			
1	35.3	35.0	35.1	1.47			
2	35.1	36.2	35.0	1.47			
3	34.8	34.7	33.2	1.47			
4	35.3	34.6	33.7	1.47			
5	34.1	35.0	33.6	1.47			
6	31.6	33.8	32.3	1.47			
7	33.2	32.4	32.6	1.47			
8	32.1	30.6	31.7	1.47			
9	30.8	29.6	30.4	1.47			
DMI[1], kg/d	25.3	25.1	24.7	0.61	0.79	0.02	<0.01
0	23.1	22.9	22.7	0.67			
1	24.2	25.5	24.9	0.97			
2	24.2	26.3	25.8	0.83			
3	27.0	26.2	24.7	0.83			
4	26.3	26.4	25.7	0.83			
5	26.5	24.8	25.6	0.83			
6	23.6	26.0	23.9	0.83			
7	25.2	22.8	24.9	0.83			
8	25.9	23.5	24.9	0.83			
9	24.8	24.0	22.3	0.83			
Feed efficiency[2,3]	1.25	1.28	1.30	0.04	0.64	0.92	0.59
0	1.34	1.28	1.27	0.03			
3	1.27	1.33	1.27	0.07			
6	1.27	1.28	1.27	0.07			
9	1.21	1.23	1.36	0.07			
Body Weight[2], kg	689.6	695.1	698.7	5.79	0.52	<0.01	0.23
0	649.1	628.2	638.8	26.07			
3	687.6	685.2	688.9	4.76			
6	694.1	698.0	699.8	6.37			
9	687.1	702.1	707.3	8.18			
BCS[2,4]	3.11	3.11	3.12	0.02	0.99	0.04	0.88
0	3.01	3.06	3.15	0.03			
3	3.11	3.08	3.08	0.04			
6	3.13	3.18	3.17	0.04			
9	3.10	3.08	3.10	0.04			

[1] Overall means are daily averages over 9 week trial.
[2] Overall means are tri weekly averages over 9 week trial.
[3]Feed efficiency = ECM/DMI, kg/d.
[4]Body Condition Score (BCS) with 1=emaciated and 5=obese (Wildman et al., 1982).

Table 7. Milk yield and composition of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item[1]	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
Milk, kg	32.4	32.8	32.0	1.37	0.91	<0.01	0.10
0	29.7	28.7	28.1	2.01			
3	34.8	34.6	33.3	1.03			
6	31.6	33.8	32.3	1.64			
9	30.8	30.0	30.5	1.89			
ECM,[2] kg	31.1	32.5	29.9	1.26	0.31	<0.01	0.05
0	30.5	28.9	28.4	1.92			
3	34.4	34.8	31.1	1.46			
6	29.4	32.9	29.7	1.46			
9	29.8	29.8	29.0	1.46			
Fat, %	3.10	3.34	3.05	0.18	0.44	0.04	0.94
0	3.37	3.21	3.17	0.19			
3	3.33	3.50	3.13	0.23			
6	2.89	3.21	2.87	0.23			
9	3.06	3.30	3.14	0.23			
Fat Yield, kg	1.00	1.06	0.93	0.04	0.03	<0.01	0.54
0	1.01	0.94	0.93	0.05			
3	1.16	1.17	0.98	0.07			
6	0.90	1.05	0.89	0.07			
9	0.95	0.96	0.90	0.07			
Protein, %	3.34	3.39	3.28	0.03	0.01	<0.01	0.86
0	3.29	3.29	3.28	0.06			
3	3.25	3.33	3.18	0.05			
6	3.35	3.41	3.29	0.05			
9	3.43	3.44	3.38	0.05			
Protein Yield , kg	1.08	1.11	1.03	0.03	0.18	0.19	0.79
0	1.00	0.96	0.93	0.07			
3	1.13	1.15	1.04	0.05			
6	1.06	1.15	1.05	0.05			
9	1.05	1.03	1.01	0.05			
Lactose, %	4.94	4.84	4.86	0.03	0.03	0.18	0.13
0	5.07	5.11	5.11	0.05			
3	4.91	4.86	4.85	0.05			
6	4.95	4.93	4.88	0.05			
9	4.96	4.72	4.86	0.05			
Lactose Yield, kg	1.60	1.60	1.55	0.07	0.85	<0.01	0.26
0	1.52	1.48	1.45	0.10			
3	1.71	1.69	1.60	0.08			
6	1.56	1.67	1.57	0.08			
9	1.53	1.43	1.48	0.08			
SNF, %	9.13	9.10	9.00	0.05	0.20	<0.01	0.07
0	9.08	9.13	9.13	0.12			
3	8.96	9.01	8.84	0.07			
6	9.13	9.19	9.02	0.07			
9	9.29	9.09	9.15	0.07			

SNF Yield, kg	2.95	2.99	2.87	0.13	0.76	0.01	0.06
0	2.74	2.65	2.60	0.18			
3	3.11	3.12	2.93	0.09			
6	2.88	3.10	2.90	0.15			
9	2.86	2.74	2.77	0.18			
MUN, mg/dL	13.02	12.49	12.22	0.59	0.61	<0.01	0.02
0	13.32	13.69	12.54	0.48			
3	14.05	13.43	12.64	0.75			
6	12.09	13.28	13.08	0.75			
9	12.91	10.76	10.95	0.75			
SCC, 10^5/mL	184.11	104.40	166.17	59.88	0.59	0.41	0.31
0	56.09	62.69	85.54	33.92			
3	174.13	96.91	292.93	113.81			
6	311.90	78.25	86.76	133.02			
9	66.31	138.04	118.83	45.92			

[1] Overall means are tri weekly averages over 9 week trial.

