The Effect of Homofermentative Lactic acid Bacteria and Exogenous Fibrolytic Enzymes on the Ensiling Characteristics and Rumen Degradability of Alfalfa, Corn Silages and Cool Season Mixed Grass Haylage

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Authorization to Submit Thesis

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Abstract

Homofermentative lactic acid bacteria (LAB) increase lactic acid concentrations, lower pH therefore LAB inoculants are used to improved silage. Fibrolytic enzymes (FE) hydrolyze structural carbohydrates and increase fiber degradation. The goal of this research was to examine the effects of a combination of four homofermentative LAB and four FE on the ensiling characteristics of alfalfa and corn silages in a laboratory setting (experiment I) as well as on ensiling characteristics of a cool season mixed grass haylage at farm scale (experiment II). Alfalfa and corn were treated with water, (control: CON) or the LAB/FE (TRT), and ensiled in tube mini silos (volume 1206 cm^3) and bucket mini-silos (volume 21,504 cm^3) for 59 d. Mixed cool season grasses were treated with water (CON), or the LAB/FE (TRT), and ensiled for 90 d in round bales. Experiment I was analyzed using a MIXED model (PROC MIXED, SAS 9.3) and experiment II was analyzed using a T-Test (PROC TTEST, SAS 9.4). Significance was declared at $P \leq 0.05$. Rate of pH decline, d 1 - 13, was greater in TRT alfalfa compared to control ($P = 0.005$) and LA, % of total organic acids, tended to be greater in the TRT alfalfa ($P = 0.06$) as did final pH ($P = 0.07$). Dry-matter degradation was greater in the TRT alfalfa ($P = 0.02$). Ammonia ($P = 0.03$) and NDF ($P = 0.02$) were reduced in the TRT grass haylage. In contrast, *in situ* NDF ($P = 0.05$), NDF ($P = 0.04$) and organic matter ($P = 0.001$) degradation were greater in the TRT grass haylage. These findings indicates that the LAB/FE additive used in this study may improve silage preservation and improve fiber degradation.

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Dedication

This work is dedicated to the people who always encouraged me and stood beside me.

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Chapter 1: Introduction

Ensiling is a method of preserving high-moisture forages with dry matters (DM) as low as 30-40%, as opposed to hay, which has a DM greater than 88%. By harvesting at a lower DM the maturity of the forages is often less than that of hay. This in turn means the plant has a lower neutral detergent fiber (NDF), acid detergent fiber (ADF), and higher crude protein (CP) content. As a result, silage is generally more digestible than more mature forages. Ensiling has been in use for over a 100 years in Europe and North America. Over the past 40 years silage has become a popular preservation method, and is frequently, used in dairies, in finishing rations of lambs and, less often, in beef operations. As a result of its popularity, there continues to be a need to research methods to improve the ensiling process.

The ensiling process is commonly divided into six phases. Phase I is the aerobic to anaerobic phase and is the time period from the closing of the container until all of the oxygen is exhausted. This period is a time of high DM loss and thus the aim is to shorten this interval as much as possible. Once an anaerobic environment has been achieved, the silage enters phase II. Phase II-IV involve the production of organic acids (OA) from bacterial fermentation of water soluble carbohydrates (WSC). Lactic acid is the predominant acid produced during bacterial fermentation, and is the cause of the rapid pH decline. Acetic acid has stronger anti-mycotic properties. In properly prepared silage, pH is generally 4.0-4.5 and the lactic acid to acetic acid ratio is greater than 3:1. The OA are produced by lactic acid-producing bacteria (LAB) and can be divided into two categories, homo- and heterofermentative LAB. Homofermentative LAB primarily produce lactic acid, whereas heterofermentative LAB produce both lactic acid and acetic acid. Stage V is the storage phase which can last as long as the silage remains anaerobic. Stage VI begins once the silage is re-exposed to oxygen. Yeast

and molds, the main causative agents of spoilage and DM loss, become active almost immediately upon exposure to oxygen. The OA produced in Phases II-IV can impede the growth of yeasts and molds. The ability for silage to resist spoilage, by yeasts and molds, is termed aerobic stability.

Adding inoculants containing LAB is the one of the most common and efficient methods used to improve the ensiling process. Inoculants may contain homo- and/or heterofermentative LAB. Homofermentative LAB inoculants decrease pH to a greater extent and produce greater amounts of lactic acid than heterofermentative LAB inoculants. Homofermentative LAB, however, have not shown consistent improvement in nutrient retention, DM loss nor aerobic stability. Heterofermentative LAB, however, increase pH, DM loss, and aerobic stability and lower lactic acid to acetic acid ratio compared to a homofermentative LAB. Neither homo- nor heterofermentative LAB inoculants have shown a consistent improvement in degradability or digestibility.

In recent years the addition of fibrolytic enzymes (FE) to inoculants has become increasingly popular. These FE are hydrolytic enzymes that cleave structural carbohydrates (CHO) into reducing sugars. Fibrolytic enzymes have increased lactic acid production and lowered pH in some studies. The use of FE increases WSC, while decreasing NDF, ADF and cellulose. Fibrolytic enzymes also improve degradability and digestibility of structural CHO, especially NDF in the first 24 h.

Summary

Bacterial inoculants increase OA production, although the type of inoculant impacts what OA are produced and the OA effect on the silage. Homofermentative LAB inoculants increase

lactic acid production and thus increase rate and extent of pH decline, and may improve DM retention. Heterofermentative LAB inoculants increase acetic acid production, pH, DM loss and aerobic stability. Fibrolytic enzymes improve the amount of WSC and decrease the amount of structural CHO, which may improve OA production when used in conjunction with LAB inoculants. Fibrolytic enzymes also improve digestibility of silages, especially fiber digestibility or rumen degradation in the first 24 h. The addition of FE to LAB inoculants is increasing and the new combination inoculants and FE effects on ensiling requires further study.

The objective of this research was to evaluate the effects of a commercially available silage treatment, Sil-All 4x4, which contains four homofermentative LAB in combination with four FE on: 1) alfalfa and corn silage in a small scale, laboratory setting and 2) a mixed cool season grass haylage in a farm scale setting.

Chapter 2: Literature Review and Hypothesis

Introduction

Ensiling is a process of preserving forage that utilizes the byproducts of anaerobic fermentation**.** During the fermentation process, LAB utilize CHO for energy which are then converted to OA. Lactic acid is one of the dominant organic acids produced and is the most responsible for the decrease in pH necessary for preservation (Woolford, 1975; Sun et al., 2012). Lactic acid is desired because it increases the rate of pH decline, which decreases the time to preservation, and possibly reduces dry matter (DM) and nutrient loss. On the other hand, other organic acids such as acetic acid are better at inhibiting yeast and mold growth (Woolford, 1975; Seglar, 2003; Kung, 2010).

Ensiling preserves forages that are less mature with greater moisture content than other preservation methods would allow. This allows the forage to be harvested with a less lignified cell wall. Lignin is indigestible and also inhibits the degradation of structural CHO in the rumen. The amount of lignin increases as the plant matures. Therefore, less mature plants should be more ruminally degradable than more mature plants of the same species (Van Soest, 1994). Crude protein is also greater in younger plants than older plants, as is sugar in grasses (van Soest, 1994), whereas starch levels increase with maturity in grains (Hunt et al., 1989). Silages, however, tend to have greater CP degradation than dried forages (Kohn and Allen, 1995).

As a method of forage preservation, ensiling has been in practice in the west since the latter 19th century. Ensiling first appeared in the United Kingdom (UK) in the 1880s but was rarely utilized until the 1940s. From the 1940s until the 1970s, the adoption of ensiling gradually increased. Today, ensiling is the most popular method of winter forage preservation in

northern Europe. Silage production is ten times greater than hay production in the UK and between a quarter and three quarter of forages fed to lactating dairy cattle are comprised of whole plant corn silages in the United States (Brassley, 1996; Bal et al., 2000; Burke et al., 2007).

Ensiling Process

The ensiling process can be divided into six different phases (Seglar, 2003). Phase I begins with the silage environment changing from aerobic to anaerobic. During this phase some of the greatest DM and nutrient loss can occur. Nutrients are lost as aerobic bacteria and yeasts convert soluble CHO to water, carbon dioxide and heat. Phase I will last until all available oxygen has been depleted. As a result, this phase needs to be as short as possible and is accomplished by ensuring adequate packing density and proper sealing of the silo, bunker or bag (Seglar, 2003).