[2]ECM = [(0.327 x kg milk) + (12.95* kg fat) + (7.2 x kg protein)] (Orth, 1992).

Table 8. Plasma metabolite concentrations of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item[1]	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
Glucose, mg/dL	64.37	62.61	63.53	0.80	0.28	0.83	0.94
0	65.56	65.19	65.79	1.02			
3	64.67	62.42	63.98	1.38			
6	64.54	62.07	62.76	1.38			
9	63.92	63.33	63.86	1.38			
Cholesterol, mg/dL	186.15	184.09	184.75	5.29	0.98	0.01	0.37
0	204.42	205.53	198.41	7.91			
3	188.80	180.50	185.97	10.77			
6	198.41	201.01	185.80	10.77			
9	171.25	170.78	182.49	10.77			
PUN, mg/dL	20.08	20.08	20.69	1.80	0.82	0.91	0.62
0	20.41	22.71	19.01	0.81			
3	20.41	20.42	19.90	1.40			
6	19.14	21.00	21.48	1.40			
9	20.70	18.81	20.68	1.40			
Triglyceride, mg/dL	14.23	15.08	14.75	0.89	0.78	0.72	0.41
0	17.78	16.87	14.82	0.98			
3	15.32	15.36	13.33	1.29			
6	13.62	14.62	14.80	1.29			
9	13.76	15.24	16.13	1.29			

[1] Overall means are tri weekly averages over 9 week trial.

Table 9. Weekly rumen fermentation characteristics of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item[1]	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
pH	6.04	6.13	6.00	0.59	0.59	<0.01	0.79
0	5.47	5.75	5.41	0.17			
3	5.77	5.94	5.78	0.10			
6	6.13	6.20	6.09	0.10			
9	6.23	6.25	6.13	0.11			
NH_3-N, mg/dL	9.27	8.95	10.36	0.65	0.26	0.40	0.43
0	11.92	11.58	15.10	3.43			
3	8.77	9.93	9.47	1.07			
6	9.42	7.58	10.04	1.07			
9	9.61	9.33	11.58	1.36			
Total, mM	77.59	69.95	78.93	4.33	0.38	0.51	0.25
0	102.98	106.04	104.62	14.22			
3	81.19	63.85	75.24	6.97			
6	74.98	74.70	72.34	6.52			
9	76.59	71.31	89.22	8.70			
Acetate, % of Total	52.34	54.07	53.20	1.21	0.64	0.26	0.62
0	48.89	48.26	46.80	2.37			
3	51.75	53.67	52.18	1.41			
6	53.33	53.83	53.28	1.41			
9	51.96	54.71	54.13	1.62			
Propionate, %	33.11	32.07	32.26	0.66	0.58	0.93	0.29
0	33.59	33.62	34.22	2.25			
3	33.50	31.13	33.19	0.92			
6	33.03	32.56	31.84	0.92			
9	32.80	32.52	31.75	1.21			
Butyrate, %	10.44	10.21	10.60	0.48	0.83	0.07	0.03
0	12.65	12.91	13.56	0.80			
3	10.58	11.33	10.62	0.58			
6	9.88	10.01	10.81	0.58			
9	10.87	9.29	10.36	0.68			
Isovalerate, %	1.60	1.71	1.85	0.08	0.08	0.48	0.14
0	1.48	1.80	1.92	0.06			
3	1.57	1.84	1.82	0.13			
6	1.46	1.56	1.95	0.13			
9	1.78	1.72	1.79	0.15			
Valerate, %	2.29	2.03	2.19	0.15	0.52	0.39	0.52
0	2.76	2.99	3.11	0.23			
3	2.38	2.07	2.29	0.18			
6	2.12	2.06	2.20	0.18			
9	2.37	1.96	2.08	0.21			
Acetate: Propionate	1.60	1.72	1.67	0.08	0.63	0.53	0.64
0	1.44	1.37	1.32	0.18			
3	1.55	1.75	1.59	0.09			
6	1.62	1.69	1.70	0.09			
9	1.64	1.71	1.72	0.11			

[1] Overall means are tri weekly averages over 9 week trial.

[2] No significant treatment by week by time point interactions were observed (P>0.05).