The second phase of ensiling is when anaerobic fermentation starts. Lactic acid and acetic acid are produced during this phase. Lactic acid and acetic acid production results in a sharp decline in pH, from a near neutral environment to a pH of approximately 5.0. Additionally, WSC decrease sharply as a result of fermentation (Bergen et al., 1991). In addition to the short chain fatty acids, CO_2 and alcohol are also produced (Seglar, 2003).

Phases III and IV are primarily marked by the continued production of lactic acid and are very similar to each other. The main difference between the two, is that heat is lost at an increased rate during third phase whereas in fourth phase the temperature of the silage remains relatively stable (Seglar, 2003). Additionally, acetic acid production stabilizes, whereas lactic acid production continues. This continuing lactic acid production decreases silage pH after

approximately d 5 (Bergen et al., 1991). The continued production of lactic acid will eventually lower the pH to an asymptote, or terminal pH, as low as 4.0 in some forages. Lactic acid content greater than 60% lactic acid (% total OA) is considered to be a good quality silage (Seglar, 2003). Additionally, the lactic acid to acetic acid ratio is greater than 3:1 in properly produced silage (Kung, 2010).

By approximately d 20 the decrease in pH and WSC, as well as lactic acid production ceased. The pH remains fairly stable after this point (Bergen et al., 1991). This asymptote is reached when pH has declined to the point where it inhibits further microbial activities (Seglar, 2003). Lactic acid bacteria are inhibited at a pH at or below 4.0 (Woolford, 1975). Once the asymptote, or terminal pH, is reached the silage enters into the fifth phase: the storage phase. This phase can last for an extended period of time, as long as the anaerobic environment is maintained (Seglar, 2003). Finally, the sixth and last phase is the feed-out stage, when the silage is exposed to air. This phase begins the process of aerobic spoilage of the silage and can result in as much as 50% DM loss (Seglar, 2003). The degradation of lactic acid in the presence of air by yeasts is the main driver of the spoilage (Kung, 2010). Additionally, the yeasts produce secondary metabolites, called mycotoxins, which can be detrimental to the health of livestock (Skladanka et al., 2013). High yeast counts may also reduce the performance of lactating dairy cows and have resulted in decreased NDF degradation (NDFD), as observed in *in vitro* trials (Santos et al., 2014).

Many factors are needed to ensure proper ensiling of forages including proper moisture levels, packing density, chop length and maintenance of an anaerobic environment (Kung, 2010). Additionally plant maturity and DM are inversely related to packing density (Muck and Holmes 2000; Johnson et al., 2002). Therefore, age of the plant, plant species and even sub-

species or hybrid type, moisture content and height of the cut may all effect the ensiling process. If any of these factors are compromised the silage may lose nutrients or be unusable (Kung, 2010). To address the many possible problems that may occur during ensiling, producers have a variety of strategies available, such as the addition of chemical additives (including ammonia, and organic acids) or the addition of biological agents such as LAB inoculants and FE. In addition to aiding preservation, an inoculant and FE combination may improve the safety of silages under certain disease conditions (Queiroz et al., 2012).

Increases in OA concentrations, and the accompanying decrease in pH, has an inhibitory effect on yeast and mold growth (Woolford, 1975). These yeasts and molds are the main contributors to aerobic spoilage (Seglar, 2003; Kung, 2010). The goal of many additives is to increase OA concentrations and decrease pH. Originally, many of the additives used to aid in the preservation of forages were selected solely on empirical evidence based upon physical properties and many were previously used in preservation of human food (Woolford, 1975).

The addition of LAB inoculants is the most popular biological method of improving the preservation of silage in the United States (Filya et al., 2007). Lactic-acid producing bacterial inoculants can contain either a single or multiple species. These bacteria can be divided into homofermentative or heterofermentative. The addition of LAB may increase the amount of lactic acid produced, which allows for a more rapid decline in pH. Additionally, heterofermentative LAB may also change the OA profile which could result in increasing aerobic stability (Ranjit and Kung, 2000). Heterofermentative bacteria are most responsible for early stage fermentation, and most active during the second phase of fermentation. They also produce other OA in addition to lactic, but are generally less efficient fermenters than homofermentative LAB (Seglar, 2003). Additionally, heterofermentative LAB are less

efficient at nutrient retention than homofermentative LAB (Ranjit and Kung, 2000). The addition of heterofermentative LAB also reduces the lactic acid to acetic acid ratio (Ranjit and Kung, 2000). Homofermentative LAB on the other hand produce primarily lactic acid. Homofermentative LAB are most active in the third and fourth phase of ensiling in silages and are more efficient fermenters than heterofermentative LAB (Seglar, 2003). Fibrolytic enzymes (FE), in addition to LAB, have also been used to improve the ensiling characteristics of lower quality foragers. Fibrolytic enzymes, especially xylanases and cellulases, increase the amount of reducing sugars when applied to pure preparations of cellulose and xylan (Columbatto et al., 2003a).

The goal of ensiling is to speed up phase I and limit the damage that occurs in phase VI. Due to the numerous factors that influence OA production and ensiling the use of LAB inoculants is popular. Homofermentative LAB inoculants produce primarily lactic acid, and are thus most effective at speeding up the preservation time. Heterofermentative LAB increase acetic acid, a stronger antimycotic agent, and therefore contribute to increased aerobic stability during phase VI. Decisions as to which inoculant type to utilize is based upon the desired outcome, faster preservation or increased aerobic stability.

Acidity

Plant maturity, dry matter and packing density. Plant maturity has multiple effects on ensiling and also effects packing density (Johnson et al., 2002). As maturity increases in corn, alfalfa and orchardgrass the pH of the silage has also been reported to increase (Doane et al., 1997; Johnson et al., 2002). Immaturity, however, especially in corn may also impede pH decline (Johnson et al., 2002). In contrast, maturity in small grains has not been observed to have a detectable effect on the final pH, except for in oats (Bergen et al., 1991). Although,

Doane et al. (1997) reported that the pH increased in alfalfa (*M. sativa*) and orchardgrass (*D. glomerata*) as maturity (and DM) increased, it should be noted that all silages tested were below a pH of 4.00, regardless of maturity level.

A drier forage may have a lower packing density (Muck and Holmes, 2000). Overly dry forages have been reported to inhibit fermentation (Kung, 2010). This inhibited fermentation results in lower lactic acid and acetic acid production (Garcia et al., 1989; Hao et al., 2015). Finally, as DM increases so does pH (Hu et al., 2009; Hao et al., 2015). Therefore moisture content of the forage has a direct impact on how well a forage packs and therefore ferments (Muck and Holmes, 2000). In addition to the effects of plant maturity and moisture, packing density itself is another area of concern. Lactic acid concentrations increase as packing density increased (Zheng et al., 2011). It should be noted that increased lactic acid concentrations are associated with decreased pH (Vlkova et al., 2012). Therefore as packing density decreases, pH should increase which is supported by research conducted by Johnson and colleagues (2002).

Plant species and hybrid. The species of plant ensiled also influences OA production and the pH of the silage. Corn and rye grass silages have been reported to have a lower pH than a wheat silage (Burke et al., 2007). Additionally ryegrass silages have been observed to have a lower pH than red clover silage (Moorby et al., 2008). In contrast, a comparison between two different legumes red clover and birdfoot trefoil, observed no differences in pH (Hojer et al., 2012). The difference in the pH values that have been reported may be related to differences in OA concentrations. Corn has been observed to have greater lactic acid concentrations than ryegrass silages whereas ryegrass had greater acetic acid concentrations (Burke et al., 2007). Additionally grass silages have greater lactic acid and acetic acid concentrations than legume

silages (Al-Mubrak et al., 2004; Moorby et al., 2008). Organic acid production differs depending on the type of plant species, which then influences the pH of the silage. Organic acid production in silage can be affected by multiple different factors (e.g. plant maturity, packing density, DM content, plant species). A variety of techniques have been developed to improve the ensiling process, including chemical and biological additives.

Chemical additives. Although ammonia may be added to improve ensiling, the use of ammonia in silage is not universally accepted as beneficial. A review of the findings of the 26 trials conducted over 17 years, led Bolsen et al. (1992) to conclude that the addition of urea or anhydrous ammonia inhibits fermentation efficiency, increased the pH of corn silage and tended to increase the pH of sorghum silage. These findings are supported by the research of others (Wannapat et al., 2013; Cao et al., 2014) who also observed that the addition of urea prior to ensiling increases the pH of the silage. Additionally, ammonia and urea effect OA concentrations (Kung et al., 2000; Wannapat et al., 2013; Cao et al., 2014). Kung et al. (2000) reported that the addition of ammonia in small amounts before fermentation increased acetic acid and lactic acid concentrations and shifted the fermentation to more heterolactic. This shift towards heterolactic fermentation was evidenced by a decrease in the lactic acid to acetic acid ratio and a corresponding increase in pH (Kung et al., 2000). In contrast, ammonia increased lactic acid, but did not change acetic acid, when applied to corn and sorghum silages (Bolsen et al., 1992). Cao and colleagues (2014) observed, however, that urea inhibited microbial activity and, as well as lactic acid and acetic acid production. Additionally, the addition of urea resulted in decreased OA concentrations (acetic, propionic and lactic; Wannapat et al., 2013). Wannapat et al. (2013) concluded that any increase in OA, with the addition of ammonia may be offset by the buffering capacity of ammonia. This may

explain why some research has reported increased OA concentrations, especially lactic acid, but no corresponding decline in pH, when silage has been treated with ammonia.