Table 10. Hourly rumen fermentation characteristics of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*[2]		
Item[1]	CON	AMY	AMYP	SEM	Treatment	Timepoint	Treatment x Timepoint
pH	6.04	6.13	6.00	0.59	0.59	<0.01	0.78
0	6.18	6.36	6.33	0.15			
2	6.24	6.27	6.24	0.13			
4	6.06	6.21	5.96	0.12			
6	6.02	5.94	5.82	0.12			
8	5.99	6.11	5.93	0.13			
10	5.86	6.02	5.85	0.16			
12	5.81	5.88	5.80	0.13			
16	5.91	5.93	5.60	0.15			
24	6.33	6.42	6.45	0.16			
NH_3-N, mg/dL	9.27	8.95	10.36	0.65	0.26	<0.01	0.59
0	8.58	7.08	8.58	1.86			
2	11.06	12.59	12.97	1.87			
4	11.14	4.38	9.73	1.84			
6	7.47	10.43	7.92	1.97			
8	6.83	5.29	9.47	1.86			
10	10.12	8.61	10.46	2.33			
12	10.01	12.90	12.66	1.90			
16	7.48	8.79	9.27	2.38			
24	10.71	10.45	12.22	2.34			
Total, mM	77.59	69.95	78.93	4.33	0.38	0.03	0.21
0	71.18	54.86	86.24	10.75			
2	74.11	63.56	76.28	10.86			
4	90.83	74.62	71.28	10.94			
6	58.92	80.07	97.48	10.75			
8	69.74	58.57	76.11	12.09			
10	83.68	73.77	66.27	14.11			
12	85.46	102.65	101.33	10.74			
16	86.62	52.93	71.12	13.76			
24	77.72	68.55	64.30	13.75			
Acetate, % of Total	52.34	54.07	53.20	1.21	0.64	0.06	0.87
0	52.78	56.54	53.19	1.79			
2	53.09	53.97	53.68	1.85			
4	53.33	52.51	53.78	1.79			
6	55.19	54.52	54.05	1.79			
8	53.96	55.73	54.11	1.79			
10	51.65	54.89	52.96	2.19			
12	51.86	52.62	51.23	1.82			
16	49.26	52.38	50.91	2.22			
24	49.99	53.50	54.90	2.26			
Propionate, %	33.11	32.07	32.26	0.66	0.58	0.18	0.32
0	33.11	30.28	32.87	1.44			
2	31.74	32.19	32.90	1.43			
4	33.15	34.16	32.09	1.45			
6	31.41	31.57	31.04	1.43			
8	30.96	29.60	32.36	1.43			
10	35.12	31.14	31.05	1.95			
12	33.30	33.22	32.38	1.44			
16	32.33	32.50	34.50	1.84			
24	36.87	33.96	31.16	1.83			
Butyrate, %	10.44	10.21	10.60	0.48	0.83	0.01	0.28

0	9.86	9.56	10.09	0.79			
2	10.98	10.10	9.80	0.86			
4	9.82	9.63	10.35	0.79			
6	9.54	10.24	10.73	0.80			
8	10.74	10.77	10.16	0.80			
10	9.25	10.59	11.33	1.00			
12	10.96	10.81	12.35	0.81			
16	13.36	11.26	10.47	0.99			
24	9.46	8.94	10.11	1.03			
Isovalerate, %	1.60	1.71	1.85	0.08	0.08	0.83	0.04
0	1.59	1.88	1.80	0.21			
2	1.65	1.84	1.78	0.21			
4	1.59	1.80	1.73	0.21			
6	1.72	1.64	1.81	0.21			
8	2.05	1.99	1.58	0.21			
10	1.11	1.69	2.40	0.27			
12	1.45	1.50	1.92	0.21			
16	2.05	1.50	1.67	0.26			
24	1.22	1.51	2.00	0.28			
Valerate, %[3]	2.29	2.03	2.19	0.15	0.52	0.02	0.83
0	2.38	1.86	2.26	0.21			
2	2.27	2.14	2.11	0.23			
4	2.10	2.02	2.14	0.22			
6	1.99	2.11	2.20	0.22			
8	2.15	1.90	2.03	0.22			
10	2.35	1.96	2.21	0.26			
12	2.30	2.01	2.40	0.22			
16	2.97	2.33	2.44	0.27			
24	2.09	1.96	1.93	0.27			
Acetate: Propionate	1.60	1.72	1.67	0.08	0.63	0.12	0.59
0	1.61	1.90	1.63	0.13			
2	1.68	1.69	1.65	0.13			
4	1.62	1.54	1.70	0.13			
6	1.78	1.74	1.75	0.13			
8	1.78	1.92	1.69	0.13			
10	1.47	1.77	1.74	0.16			
12	1.56	1.59	1.59	0.13			
16	1.53	1.64	1.48	0.16			
24	1.39	1.63	1.79	0.16			

[1] Overall means are tri weekly averages over 9 week trial.
[2] No significant treatment x week x timepoint interactions were observed (P>0.05).
[3] A tendency for week x timepoint was observed (P=0.08).