Another strategy to improve OA production and thus pH decline is to add materials rich in WSC such as molasses (Alli et al., 1984; Wanapat et al., 2013). The addition of molasses increases lactic acid and acetic acid concentrations (Alli et al., 1984), as well as other VFA including butyric acid (Wannapat et al. 2013). Despite increasing OA, no change in pH was detected when molasses was added to whole rice silage (Wannapat et al., 2013). This may have been the result of the buffering capacity of the ammonia that was also utilized, according to the researchers (Wannapat et al., 2013). In contrast, when molasses was added to leucaena, a legume that demonstrates slower fermentation rates compared to other legumes, a decrease in pH was observed (Alli et al., 1984). Increasing WSC concentrations with the addition of molasses may increase OA concentration, and thus improve pH decline in silages.

The direct addition of OA is another strategy utilized to decrease silage pH. Propionic acid has been utilized because of its strong anti-mycotic properties, though it does not lower pH to the extent that lactic acid does (Woolford, 1975). The addition of propionic acid, during ensiling has been reported to correspond with a linear decrease in pH as propionic acid concentration increased (Kung et al., 2000). Additionally, the use of propionic acid in ensiling, has been observed to numerically lower lactic acid, though, the difference was not significant (Kung et al., 2000). The use of propionic acid may prove beneficial in preserving forages during the early stages of fermentation, until LAB can contribute to preservation (Woolford, 1975). Direct addition of OA may contribute to a faster rate of pH decline, but it can be very hard on equipment and costly (Kung et al., 2000).

Inoculants. Bacterial inoculants, containing LAB, are the most common biological method utilized to improve OA production and thus pH decline (Filya et al., 2007). Lactic acid is the most efficient acid at decreasing silage pH (Woolford, 1975). The addition of homofermentative LAB inoculants resulted in a greater lactic acid concentrations and an increased lactic acid to acetic acid ratios compared to a control (Filya et al., 2007) and to heterofermentative LAB (Filya et al., 2006; Tabacco et al., 2011). Additionally homofermentative LAB decreased pH, compared to either heterofermentative LAB or a control, for alfalfa silage (Filya et al., 2007). In contrast, Filya et al. (2006) and Tabacco et al. (2011) observed no detectable difference between corn silages treated with homofermentative LAB and a control. Heterofermentative LAB, such as *L. buchneri*, however, have increased pH in comparison to homofermentative LAB (Driehuis et al., 1999; Filya et al., 2007; Tabacco et al., 2011). Additionally, heterofermentative LAB decreased lactic acid and increased acetic acid concentration as the concentration of LAB increases (Driehuis et al., 1999). Homo- and heterofermentative LAB may also be used in combination. Combination inoculants of this nature increased lactic acid concentrations through d 7 and acetic acid concentration by d 95 (Addah et al., 2013). Combination inoculants have also decreased pH compared to a control (Adesogan et al., 2015; Arriola et al., 2015). Overall, the selection of an inoculant will impact the type of OA produced and thus impact the rate and extent of pH decline.

Fibrolytic enzymes. Fibrolytic enzymes, especially those containing microbial derived xylanases and cellulases, increase soluble CHO from xylan and cellulose (Columbatto et al., 2003a). Organic acids are derived from the fermentation of WSC (Bergen et al., 1991, Seglar, 2003). Therefore, FE may increase acidification of the silage. Fibrolytic enzymes increase

lactic acid concentrations and thus decrease pH in high fiber silages such as shrubby sweetvetch (*H. fruitcosum*; Sun et al., 2012). The increase in lactic acid concentrations may be delayed though, as demonstrated by Lynch et al. (2015), where the pH, lactic acid and acetic acid concentrations in corn silage were not different during the first seven days of ensiling. By d 14 continuing through d 70, however, the FE increased lactic acid and acetic acid concentrations, which lowered the pH (Lynch et al., 2015). Fibrolytic enzymes, in combination with homofermentative and heterofermentative LAB inoculants have also been successful in overcoming delayed acidification in corn that was infected with southern rust (Queiroz et al., 2012). In contrast, not all FE decrease pH as observed when four FE were applied to Bermuda grass silage and only one resulted in a decreased pH (Dean et al., 2005). Fibrolytic enzymes increase the availability of sugars, which may contribute to greater OA production and a lower pH, although the effect may not occur until after the first seven days of ensiling.

Increased maturity, DM and decreased packing density may inhibit OA production; especially lactic acid production. This could inhibit pH decline. Differences in plant species can result in different concentrations of OA in silage, and differences in pH. Homofermentative LAB increase lactic acid concentration and generally increase pH decline. Fibrolytic enzymes may improve OA production, especially in forages with high structural CHO content resulting in a greater pH decline.

Dry Matter Loss

Plant maturity, dry matter and packing density. Dry matter loss during fermentation may also be effected by maturity, DM and packing density of the plants to be ensiled. Increased

maturity increases DM losses (Johnson et al., 1999). Additionally, increased DM also increased DM loss (Hu et al., 2000; Kung, 2010). Finally, DM losses decreases at increased packing densities (Ruppel et al., 1995; Kung, 2010). Therefore, increases in DM, maturity and the related decrease in packing density may increase DM losses.

Inoculants. Dry matter losses are partially the result of the microbial fermentation of soluble CHO (Bergen et al., 1991, Seglar, 2003). Therefore, the addition of LAB inoculants may affect DM losses. Dry matter losses increased or tended to increase with the use of homofermentative LAB inoculant added to corn silage (Hu et al., 2009, Tabacco et al, 2011). In contrast, no difference was detected, from a control, when a homofermentative LAB was added to sorghum silages (Tabacco et al., 2011). Heterofermentative LAB inoculants, however, increased DM loss to a greater extent than homofermentative LAB (Tabacco et al., 2011) and non-inoculated silages (Driehuis et al., 1999; Tabacco et al., 2011). Overall, both homo- and heterofermentative LAB generally increase DM loss.

Fibrolytic enzymes. Fibrolytic enzymes convert structural CHO to soluble CHO, which may affect DM loss during fermentation. The use of FE in combination with an LAB inoculant did not change the DM loss (Queiroz et al., 2012; Lynch et al., 2015). In contrast, Adesogan et al. (2004) reported that a combination inoculant containing LAB and FE decreased DM loss. Dry matter losses were increased when ferulic acid esterases were added to a mixture of FE containing cellulases and xylanses (Lynch et al., 2015). Any effect on DM losses, then, may depend less on the FE and more on other additives that the FE may be paired with.

Overall, increased DM and maturity in addition to decreased packing density contribute to increased DM loss. Inoculants have mixed impacts on DM recovery. Homofermentative LAB

inoculants increased or did not change DM loss, whereas heterofermentative LAB increase DM losses. Fibrolytic enzymes also appear not to have any impact on DM loss, although when combined with LAB inoculants, they may decrease DM loss under certain circumstances.

Nutrients

Plant maturity. Nutrient content of plants change as the plant matures and this will impact the nutrient levels of the silage as well. Crude protein and sugar content, as a percentage of DM, decrease whereas NDF and ADF increase, in grass silage as maturity of the grass increased, (Kohn and Allen, 1995; Jalali et al., 2012). In addition to having greater CP and WSC, metabolizable energy (ME) was also greater in less mature grass silages compared to mature grass silages (Keardy and Hanrahan, 2013). In contrast, starch levels increase in whole plant corn silages as the plant matures (Hunt et al., 1989). Additionally, ADF and NDF decrease as plant maturity increases, in whole plant corn silage (Hunt et al., 1989; Bal et al., 2000). Whereas, ADF, NDF and lignin increase as the plant matures in corn stovers (Hunt et al., 1989). In small grain silages, such as wheat, barley and oats, WSC, NDF and ADF increase as the plant matures (Bergen et al., 1991). The effects of maturity on nutrients vary depending on the plant species.