Table 11. Relative abundance of phylogenic taxonomy of OTU[1] of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
Phylum							
Actinobacteria	0.05	0.04	0.05	0.01	0.75	0.29	0.68
0	0.11	0.08	0.17	0.08			
4	0.05	0.04	0.04	0.01			
8	0.05	0.05	0.06	0.01			
Bacteroidia	0.85	0.80	0.84	0.05	0.80	0.94	0.50
0	0.76	0.71	0.63	0.07			
4	0.81	0.82	0.86	0.07			
8	0.89	0.78	0.81	0.07			
Clostridia	0.64	0.68	0.61	0.05	0.66	0.86	0.52
0	0.65	0.77	0.80	0.08			
4	0.67	0.68	0.57	0.07			
8	0.60	0.69	0.65	0.07			
Saccharibacteria	0.13	0.12	0.19	0.03	0.39	0.67	0.72
0	0.09	0.12	0.16	0.02			
4	0.15	0.10	0.22	0.05			
8	0.12	0.13	0.17	0.05			
Unclassified Bacteria	0.21	0.21	0.23	0.02	0.45	0.54	0.56
0	0.18	0.13	0.18	0.09			
4	0.22	0.20	0.24	0.02			
8	0.19	0.21	0.23	0.02			
Class							
Actinobacteridae	0.03	0.02	0.04	0.00	0.24	0.56	0.97
0	0.11	0.07	0.16	0.09			
4	0.03	0.02	0.04	0.01			
8	0.04	0.03	0.04	0.01			
Bacteroidales	0.84	0.80	0.83	0.05	0.84	0.98	0.51
0	0.76	0.71	0.63	0.07			
4	0.79	0.82	0.85	0.07			
8	0.89	0.77	0.12	0.07			
Clostridiales	0.64	0.68	0.61	0.05	0.65	0.89	0.53
0	0.66	0.77	0.80	0.08			
4	0.67	0.68	0.57	0.07			
8	0.60	0.69	0.64	0.07			
Unclassifed bacteria	0.27	0.24	0.34	0.01	0.02	0.47	0.55
0	0.19	0.17	0.24	0.03			
4	0.31	0.22	0.37	0.04			
8	0.23	0.26	0.41	0.04			
Order							
Bifidobacteriales	0.03	0.24	0.04	0.01	0.45	0.80	0.99
0	0.10	0.07	0.16	0.09			

4	0.03	0.02	0.04	0.01			
8	0.03	0.03	0.04	0.01			
Lachnospiraceae	0.34	0.36	0.34	0.02	0.72	0.85	0.62
0	0.39	0.43	0.42	0.04			
4	0.36	0.36	0.34	0.03			
8	0.32	0.37	0.35	0.03			
Prevotellaceae	0.69	0.64	0.65	0.02	0.43	0.30	0.05
0	0.66	0.54	0.48	0.07			
4	0.58	0.68	0.66	0.03			
8	0.80	0.60	0.64	0.03			
Ruminococcaceae	0.34	0.39	0.33	0.04	0.51	0.62	0.55
0	0.43	0.47	0.50	0.18			
4	0.40	0.39	0.30	0.04			
8	0.37	0.40	0.36	0.04			
Unclassified Bacteria	0.60	0.60	0.64	0.05	0.80	0.09	0.06
0	0.58	0.63	0.64	0.01			
4	0.68	0.58	0.66	0.06			
8	0.51	0.62	0.62	0.06			
Family							
Bifidobacteriaceae	0.03	0.02	0.04	0.01	0.45	0.80	0.99
0	0.10	0.07	0.16	0.09			
4	0.03	0.02	0.04	0.01			
8	0.03	0.03	0.04	0.01			
Prevotella	0.62	0.57	0.57	0.02	0.27	0.16	0.02
0	0.61	0.49	0.44	0.06			
4	0.50	0.61	0.58	0.02			
8	0.74	0.53	0.55	0.02			
Unclassified Bacteria	0.90	0.95	0.95	0.02	0.38	0.05	0.01
0	0.89	1.02	1.04	0.06			
4	1.01	0.92	0.94	0.02			
8	0.79	0.97	0.96	0.02			

[1] OTU=Operational Taxonomic Unit

Table 12. Relative abundance of individual OTU[1] of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
OTU 1	0.46	1.68	0.57	0.21	0.13	0.65	0.68
0	1.11	1.41	1.24	0.09			
4	0.64	1.66	0.94	0.27			
8	0.28	1.70	0.97	0.27			
OTU 2	1.13	0.92	1.54	0.28	0.41	0.66	0.68
0	0.62	0.92	1.20	0.14			
4	1.26	0.48	1.81	0.42			
8	1.01	1.05	1.28	0.42			
OTU 3	1.23	1.21	0.71	0.23	0.32	0.02	0.01
0	0.92	1.30	0.69	0.10			
4	0.86	1.32	0.66	0.24			
8	1.61	1.11	0.75	0.24			
OTU 4	1.32	0.78	0.92	0.15	0.30	0.71	0.35
0	1.32	0.74	0.94	0.06			
4	1.09	0.84	1.00	0.20			
8	1.54	0.73	0.85	0.20			
OTU 5	0.93	0.92	0.89	0.12	0.95	0.72	0.05
0	0.96	0.66	0.75	0.06			
4	0.75	1.07	0.88	0.13			
8	1.11	0.77	0.90	0.13			
OTU 6	0.92	0.70	0.99	0.30	0.79	0.92	0.48
0	1.40	1.06	1.21	0.28			
4	1.08	0.69	0.79	0.35			
8	0.75	0.70	1.19	0.35			
OTU 7	0.76	0.93	0.56	0.59	0.82	0.87	0.48
0	0.44	1.09	0.75	0.09			
4	1.01	0.85	0.45	0.62			
8	0.51	1.01	0.66	0.62			
OTU 8	0.80	0.76	0.87	0.08	0.71	0.74	0.43
0	0.77	0.70	0.55	0.12			
4	0.67	0.81	0.89	0.12			
8	0.93	0.72	0.84	0.12			
OTU 9	0.81	1.01	0.52	0.16	0.35	0.28	0.01
0	1.02	0.99	0.74	0.16			
4	0.69	1.17	0.54	0.16			
8	0.94	0.86	0.49	0.16			
OTU 10	0.92	0.58	0.72	0.08	0.20	0.28	0.29
0	0.73	0.49	0.50	0.54			
4	0.65	0.54	0.76	0.15			
8	1.20	0.62	0.68	0.15			
OTU 11	0.82	0.77	0.84	0.02	0.22	0.93	0.34