Plant species. Different species of plants may differ considerably on the nutrient content and may result in the silage differing in nutrient content. Ryegrass silages have greater CP, NDF, ether extract and ash levels than whole wheat or corn silages (Burke et al., 2007). In contrast corn silages have higher starch levels compared to wheat silages, whereas grass silages have no detectable starch (Burke et al., 2007). Red clover silages (a legume) have higher CP

content compared to grass silages, whereas grass silages have greater organic matter (OM), WSC, ether extract, ME and NDF (Al Mubrak et al., 2004; Moorby et al., 2008).

Inoculants. Inoculants have an inconsistent effect on nutrient levels of silage. The difference may be related to the plant species ensiled. Crude protein increase with the addition of either hetero or homofermentative LAB (Tabacco et al., 2011). In contrast, the addition of the homofermentative LAB, *L. plantarum* had no detectable effect on CP, whereas it did lower ammonia (Hu et al., 2009). Additionally, both a homofermentative LAB inoculant and a combination homo- and heterofermentative LAB inoculant did not change the CP content in mixed cool season grass silages (Guo et al., 2013). Ammonia levels, in contrast, were greater in a first cut grass silage treated with either inoculant, whereas in later cuts the silages treated with homofermentative inoculant had the lowest ammonia level (Guo et al., 2013). The addition of a homofermentative LAB inoculant did not change the starch or WSC content of a corn silage (Hu et al., 2009; Tabacco et al., 2011). In contrast, homofermentative LAB decreased WSC to a greater extent than a control, in alfalfa silage (Filya et al., 2007). Additionally, a combination of hetero and homofermentative LAB inoculant applied to Bermuda grass decreased WSC (Adesogan et al., 2004). Finally, Hu et al. (2009) observed that the addition of a homofermentative LAB inoculant decreased ADF whereas NDF was not changed.

Fibrolytic enzymes. When FE (xylanases and cellulases) are added to a pure mixture of cellulose and xylan, the amount of reducing sugars increased and increased in proportion to the FE dose level whereas cellulose and xylan decreased (Columbatto et al., 2003a). Cellulases may also cleave hemicellulose (Van Soest, 1994). The addition of FE containing cellulases to plants high in structural CHO, such as shrubs, decreased NDF and ADF content

(Sun et al., 2012). Additionally, when added to corn silages, FE reduced NDF and ADF whereas WSC were increased (Thomas et al., 2013; Lynch et al., 2015). When applied to bermudagrass silage, three of four FE examined decreased NDF and ADF (Dean et al., 2005). Additionally there was a corresponding increase in WSC in two of the four FE tested and a third demonstrated a trend towards increased WSC. It should be noted that the greatest level of WSC corresponded with the lowest NDF, ADF and pH (Dean et al., 2005). Finally, the addition of FE had did not change the CP content of silages (Dean et al., 2005; Thomas et al., 2013).

Plant species and maturity both effect the nutrient content of silages. Although, homofermentative LAB may decrease ammonia and WSC content, research has reported inconsistent effects of inoculants on the nutrient content of silages. Fibrolytic enzymes reduce NDF and ADF, whereas increasing WSC, with little detectable effect on CP.

Feed Performance

Plant maturity. In addition to inhibiting OA production, the maturity of the plant at the time of ensiling may also affect feed intake, digestibility and performance. Increased plant maturity results in a lower DM intake (DMI; Jalali et al., 2012). Additionally, as the maturity level of grass silages increases, not only does DMI decrease, ME and NDF intake also increases (Jalali et al., 2012). Maturity also decreases DM and NDF degradation in the rumen (Hunt et al., 1989; Bal et al., 2000; Keardy and Hanrahan, 2013). Additionally as maturity level increases, the body condition score of lactating ewes' decreases (Jalali et al., 2012).

Plant species, hybrid and cutting height. Since plant species affects the nutrition content of silages, differences in species may also impact feed intake, digestibility and performance of

the resulting silage. Corn silages have a greater rate of rumen DM degradation (DMD) and OM degradations (OMD) than ryegrass silage (Burke et al., 2007). Rye grass silage, however, have greater DMD, OMD, nitrogen degradation and NDF degradation compared to red clover silage (Moorby et al., 2008). Additionally, DMI was greater for grain silages, such as whole wheat and corn silage, than grass silages (Burke et al., 2007). Silages containing birdsfoot trefoil have greater DMI than those containing red clover (Hojer et al., 2012). Al Mubrak et al. (2004) reported that red clover silage increased DMI, milk yield and milk protein in Holstein cows than a grass silage. Milk yield (26.5 vs. 33.2 ±0.85 kg/d; for ryegrass and corn silage; respectively) and solid corrected milk yield (255.2 vs. 31.6 ±0.82 kg/d; for ryegrass and corn silage, respectively) was greater in cows fed corn silage compared to ryegrass silages, whereas BCS was unchanged regardless of feed type (Burke et al., 2007). Additionally, multiparous Holstein-Friesian cows fed ryegrass silage had a greater weight gain (35.0 vs. 15.4 ± 1.75 kg; for ryegrass and red clover silage, respectively) than those fed red clover silage (Moorby et al., 2008). In contrast the calf weights (40.8 vs. 42.5 ± 1.03 kg; for ryegrass and red clover silage, respectively) were greater in those cows fed red clover silage (Moorby et al., 2008); however, subsequent feed intake during lactation, rumen degradation and milking performance was unaffected by either treatment. Milk yield and composition were also not affected when two different legume-grass mixed silages were fed to cattle (Hojer et al., 2012). The fatty acid profile of milk also differed in relationship to the type of silage fed, (red cloves silage increased $C_{18:2}$ content by 30% and doubled $C_{18:3}$ content; Al Mubrak et al., 2004). Overall, feed intake, digestion and performance are directly impacted by the plant species ensiled.

Feed intake, digestion and subsequent animal performance may also be impacted by the type of plant hybrid ensiled. Some researchers have observed a difference in feed intake, OM and starch intake, related to hybrid type whereas no difference was detected for NDF or ADF intake (Kennington et al., 2005). In contrast, Holt et al., (2013) reported no differences in DMI or OM, whereas NDF and ADF intake did differ related to hybrid type. Additionally, research by Akins and Shaver (2014) reported no difference in DMI between different hybrid types of whole plant corn silage, when silage was 40% of a total mixed ration (TMR). There was a differences observed, however, when the percentage was increased to 65% of the TMR (Akins and Shaver, 2014). Different hybrids have also demonstrated different DM degradation and NDF degradation (Kennington et al., 2005; Kung et al., 2008). This is contrary to Holt et al. (2013), who reported no difference detected for ADF or NDF degradation related to hybrid species of corn. Additionally total-tract digestibility of DM and OM have been observed to differ between corn hybrids (Kennington et al., 2005). Hybrid may affect feed intake and digestion, most notably fiber digestion, although the results are not consistent from trial to trial.

Differences in cutting height may alter fiber intake, starch intake and rumen degradation of fiber in silage. It is reported that a lower cutting height decreases starch intake, whereas increasing NDF and ADF intake, and no difference was detected for OM intake (Kennington et al., 2005). A higher cutting height has been observed to increase DMD at 24 h and 72 h (Kennington et al., 2005). A lower cutting height, however, has been reported to increase NDFD and ADFD in the first 24 h (Kennington et al., 2005; Lynch et al., 2015), although at 48 h the results differ. Kennington et al. (2005) reported that NDFD tended to be greater at 48 h with a lower cutting height, whereas Lynch et al. (2015) reported that no difference was

detected. Neither reported a difference for ADFD at 48 h related to cutting height (Kennington et al., 2005; Lynch et al., 2015). In contrast, Kung et al. (2008) observed that no difference was detected for NDFD with different cutting heights. Kennington et al. (2005) reported, that ruminal pH was not affected by cutting height, whereas cutting height did affect the ruminal VFA levels. A lower cutting height decreased ruminal acetate and propionate concentrations, but increased ruminal valerate and butyrate concentrations (Kennington et al., 2005). The effect of cutting height appears to be largest on fiber intake, whereas impact on fiber degradation is inconsistent and any improvement may abate after 24 h of incubation.

Chemical additives. Multiple different chemical additives, such as ammonia, molasses and propionic acid, have been utilized to improve silage preservation, and each has different effect on feed intake and performance. Ammonia increased CP intake, ME intake, DMD and OMD (Cao et al., 2014). Additionally, molasses combined with urea increased OMD and DMD (Wannapat et al., 2013). Overall, the addition of ammonia and molasses, during ensiling appears to increase the feed value of the silage.