0	0.68	0.54	0.60	0.06			
4	0.85	0.87	0.73	0.09			
8	0.80	0.67	0.95	0.09			
OTU 12	0.74	0.91	0.68	0.10	0.32	0.33	0.61
0	0.73	0.86	0.82	0.17			
4	0.76	0.97	0.67	0.11			
8	0.71	0.85	0.68	0.11			
OTU 13	0.86	0.61	0.69	0.06	0.15	0.12	0.18
0	0.57	0.42	0.42	0.48			
4	0.56	0.53	0.72	0.11			
8	1.16	0.69	0.66	0.11			
OTU 14	0.57	0.64	0.60	0.12	0.94	0.90	0.35
0	0.65	1.05	0.83	0.13			
4	0.64	0.70	0.49	0.15			
8	0.51	0.57	0.71	0.15			
OTU 15	0.78	0.82	0.61	0.01	0.66	0.53	0.08
0	0.64	0.72	0.54	0.07			
4	0.62	0.91	0.62	0.05			
8	0.94	0.74	0.59	0.05			
OTU 16	0.69	0.72	0.65	0.07	0.81	0.69	0.46
0	0.64	0.74	0.82	0.11			
4	0.62	0.78	0.60	0.10			
8	0.76	0.65	0.71	0.10			
OTU 17	0.89	0.58	0.32	0.21	0.33	0.58	0.93
0	1.52	1.69	1.54	0.46			
4	0.80	0.55	0.30	0.24			
8	0.97	0.61	0.34	0.24			
OTU 18	0.62	0.74	0.66	0.10	0.75	0.26	0.45
0	0.59	0.79	0.77	0.08			
4	0.56	0.76	0.50	0.13			
8	0.69	0.73	0.83	0.13			
OTU 19	0.57	0.75	0.87	0.05	0.11	0.14	0.77
0	0.80	0.71	0.66	0.11			
4	0.47	0.71	0.80	0.08			
8	0.67	0.78	0.95	0.08			
OTU 20	0.88	0.64	0.44	0.21	0.33	0.24	0.12
0	0.88	0.68	0.71	0.04			
4	0.84	0.84	0.43	0.21			
8	0.92	0.44	0.44	0.21			
OTU 21	0.79	0.55	0.42	0.08	0.17	0.08	0.35
0	0.58	0.33	0.31	0.79			
4	0.50	0.47	0.35	0.13			
8	1.08	0.62	0.50	0.13			
OTU 22	0.73	0.57	0.53	0.08	0.39	0.09	0.01
0	0.96	0.60	0.51	0.13			
4	0.59	0.60	0.57	0.08			

8	0.87	0.55	0.48	0.08			
OTU 23	0.77	0.53	0.75	0.07	0.22	0.66	0.51
0	0.52	0.59	0.40	0.09			
4	0.73	0.55	0.74	0.08			
8	0.81	0.51	0.76	0.08			
OTU 24	0.51	0.45	0.76	0.07	0.15	0.85	0.50
0	0.77	0.78	0.79	0.76			
4	0.67	0.46	0.64	0.17			
8	0.35	0.44	0.88	0.17			
OTU 25	0.67	0.64	0.73	0.11	0.84	0.92	0.50
0	0.53	0.39	0.59	0.04			
4	0.64	0.70	0.70	0.12			
8	0.70	0.58	0.77	0.12			
OTU 26	0.38	0.92	0.46	0.10	0.11	0.32	0.64
0	0.48	0.74	0.65	0.06			
4	0.28	0.93	0.32	0.15			
8	0.48	0.90	0.60	0.15			
OTU 27	0.29	0.92	0.05	0.71	0.60	0.64	0.26
0	0.74	1.59	0.73	0.26			
4	0.45	0.84	0.04	0.72			
8	0.12	1.00	0.05	0.72			
OTU 28	0.41	0.64	0.62	0.15	0.62	0.66	0.80
0	0.45	0.80	0.64	0.08			
4	0.36	0.67	0.56	0.19			
8	0.47	0.61	0.69	0.19			
OTU 29	0.61	0.68	0.62	0.07	0.75	0.45	0.18
0	0.51	0.43	0.44	0.06			
4	0.54	0.77	0.51	0.09			
8	0.67	0.59	0.74	0.09			
OTU 30	0.58	0.56	0.67	0.12	0.79	0.79	0.36
0	0.24	0.18	0.28	0.30			
4	0.44	0.64	0.68	0.15			
8	0.73	0.48	0.66	0.15			
OTU 31	0.43	0.71	0.35	0.17	0.44	0.84	0.67
0	1.52	1.63	1.19	0.25			
4	0.44	0.63	0.47	0.22			
8	0.42	0.78	0.23	0.22			
OTU 32	0.49	0.58	0.53	0.06	0.54	0.79	0.49
0	0.57	0.26	0.82	0.14			
4	0.56	0.55	0.44	0.10			
8	0.41	0.61	0.62	0.10			
OTU 33	0.53	0.64	0.50	0.11	0.61	0.39	0.18
0	0.76	0.47	0.38	0.13			
4	0.52	0.73	0.49	0.11			
8	0.55	0.55	0.52	0.11			
OTU 34	0.49	0.52	0.41	0.01	0.06	0.56	0.57