Inoculants. The effect of LAB on digestibility and degradation of fiber is inconsistent. In research conducted by Arriola et al. (2011), neither a combination hetero- and homofermentative LAB inoculant nor a heterofermentative LAB inoculant improved total tract digestion of DM or CP. Additionally the inoculants were reported to have decreased NDF and ADF digestion (Arriola et al., 2011). This is in contrast to others (Filya et al., 2006; Queiroz et al., 2013) who reported no differences in *in situ* rumen degradation, or *in vitro* digestion of DM, OM or NDF in silages treated with homo- or heterofermentative LAB inoculants. Jalc et al. (2009), however, did report an increase in *in vitro* digestibility of corn silage treated with homofermentative LAB inoculants. Finally, Aksu et al. (2004) also

reported an increase in total tract DM digestibility and NDF digestibility, as measured in sheep feces, of corn silage treated with a combination homo- and heterofermentative LAB inoculant. The effectiveness of LAB inoculants to improve fiber degradation is inconsistent, and neither homo- nor heterofermentative LAB have been reported to consistently improve DM, NDF or ADF degradation or digestibility.

Fibrolytic enzymes. Although forages are important and a large source of feed for cattle worldwide, less than 50% of the cell wall is digestible in ruminants; therefore, small increases in fiber degradation can increase production in dairy and beef cattle and greatly reducing manure output (Hatfield et al., 1999). Fibrolytic enzymes, containing cellulases and xylanses, have increased rumen degradation of cellulose in treated alfalfa and barley straw (Badhan et al., 2014). This increase in cellulose degradation was related to removal of xylan, which forms a complex around cellulose and inhibits cellulose degradability (Badhan et al., 2014). Multiple studies also report that FE increase both *in vitro* digestibility and *in situ* degradation of fiber in silages, although most of the increase in digestion of silages occurs during the first 24 h of incubation (Columbatto et al., 2003b; Lynch et al., 2015; Romero et al., 2015). In contrast, Ribeiro et al. (2015) reported that 24 h *in vitro* DM, ADF and NDF disappearance was not detectably different in warm season grass silages with the addition of FE. There was, however, a dose effect observed. As the FE dose increased, disappearance of DM, ADF and NDF improved at 24 and 48 h (Ribeiro et al., 2015). Additionally, FE have been reported to increase ruminal VFA concentration over a control (61.2 vs. 55.4 m*M*; respectively), while lowering the ruminal acetic to propionic ratio (3.03 vs. 3.24; respectively; Romero et al., 2015). Finally, the kinetics of *in situ* disappearance of DM, NDF, ADF and CP have also been reported to increase with the addition of FE at the time of ensiling (Thomas et al., 2013).

Fibrolytic enzymes appear to improve the degradation of DM and NDF, and possibly improve the degradation of ADF, although the improvement is greatest during the first 24 h of incubation.

Aerobic Stability and Yeasts

Plant maturity, dry matter and packing density. Packing density, DM and maturity are related, and impact OA concentrations and pH decline, thus they may also impact silage aerobic stability. Johnson et al. (2002) reported that plant maturity has such variable effect on silage aerobic stability that they were unable to draw a conclusion. In contrast Xie et al. (2012) reported that aerobic stability increased as plant maturity increased. Additionally aerobic stability has been reported to increases as packing density increased in other research (Ruppel et al., 1995). Aerobic stability, however, has been observed to increase as the DM of corn silage increases (Hu et al., 2009), even though an increase in DM contributes to a decreased packing density (Muck and Holmes, 2000).

Chemical additives. The direct application of OA, especially propionic acid may decrease yeast and molds and improve aerobic stability. As pH decreases, to between 4.0 and 5.0 acetic, formic and propionic acids inhibit spore forming bacteria and yeast/molds, and at pH below 4.0 LAB are also inhibited (Woolford, 1975). Additional research has demonstrated a linear relationship between propionic acid concentrations, when paired with ammonia, and aerobic stability (Kung et al., 2000). Propionic acid has been reported to have the strongest anti-mycotic properties, whereas formic and lactic acid decrease pH at the fastest rate (Woolford, 1975). The addition of propionic acid increased aerobic stability to as great as 69 hours, as well as decreased the pH and peak temperature of propionic acid treated silage after

24 hours (Kung et al., 2000). Woolford (1975) concluded that as long as an additive was capable of suppressing microbial growth long enough to give preference to LAB growth, then it was effective. On the other hand subsequent researchers have concluded, that while the anti-mycotic properties of propionic acid are advantageous in improving aerobic stability and decreasing yeast growth, propionic acids high costs, the need for high rate of application and its highly corrosive nature, especially on farm equipment, its use is limited (Kung et al., 2000).

Inoculants. Homofermentative LAB may decrease the time to preservation, their effect on aerobic stability are more debatable. In a meta-analysis of LAB inoculants, Muck and Kung (1997), reported that inoculants containing homofermentative LAB only improved aerobic stability in 30% of the research. The inability to consistently improve aerobic stability was attributed to the yeasts, that are most responsible for aerobic spoilage, being acid tolerant and being able to utilize lactic acid as an energy substrate (Muck and Kung, 1997). Ranjit and Kung (2000) though, reported that two strains of *L. plantarum,* a species of homofermentative LAB commonly used as inoculants, decreased yeast counts and improved aerobic stability. Guo et al., (2013) also reported that *L. plantarum* inoculants improved aerobic stability. In contrast, Hu et al. (2009) reported no difference for aerobic stability in corn silages treated with homofermentative LAB. Heterofermentative LAB however, have been observed to improve the aerobic stability of silages compared to a non-inoculated silage (Driehuis et al., 1999, Tabacco et al., 2011). Additionally, heterofermentative LAB have been reported to improve aerobic stability over homofermentative LAB (Arriola et al., 2011; Tabacco et al., 2011). Furthermore, combination homo- and heterofermentative LAB inoculants have also been reported to increase aerobic stability (Adesogan et al., 2004; Reich and Kung; 2010;

Addah et al., 2013 Guo et al., 2013). Whereas some research reports that homofermentative LAB may improve aerobic stability, the findings are inconsistent. Heterofermentative LAB and a combination of homo- and heterofermentative LAB have been shown to consistently improve aerobic stability.

Fibrolytic enzymes. The effect of FE on the aerobic stability of silages is inconsistent. Fibrolytic enzymes have been reported to increase aerobic stability in corn silage infected with Southern Rust fungi (Queiroz et al., 2012). The addition of FE to a combination LAB inoculant, has also been reported to improve aerobic stability greater than the inoculant alone in Bermuda grass silage (Adesogan et al., 2004). Fibrolytic enzymes by themselves have not been reported to demonstrate a detectable differences for yeast counts in sorghum silage (Thomas et al., 2013). Whereas, others have reported an increase, with the use of FE, in aerobic stability in warm season grasses, even though yeasts counts were not different (Dean et al., 2005). In contrast to other research, Lynch and colleagues (2015) reported the addition of FE increased yeast cultures at 28 d and 70 d ensiling. Research does suggest that FE may enhance the ability of LAB inoculants to improve aerobic stability.

Summary

Multiple factors impact how well a silage performs. Whereas, there are multiple different strategies to improving silage preservation, the use of bacterial inoculants is the most popular biological method, and possibly one of the most effective. Homofermentative LAB inoculants have been shown to decrease pH by increasing lactic acid production in silages. The rapid decrease in pH may result in less DM loss and greater nutrient retention, although the effect of homofermentative LAB on digestion and feed performance is inconsistent. Homofermentative

LAB may also improve aerobic stability, whereas heterofermentative LAB inoculants are much more efficient at improving the aerobic stability of silages. Fibrolytic enzymes are increasingly added to inoculants, and the ability to free reducing sugars may improve OA, especially lactic acid, production and thus pH decline and possibly aerobic stability. In addition FE have been shown to increase fiber degradation and the kinetics of fiber degradation, especially in the first 24 h, while decreasing overall fiber content and increasing WSC content, which could prove beneficial in a high production system.

Hypothesis:

1. A combination additive, comprised of four species of homofermentative LAB, - *L. plantarum, P. acidilactici, P. pentosaceus, and L. acidophilus*- in combination with exogenous fibrolytic enzymes (FE) containing cellulase, xylanase, β-glucanase, and α-amylase decreases the rate of pH decline and increases lactic acid production, WSC and DM degradation (DMD), decrease DM loss and fiber content in a corn silage and alfalfa silage compared to a non-treated (control) silage of the same forage type.

2. The same treatment decreases DM loss and fiber content, increases the amount of WSC, and DM degradation, NDF degradation and ADF degradation compared to a control in cool season grass silage.