0	0.57	0.65	0.82	0.14			
4	0.60	0.50	0.41	0.08			
8	0.37	0.53	0.41	0.08			
OTU 35	0.69	0.43	0.37	0.29	0.73	0.55	0.65
0	0.23	0.63	0.44	0.38			
4	0.89	0.408	0.39	0.34			
8	0.48	0.448	0.36	0.34			

[1] OTU=Operational Taxonomic Unit

Table 13. Apparent total tract digestion of nutrients of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				*P-values*		
Item, % Digested	CON	AMY	AMYP	SEM	Treatment	Week	Treatment x Week
DM	54.26	59.69	55.25	1.20	<0.01	0.09	0.95
0	51.18	49.80	52.99	1.46			
3	54.02	60.68	55.16	2.07			
6	56.88	60.72	56.75	2.07			
9	51.88	57.68	53.84	2.07			
OM	56.26	61.61	57.33	1.17	<0.01	0.10	0.94
0	53.00	51.57	54.97	1.45			
3	55.92	62.44	57.10	2.03			
6	58.93	62.56	58.80	2.03			
9	53.94	59.83	56.09	2.03			
CP	55.46	57.95	54.78	1.24	0.15	0.01	0.86
0	49.73	47.91	52.13	1.70			
3	56.35	59.32	56.21	2.15			
6	57.67	58.59	57.62	2.15			
9	52.35	55.95	50.52	2.15			
NDF	32.18	39.25	32.80	2.81	0.09	0.01	0.25
0	11.55	8.35	8.75	3.66			
3	29.25	39.09	32.78	4.27			
6	37.87	46.27	33.66	4.27			
9	29.41	32.37	31.96	4.27			
ADF	24.88	33.31	28.07	3.30	0.14	0.00	0.50
0	7.80	4.14	4.63	3.80			
3	22.34	34.32	26.70	4.87			
6	32.57	39.51	30.97	4.87			
9	19.73	26.09	26.55	4.87			
Starch	96.19	96.33	95.81	0.34	0.52	0.06	0.34
0	97.31	96.37	97.64	0.78			
3	95.87	95.88	95.47	0.63			
6	96.73	95.91	95.66	0.63			
9	95.96	97.21	96.29	0.63			

Table 14. Ruminal degradation of TMR of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				
Item[1]	CON	AMY	AMYP	SEM	*P-value*
DM dis., %					
A[2]	40.99	35.88	37.45	1.25	0.13
B[3]	46.32	51.81	39.88	2.99	0.14
C[4]	12.69	12.31	22.67	3.08	0.16
K_d[5], % h	4.95	2.88	5.17	0.40	0.05
RDDM[6]	59.20	55.21	54.29	1.47	0.18
CP dis, %					
A	30.22	24.59	21.50	3.20	0.29
B	66.63	75.41	63.01	5.83	0.41
C	3.15	0.00	15.49	5.19	0.23
K_d, % h	4.41	2.22	4.00	0.68	0.20
RDP[7]	54.11	48.27	44.30	2.39	0.13
RUP[8], % of CP	45.89	51.73	55.70	2.39	0.13
NDF dis, %					
A	11.61	8.77	11.13	1.29	0.38
B	67.80	71.18	47.55	12.44	0.45
C	20.06	20.05	41.32	11.60	0.44
K_d, % h	2.82	1.47	2.54	0.48	0.26
RDNDF[9]	29.20	24.42	23.66	2.86	0.44
ADF dis, %					
A	7.31	6.23	8.09	0.95	0.47
B	82.05	93.77	67.32	8.17	0.22
C	10.64	0.00	24.59	8.07	0.24
K_d, % h	2.16	0.93	1.44	0.37	0.20
RDADF[10]	24.65	21.15	19.55	2.67	0.48
Starch dis, %					
A	57.67	48.00	50.74	3.49	0.28
B	42.33	47.03	49.26	6.10	0.74
C	0.00	4.98	0.00	2.87	0.46
K_d, % h	5.74	7.42	9.01	1.65	0.47
RDS[11]	75.87	75.40	78.46	0.93	0.18

[1]Units expressed in % DM unless otherwise noted.