Objective:

1) Study the effects of a combination silage additive, comprised of four species of homofermentative LAB in combination with exogenous FE on the ensiling characteristics, to include pH, OA production, DM loss, nutrient loss, of corn and alfalfa silage ensiled for 59 d in mini-silos and on cool season grasses ensiled for 90 d in round bales

2) Study the impact of the treatment on *in situ* DMD of corn and alfalfa silage and the impact of the treatment on *in situ* DM, NDF, ADF, CP and organic matter degradation of cool season grass silage.

3) Study the impact of the treatment on the aerobic stability of the cool season grass silage after 90 d of ensiling.

Chapter 3: Experiment I

Abstract

Prior to ensiling, inoculants containing lactic-acid bacteria (LAB) may be applied to promote greater, more rapid lactic acid production causing a faster pH decline, possibly resulting in less DM and nutrient loss during storage. Inclusion of fibrolytic enzymes (FE) to the forage at time of storage may increase simple carbohydrates (CHO) availability increasing fermentation rate and pH decline. To test the effects of an additive containing four species of LAB in combination with four FE, green chopped whole plant corn and alfalfa were treated with water (CON) or with water containing the inoculant + enzyme (TRT). Each forage was packed into mini-silos (1206 cm^3 volume; n = 3 / treatment per date) to monitor rate of fermentation. The pH was measured on d 1, 2, 3, 7, 13, 17, 21, 28, and 45 for alfalfa and on d 1, 2, 3, 7, 10, 17, 28, and 45 for corn. Data were analyzed using PROC NLIN and PROC MIXED of SAS with significance declared at $P \leq 0.05$. The rate of decline to terminal pH was faster ($P < 0.01$) in the TRT than CON in alfalfa, but was not different for corn ($P = 0.91$). Additionally, buckets (n = 5 per treatment; 21,504 cm^3 volume) of each forage were ensiled to measure effect on nutrient composition (DM, crude protein, ammonia, starch in corn, and sugar, in alfalfa) and pH after 59 d of fermentation. The pH of the TRT tended to be lower than CON in alfalfa buckets ($P = 0.07$) on d 59 but did not differ in corn ($P = 0.92$). The percent sugar and starch did not differ ($P \geq 0.58$) between treatments for alfalfa and corn, respectively. Crude protein (CP) content of alfalfa was lower in the TRT compared with CON ($P = 0.001$; 13.3 vs. 13.0 SEM 0.1%; respectively) but treatments did not differ ($P = 0.11$) in corn. *In situ* DM degradation was greater in the TRT alfalfa silage ($P = 0.02$; 46.7 vs. 44.2 SEM 0.8%; respectively) than CON, but there was no difference detected between

treatments in corn silage ($P = 0.71$). The treatment additive appeared to increase the rate of decline to terminal pH in alfalfa, but not in corn, but it had minimal effect on nutrient composition of either ensiled forage.

Introduction

Ensiling is one of the most popular methods of preserving forages in the United States, Canada and Europe. Silage is fed extensively to dairy cattle and finishing lambs (Brassley, 1996; Burke et al., 2007). The anaerobic fermentation of water soluble carbohydrates (WSC) during ensiling results in the formation of organic acids (OA; Bergen et al., 1991, Seglar, 2003). Of these OA, lactic acid is the most responsible for the pH decline of the silage, although it is not considered a strong antimycotic (Woolford, 1975). The addition of inoculants containing either homo- or heterofermentative LAB, is the one of the most common methods used (Filya et al., 2007). Homofermentative LAB inoculants decrease pH to a greater extent and produce greater amounts of lactic acid than those treated with heterofermentative LAB, although, compared to untreated silages the results are not consistent (Filya et al., 2006, 2007; Tabacco et al., 2011). Additionally, homofermentative LAB have not shown consistent improvement in nutrient retention, DM loss, rumen degradation or aerobic stability (Muck and Kung, 1997; Filya et al., 2006, 2007).

Exogenous FE are also increasingly used to improve silage preservation. These FE hydrolyze the bonds of structural CHO and increase WSC (Thomas et al., 2013; Lynch et al., 2015). The use of FE also increases LA production and lower pH, especially in forages high in structural CHO (Sun et al., 2012). Additionally, FE have also been reported to improve digestibility of structural CHO, especially NDF in the first 24 h of incubation (Columbatto et al., 2003a; Romero et al., 2015).

The objective of this research was to evaluate the effects of a silage treatment, which contained four homofermentative LAB in combination with four FE on alfalfa and corn silage in a small scale, laboratory setting.

Materials and Methods

Silage Preparation: Mature alfalfa (*M. sativa*) was harvested and chopped to an approximate length of 2.5 cm, at the University of Idaho Dairy Center (Moscow, ID, 83844). Fresh cut whole plant corn (*Z. mays*) was obtained from a private farm near Colfax, WA (99111), in late summer, 2013, harvested at 2/3 milk line maturity. Dry matter was determined using Koster moisture tester C (Koster Crop Tester, Inc., Medina, OH, 44256) and microwave prior to ensiling. Alfalfa was determined to contain approximately 57% DM and corn was determined to contain approximately 33% DM.

Each forage was ensiled in both PVC tube mini-silos (average volume-1206 cm^3) and bucket silos (volume 21,804 cm^3). The mini-silos were made of 10 cm diameter by 15 cm in height PVC tubing closed at both ends with plastic PVC caps, one of which was vented using an 18 gauge needle and pipette bulb. The plastic buckets were similarly vented with three 18 gauge needles and pipette bulbs inserted into the lid. Empty weights were recorded on all mini-silos and buckets. Each bucket and mini-silo were then assigned a treatment group, CON or TRT. The alfalfa was divided into six equal-sized portions of 22.69 kg; each of these portions was hydrated to an approximate 40% DM by adding 9.5L of water to 22.69 kg of alfalfa. The TRT group was treated with Sil-All 4x4, a commercially available inoculant enzyme mixture from Lallemand Animal Nutrition, North America (Milwaukee, WI) that contains four homofermentative LAB (*L. plantarum, L. acidophilus*, *P. acidilactici* and *P. pentosaceus*) plus four FE (cellulase, xylanase, β-glucanase and α-amylase).The additive was reconstituted

per manufacturers direction 5.3 g of additive per liter of water and applied at a concentration of 2×10^5 CFU (of LAB)/g of silage. The CON was treated with water. Both treatments were applied using a common garden sprinkler and the forage was mixed by hand using the four corners mixing method. After mixing, 520 g of rehydrated alfalfa was packed into 64 pre-weighed mini-silos for a density of approximately 168 kg (DM)/m^3. Mini-silos were assigned to treatment and repetition within group, i.e. silo one contained CON repetition one. The PVC mini-silos where then capped and sealed using aluminum tape. Additionally, 9.14 kg of alfalfa was added to each bucket silo for a density of approximately 168 kg (DM)/m^3. Each bucket contained only one repetition from one group, i.e. bucket #1 contained repetition one of CON. Whole plant corn was prepared in a similar manner, except no extra water was added. Instead, Sil-All 4x4 (*L. plantarum, L. acidophilus, P. acidilactici* and *P. pentosaceus* in combination with cellulase, xylanase, β-glucanase and α-amylase) was applied to the TRT at 2×10^5 CFU (of LAB)/g of silage by applying 10 mL of Sil-All 4×4 reconstituted at a concentration of 5.3 g of additive per liter to 22.69 kg of chopped, whole plant corn by hand. The CON group was treated with water 10 mL of water per 22.69 kg of corn. To each PVC mini-silo, 668 grams whole plant corn was added for a density of approximately 175 kg (DM)/m^3. Additionally, 11.77 kg of chopped corn was added to each bucket for a density of approximately 175kg (DM)/m^3.

Sampling and analysis: On d 0 fresh samples of all 3 repetitions for both treatments, were obtained to measure for pH, titratable acidity, DM. Additionally samples were frozen for future analysis of NDF, ADF, cellulose, ash, CP, NH_3-N, sugar for alfalfa silage and starch for corn silage.

The PVC mini-silos were opened six at a time, three mini silos per treatment. Alfalfa silage mini-silos were opened on d 1, 2, 3, 7, 13, 17, 21, 28 and 45. Samples were collected for pH and titratable acidity on each d of sampling.

Corn silage PVC mini-silos were opened on d 1, 2, 3, 7, 10, 17, 28 and 45. Sampling days differed from alfalfa related to logistics. Samples were collected to measure pH and titratable acidity.

Bucket silos were opened for both corn silage and alfalfa silage on d 59 and samples were taken to measure pH, titratable acidity, DM, VFA, NDF, ADF, cellulose, ash, CP, NH_3-N, sugar for alfalfa silage, starch for corn silage, *in situ* (*IS*) degradation, LAB cultures and yeast-mold cultures. Weights were obtained on d 59 for the bucket silos for alfalfa and corn silages; this weight on a DM basis was then compared to the initial DM weight and used to determine DM loss.