[2]Soluble fraction

[3] Potentially degradable fraction

[4] Undegradable fraction

[5] Rate of degradation.

[6] Ruminally degradable DM

[7] Ruminally degradable protein

[8] Ruminally undegradable protein

[9] Ruminally degradable NDF

[10] Ruminally degradable ADF

[11] Ruminally degradable starch

Table 15. Ruminal degradation of corn of lactating cows fed control (CON), amylolytic enzyme (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				
Item[1]	CON	AMY	AMYP	SEM	*P-value*
DM dis., %					
A[2]	31.50	25.71	27.88	2.43	0.36
B[3]	68.50	72.85	66.54	2.80	0.39
C[4]	0.00	1.44	5.58	3.32	0.54
K_d[5], % h	4.73	3.84	6.75	1.07	0.29
RDDM[6]	57.79	56.51	60.16	0.64	0.06
CP dis, %					
A	26.21	21.82	26.84	2.27	0.36
B	73.79	78.18	73.16	2.27	0.36
C	.	.	.	.	.
K_d, % h	4.15	3.14	4.37	0.52	0.33
RDP[7]	52.26	52.33	54.81	0.90	0.22
RUP[8], % of CP	47.74	47.67	45.19	0.90	0.22
Starch dis, %					
A	38.94	28.09	31.15	4.68	0.37
B	53.50	55.43	52.87	11.85	0.99
C	7.55	16.48	15.98	12.41	0.85
K_d, % h	7.08	15.25	17.40	8.75	0.71
RDS[9]	63.48	59.28	65.94	1.23	0.07

[1]Units expressed in % DM unless otherwise noted.
[2]Soluble fraction
[3] Potentially degradable fraction
[4] Undegradable fraction
[5] Rate of degradation.
[6] Ruminally degradable DM
[7] Ruminally degradable protein
[8] Ruminally undegradable protein
[9] Ruminally degradable starch

Table 16. Ruminal degradation of CS from wk 1 to 3 of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				
Item[1]	CON	AMY	AMYP	SEM	*P-value*
DM dis., %					
A[2]	45.76	39.90	45.15	2.69	0.36
B[3]	30.09	32.82	29.16	3.90	0.80
C[4]	24.14	27.28	25.69	4.33	0.88
K_d[5], % h	7.19	4.56	5.56	1.40	0.49
RDDM[6]	60.42	55.73	57.05	1.07	0.11
CP dis, %					
A	64.65	57.83	61.99	3.21	0.43
B	22.15	27.15	35.93	7.01	0.47
C	13.20	15.02	2.07	9.22	0.61
K_d, % h	2.70	1.86	1.33	0.79	0.54
RDP[7]	70.27	63.19	67.62	2.90	0.35
RUP[8], % of CP	29.73	36.81	32.38	2.90	0.35
NDF dis, %					
A	5.31	5.41	5.43	1.66	1.00
B	35.73	34.84	56.09	21.82	0.76
C	58.96	59.76	38.48	22.35	0.77
K_d, % h	6.61	2.81	3.89	1.75	0.40
RDNDF[9]	21.87	18.19	15.18	1.26	0.07
ADF dis, %					
A	1.41	3.03	3.55	0.89	0.34
B	35.85	38.06	55.86	23.25	0.81
C	62.74	58.92	40.58	23.50	0.79
K_d, % h	6.92	2.28	4.44	2.11	0.41
RDADF[10]	18.41	15.21	12.64	1.18	0.09
Starch dis, %					
A	61.39	54.72	58.20	5.19	0.69
B	38.61	45.28	38.48	5.82	0.68
C	0.00	0.00	3.32	1.91	0.46
K_d, % h	10.67	7.65	11.29	2.46	0.59
RDS[11]	84.06	81.05	81.76	2.11	0.62

[1]Units expressed in % DM unless otherwise noted.
[2]Soluble fraction
[3] Potentially degradable fraction
[4] Undegradable fraction
[5] Rate of degradation.
[6] Ruminally degradable DM
[7] Ruminally degradable protein
[8] Ruminally undegradable protein
[9] Ruminally degradable NDF
[10] Ruminally degradable ADF
[11] Ruminally degradable starch