The pH of each silage sample was determined using an Oakton pH 11 series pH/mV/^{0}C meter (Cole-Parmer, Court Vernon Hills, IL). Samples were prepared as described by Kung et al. (2000) first by removing, and setting aside, the top 2.5 cm of from mini-silos and 7.5 cm from bucket silos. After the top layer had been removed, the silos were emptied and mixed thoroughly, 50 g were removed and mixed with 450 ml of distilled water and blended for one min on liquefy setting in a Rival 6 speed mixer (Jarden Corp., New York, NY) . The slurry was then filtered through 2 layers of cheese cloth and the pH of the filtered liquid was immediately measured. After pH was determined, 70 mL of the filtered fluid was removed and placed into a clean beaker for titratable acidity, which was measured using the same pH probe by adding 1N NaOH, via pipet, to the 70 ml of filtered fluid until pH was raised to 6.80.

The volume added, in μL, was recorded and used to calculate titratable acidity in mL/kg (DM).

Dry matter determination: Dry matter for mini-silos was determined by adding the top 2.5 cm layer back that was removed prior to pH and titratable acidity testing, into the remaining silage and thoroughly mixed. A sub-sample was then placed into an oven set at 60^0C for 48 h and then re-weighed. Dry matter for the bucket silos was measured for the top 7.5 cm and for the remaining silage separately. Dried samples were then placed into sealable bags and stored in refrigerator for further nutrient analysis.

Nutrient analysis: Samples were dried at 60° C and ground twice using a Wiley mill (Thermo Fisher, Waltham, MA), first using a 2 mm screen then finishing with a 1 mm screen for approximately 1 min. The DM was determined by placing 1 g of ground silage sample into a pre-weighed 50 mL beaker. The beaker was then placed into an oven set at 105^0 C for 24 h. After this the beaker was transferred to a desiccator and allowed to dry for 2 h. Following determination of DM, the dried sample plus beaker were transferred to a muffle furnace ashed for 6 h at 600^0 C. The beaker and ashed sample was transferred to the desiccator, and allowed to cool for 2 h before weighing. Weight was recorded and used to determine ash as a percentage of DM (AOAC, 1999).

The ground samples from d 0 and the bucket samples on d 59 were then used to determine NDF, ADF and Cellulose. Neutral detergent fiber, ADF and cellulose analysis were performed as described by Van Soest et al. (1991) in an Ankom 200 (ANKOM Technologies, Macedon, NY) fiber digester with the addition of 5 mL of amylase during NDF. Initial sample weight was adjusted to 105°C DM using the laboratory DM calculated above.

Frozen samples from d 0 and buckets on d 59, were shipped to Dairyland Laboratories (Arcadia, WI) and analyzed for CP (method described in AOAC, 1999), ammonia (as part of CP; method described in AOAC, 1999), and sugar (alfalfa) or starch (corn; as per method described in Method workshop from 30th Annual MW AOACI Meeting and Exposition, awaiting approval).

Microbial analysis: Cultures were performed on d 59 from samples of silage obtained from the bucket silos. To prepare the samples for culturing, 25 g of silage were added to 225 mL of sterile physiological saline and blended on liquefy for 1 minute, modified from the procedure described by Reich and Kung (2010). The slurry was then filtered through two layers of cheesecloth and serial dilutions in physiological saline were made from the filtered liquid. Serial dilutions were then plated on MRS agar (Difco laboratories, Detroit, MI) for LAB. One half of a mL of solution was applied to the agar and spread using a glass spreader, which had been sterilized by first rinsing in 70% isopropyl alcohol, then rinsed a second time in 90% anhydrous alcohol and flame sterilized. The spreader was then allowed to cool and then used to spread the diluted sample over the agars surface without penetrating the agar. The plates were then placed into an incubator set at 35^0C for 72 h and only plates between 30 and 200 CFU were counted, and reported on a $\log_{10}$ CFU/g of DM basis.

Yeast-mold cultures were also plated on d 59 from the same silage samples as the LAB cultures. Sample was prepared for culturing similar to the method as used for LAB cultures. After preparation 1 mL of diluted sample were applied to 3M petri-film yeast-mold plates (3M, St Paul, MN) per manufacturer's directions and incubated at room temperature for 5 d. Only plates with 10-200 CFU were counted and reported on a $\log_{10}$ CFU/g of DM.

Organic acids: Volatile fatty acids were determined using gas chromatography (GC). Samples were prepared by mixing 100 g of silage with 400 mL of water. Silage was added at approximately 33 g at a time to 400 mL of distilled water, blended on liquefy for 15 s, then another 33 g added and blended for 15 s, and then the remaining silage added and blended for 1 min. The blended slurry was then filtered through two layers of cheese cloth and 40 mL were poured into a 50 mL conical tube and centrifuged at 2400 x *g*, at 4° C, for 20 min on a Sorvall ST 16R centrifuge (Thermo Scientific, Sunnyvale, CA). After centrifuging, the supernatant was poured into a new 50 ml labeled conical tube and frozen at -20° C. Samples were then thawed at room temperature, and centrifuged, using the same centrifuge, at 2400 x *g*, at 4° C, for 30 min. The supernatant was then transferred into a new 50 mL conical tube. Five mL of supernatant were then transferred into a 15 mL conical tube and 1 ml of 25% metaphosphoric acid was added to remove any remaining proteins and placed in the -20°C freezer overnight. After thawing at room temperature, the alfalfa samples were centrifuged on a Sorvall Evolution RC centrifuge (Thermo Scientific, Sunnyville, CA) at 1000 x *g*, for 10 min at 23° C, and the corn samples were centrifuged on the same machine at 750 x *g*, for 10 min at 23° C. Using a transfer pipette, the supernatant was transferred to a 5 mL conical tube, and 1 mL was pipetted from the 5 mL conical tube and placed into a gas chromatography vial. One-hundred μL of 2-ethylbuteric acid was added to the GC vial as an internal standard and then the vials were capped. Gas chromatography was conducted on a Hewlitt-Packard 6890 series GC using an Agilent DB-FFAP column (Hewlitt-Packard, Avondale, PA). Oven temperature was initially 100°C, increased to 150°C for 10 min, then 175°C for 1.50 min, with a constant pressure of 2.00 psi and a 1:10 split. Hydrogen was used as the carrier gas.

Lactic acid was analyzed using anion chromatography (AC) using a Dionex ED 40 detector and a GP40 pump (Thermo Scientific, Sunnyville, CA), using a modified version of the procedure described by Dionex corporation (Dionex, Thermo-Scientific) for analysis of OA. One hundred milli-mole of sodium hydroxide was used as the effluent and ran at 0.75 mL/min at 5% for the first 5 min, then increased from 5-8% from 5 min to 8 min, increased to 50% at 8.00 minutes, then the rate was increased to 1.5 mL/minutes, and NaOH increased to 70% from 8.01 to 12 min, then decreased to 5% from 12 to 12.5 min at the same rate and maintained at this rate and concentration for 1 min. Then the rate was decreased to 0.75 mL/min at 5% NaOH for 5 min. Oven temperature remained at 30^{o} C throughout the run and the carrier fluid was distilled water filtered to 18 micro-ohms. Samples were prepared by mixing 100 g of silage with 400 mL of water. The blended slurry was then filtered through two layers of cheese cloth and 40 mL were poured into a 50 mL conical tube and centrifuged at 2400 x *g*, at 4° C, for 20 min on a Sorvall ST 16R centrifuge (Thermo Scientific, Sunnyvale, CA). After centrifuging, the supernatant was poured into a new 50 ml labeled conical tube and frozen at -20° C. Samples were then thawed at room temperature, and centrifuged, using the same centrifuge, at 2400 x *g*, at 4° C, for 30 min. The supernatant was then transferred into a new 50 mL conical tube. Samples were then vacuum filtered through Whattman 1 filter paper and then diluted to 1:10,000.

In situ degradation: All animal trials were performed in accordance to procedures approved by the University of Idaho's Institutional Animal Care and Use Committee (Protocol #2014-2).

Dried (60° C) silage samples, from the buckets on d 59, were ground through a 2mm screen, weighed (4.5 g), into Dacron bags (50 μm pores, 10 cm by 20 cm) and double sealed. These

were then loaded into large mesh bags and introduced into the rumen of a cannulated multiparous crossbred cow, being fed ad lib oat hay and 0.5 kg/day of dried cracked corn, in the peripartum period at 4, 12, 24 and 48 h. Samples were prepared and weighed as per the procedure described by Stern et al. (1997).