Table 17. Ruminal degradation of CS from wk 4 to 6 of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				
Item[1]	CON	AMY	AMYP	SEM	*P-value*
DM dis., %					
A[2]	44.17	39.94	44.59	2.82	0.52
B[3]	32.99	45.96	27.26	9.16	0.44
C[4]	22.84	14.10	28.15	8.58	0.57
K_d[5], % h	6.09	3.27	6.76	1.91	0.48
RDDM[6]	58.74	54.58	57.55	0.88	0.09
CP dis, %					
A	66.50	61.87	63.81	2.22	0.44
B	21.69	12.06	28.76	15.42	0.76
C	11.81	26.07	7.43	17.07	0.74
K_d, % h	6.36	9.86	1.69	6.24	0.68
RDP[7]	71.64	58.14	68.70	4.90	0.27
RUP[8], % of CP	28.36	41.86	31.30	4.90	0.27
NDF dis, %					
A	6.77	4.18	8.24	1.58	0.32
B	56.67	63.83	57.94	26.66	0.98
C	36.56	31.99	33.82	26.88	0.99
K_d, % h	2.21	2.72	1.34	1.18	0.73
RDNDF[9]	19.55	19.15	15.82	2.29	0.53
ADF dis, %					
A	2.26	0.10	0.70	0.41	0.07
B	53.47	64.00	49.90	29.72	0.94
C	44.28	35.90	49.40	29.47	0.95
K_d, % h	2.15	2.63	1.71	1.18	0.87
RDADF[10]	14.03	14.15	7.80	2.46	0.26
Starch dis, %					
A	63.65	52.24	60.32	4.82	0.36
B	36.35	42.19	37.30	6.36	0.80
C	0.00	5.57	2.38	3.22	0.54
K_d, % h	11.31	15.30	16.05	6.59	0.87
RDS[11]	85.45	79.88	85.98	1.92	0.19

[1]Units expressed in % DM unless otherwise noted.
[2]Soluble fraction
[3] Potentially degradable fraction
[4] Undegradable fraction
[5] Rate of degradation.
[6] Ruminally degradable DM
[7] Ruminally degradable protein
[8] Ruminally undegradable protein
[9] Ruminally degradable NDF
[10] Ruminally degradable ADF
[11] Ruminally degradable starch

Table 18. Ruminal degradation of CS from wk 7 to 9 of lactating cows fed control (CON), amylolytic (AMY), and amylolytic and proteolytic enzyme (AMYP) diets.

	Treatment				
Item[1]	CON	AMY	AMYP	SEM	*P-value*
DM dis., %					
A[2]	47.31	43.25	47.42	1.97	0.36
B[3]	25.20	43.87	23.23	9.59	0.37
C[4]	27.49	12.88	29.35	8.33	0.42
K_d[5], % h	7.16	2.45	3.88	0.73	0.04
RDDM[6]	59.45	56.48	55.54	1.51	0.30
CP dis, %					
A	61.71	59.61	64.28	2.07	0.40
B	14.78	40.39	20.05	7.32	0.17
C	23.51	0.00	15.66	9.05	0.31
K_d, % h	9.51	0.50	3.97	2.68	0.20
RDP[7]	69.61	62.84	67.67	2.10	0.21
RUP[8], % of CP	30.39	37.16	32.33	2.10	0.21
NDF dis, %					
A	6.42	5.23	2.20	1.44	0.25
B	63.48	74.98	55.23	30.49	0.90
C	30.10	19.78	42.57	31.35	0.88
K_d, % h	2.11	1.08	10.81	5.82	0.51
RDNDF[9]	16.70	18.10	12.51	3.93	0.63
ADF dis, %					
A	4.92	4.42	1.61	1.27	0.29
B	70.59	72.28	54.34	30.99	0.59
C	24.50	23.30	44.05	32.06	0.88
K_d, % h	1.13	0.83	8.90	5.01	0.51
RDADF[10]	13.19	14.61	7.49	2.91	0.32
Starch dis, %					
A	72.25	63.77	69.63	2.29	0.16
B	27.75	33.76	26.70	3.93	0.48
C	0.00	2.47	3.67	2.56	0.63
K_d, % h	11.63	13.91	19.63	8.02	0.78
RDS[11]	89.08	86.40	87.57	1.12	0.36

[1]Units expressed in % DM unless otherwise noted.

[2]Soluble fraction

[3] Potentially degradable fraction

[4] Undegradable fraction

[5] Rate of degradation.

[6] Ruminally degradable DM

[7] Ruminally degradable protein

[8] Ruminally undegradable protein

[9] Ruminally degradable NDF

[10] Ruminally degradable ADF

[11] Ruminally degradable starch

OVERALL CONCLUSIONS

The research presented provides further knowledge on the influence of supplementing exogenous amylolytic and proteolytic enzymes to dairy cows fed freshly ensiled corn silage based diets. We observed no improvements in milk yield and feed efficiency; unlike previous reports with amylolytic and proteolytic supplementation (Tricarico et al., 2002; Harrision and Tricarico, 2007, Klingerman et al., 2008). Despite a lack of improvement in lactation performance, ruminal percentage butyrate concentrations were increased for cows fed the diet supplemented with the amylolytic enzyme during week 3, similar to results presented by Tricarico et al. (2002), Hristov et al. (2000), and Tricarico et al. (2005). In addition, ruminal *Prevotella* populations decreased in the amylolytic enzyme diet overtime while populations increased in the control diet; however, this directly contradicts previous reports from Noziere et al. (2014). As previous research on exogenous amylolytic and proteolytic enzymes is limited, further research is warranted to facilitate our understanding of exogenous enzymes can influence fermentation pathways and, subsequently, lactation performance. Based on the conditions of this study, we conclude the addition of exogenous enzymes altered the rumen microbiome and fermentation pathways, but maintained lactation performance compared to cows fed the control diet with no enzyme supplementation.

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