Statistical analysis: Statistical analyses for experiment I was conducted using SAS, version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA). Chemical analysis, VFA, microbial cultures and *in situ* data were analyzed using a MIXED model (PROC MIXED). Repeated measurements were subjected to a correlation of variance (COV), any value greater than 15% were repeated for verification. Volatile fatty acids were checked for outliers by a studentized T-Test and any with a value greater than 3 were considered as possible outliers. Rate of pH decline was analyzed using a non-linear regression (PROC NLIN, SAS 9.3) using the model $pH = B0^{(-B1 \times date)} + C$, where B0 is the intercept, B1 is the slope (rate of decline) and C is the asymptote or minimum pH value, related to the asymptotic rate of pH decline in silage.

Rate of DM degradation was analyzed using a nonlinear regression (PROC NLMIXED, SAS 9.4), and the model $mu = m \times (1-exp^{(-B \times h)})$, where mu equals the mean of the treatment, -B is the slope of the line (rate of increase) and m is the maximum value. Significance was set at $P \leq 0.05$ and trend at $P \leq 0.10$.

Results

Chemical and nutrient analyses for alfalfa and corn prior to ensiling are shown in Table 3-1.

The decline in pH for d 1 to d 13 of ensiling for alfalfa silage was analyzed using a non-linear regression and the results are shown in Table 3-2. The plot of the rates of pH decline of both TRT and CON alfalfa silage is presented in Figure 3-1. The TRT alfalfa silage demonstrated a

greater rate of pH decline than the CON alfalfa silage (P = 0.005; -2.22 vs. -1.17, SEM 0.32; respectively) and there was a difference in lines (treatments; P = 0.0001). No difference was detected for terminal pH between treatments (P = 0.79).

The rate of pH decline of the corn silages, from d 1 to d 17, was also analyzed using a similar non-linear regression model and the results are given in Table 3-2. The plots of the rates of pH decline in both TRT and CON corn silage is presented in Figure 3-2. No difference was detected between treatments for rate of pH decline, lines (treatment) or terminal pH ($P \geq$ 0.16).

Chemical analyses of the silage on d 59 are shown in Table 3-3. No difference was detected for DM or DM loss between treatments in the alfalfa silage ($P \geq 0.53$). The pH of alfalfa silage on d 59 tended to be lower in the TRT than the CON (P = 0.07; 4.66 vs. 4.69 ± 0.2; respectively), whereas titratable acidity was lower in the TRT than the CON (P = 0.02; 310.9 vs. 328.5 ± 5.1 mmol NaOH/g DM; respectively). No differences were detected for ammonia, sugar, ash, number of yeast cultures or number of LAB cultures between treatments in the alfalfa silage ($P \geq 0.73$). Crude protein was lower in the TRT than the CON alfalfa silage (P = 0.001; 13.0 vs. 13.4 ± 0.1%; respectively), but no difference was detected between treatments for the change in CP, the difference between the CP on d0 and on d 59 (P = 0.14).

Furthermore, no differences were detected for DM, DM loss, final pH, titratable acidity, yeast cultures or LAB cultures between treatments ($P \geq 0.53$) or in CP, ammonia, starch, or ash, between treatments ($P \geq 0.11$) in the corn silage. The TRT corn silage, however, had less increase in ammonia than the CON corn silage, (d 0 and on d 59; P = 0.07; 0.3 vs. 0.4 ± 0.01% unit; respectively).

Organic acid concentrations of the silages on d 59 are shown in Table 3-4. The TRT alfalfa silage had a greater concentration of lactic acid than the CON (P = 0.02; 2367.6 vs. 1680.3 ± 183.7 µmol/g DM; respectively) and TRT alfalfa silage also had a tendency for lactic acid to be a greater as percent of all OA than the CON (P = 0.06; 88.49 vs. 82.18 ± 2.03%; respectively). No difference was detected in acetic acid concentration between treatments in the alfalfa silage (P = 0.23), but acetic acid tended to be lower as a percent of total OA in the TRT than the CON (P = 0.06; 11.35 vs. 17.63 ± 2.03%; respectively). Isobutyric acid concentration was also greater in the TRT than the CON alfalfa silage (P = 0.03; 1.9 vs. 1.1 ± 0.2 µmol/g DM; respectively), although there was no difference detected between treatments in isobutyric acid, as a percent of total OA (P = 0.14). Valeric acid concentrations (P = 0.006; 0.1 vs. 0.7 ± 0.1 µmol/g DM; respectively) and valeric acid as a percent of total OA (P = 0.001; 0.01 vs. 0.05 ± 0.01%; respectively) were less in the TRT alfalfa silage compared to the CON alfalfa silage.

Lactic acid concentrations and lactic acid as a percent of total OA for the corn silage were numerically greater in the TRT, but not different than CON ($P \geq 0.14$). Acetic acid concentrations in the corn silage were greater in the TRT than CON (P = 0.03; 131.0 vs. 119.2 ± 3.2 µmol/g DM; respectively), but no difference was detected for acetic acid as a percent of total OA between treatments (P = 0.34). Both butyric and isobutyric acid concentrations were not detectable in the corn silage on d 59 for either treatment. Valeric acid concentrations in the corn silage tended to be greater in the TRT than the CON (P = 0.09; 7.9 vs. 6.6 ± 0.5 µmol/g DM; respectively); however, valeric acid content, as a percent of total OA, was lower for the TRT than the CON (P = 0.005; 0.37 vs. 0.59 ± 0.04%; respectively).

Fiber content, in the alfalfa silage, of the TRT was lower for all categories than the CON (Figure 3-3) including NDF (P = 0.04; 44.4 vs. 45.8 ± 0.5%; respectively), ADF (P =0.001; 30.5 vs. 31.8 ± 0.2%; respectively) and cellulose (P = 0.05; 19.9 vs.20.6 ± 0.2%; respectively). In the corn silage (Figure 3-4), however, there was no detectable difference between treatments for NDF (P = 0.39; 40.4 vs. 41.3 ± 0.7%), ADF (P = 0.28; 21.4 vs. 22.1 ± 0.4%) or cellulose (P = 0.74; 17.7 vs. 17.9 ± 0.3%).

In situ rumen DMD (*IS*DMD) is shown on Table 3-5 for both corn and alfalfa silage. Overall, the TRT demonstrated a greater *IS*DMD than the CON in the alfalfa silage (Figure 3-5; P = 0.02; 60.3 vs. 59.0 ± 0.4%; respectively). Although *IS*DMD in the alfalfa demonstrated a time effect (P < 0.0001), no interaction was detected (P = 0.49). Dry matter degradation greater at the 4 h time point in the TRT than the CON (P = 0.02; 46.7 v 44.2 ± 0.8%; respectively).

No difference was detected between treatments in *IS*DMD in the corn silage (P = 0.71). Although *IS*DMD for corn silage demonstrated a time effect (P < 0.0001), no interaction was detected (P = 0.29).

The parameters for the rate of *IS*DMD over 48 h for the alfalfa silage are in Table 3-6 and Figure 3-6 presents the contrast between rates of *IS*DMD over 48 h for TRT and CON. No differences were detected in rate or maximum *IS*DMD between treatments in the alfalfa silage ($P \geq 0.29$).

The parameters for the rate of *IS*DMD over 48 h for the corn silage, are in Table 3-6 and Figure 3-7 presents the contrast between the rates of *IS*DMD over 48 h for TRT and CON. No differences were detected between treatments for the rate or maximum *IS*DMD ($P \geq 0.28$).

Discussion

Lactic acid is the greatest driver of pH decline and should be greater than 60% of total OA content (Woolford, 1975; Seglar, 2003; Kung, 2010). A rapid pH decline is desirable because, as the pH approaches 4.0, yeasts and molds are inhibited (Seglar, 2003). These yeast and molds are the main driver of DM and nutrient loss, as well as a possible source of illness to livestock (Kung, 2010; Skladanka et al., 2013). As Figure 3-1 demonstrates the rate of pH decline in the alfalfa silage was more rapid in the TRT, and is consistent with the increased lactic acid concentrations in the TRT. These findings are consistent with Filya et al. (2007) who observed greater lactic acid concentrations and lower pH with the use of homofermentative LAB inoculants. In addition, Sun et al. (2012) demonstrated that FE increased lactic acid concentrations. This may be the result of increased availability of WSC as a result of the FE, as was observed in Sun et al. (2012). The pH on d 59 in the TRT alfalfa silage tended to have a lower pH, which also is in line with the findings of other reports (Filya et al., 2007; Sun et al., 2012). This is expected, since pH decline is the result of OA produced during fermentation of WSC, especially lactic acid (Woolford, 1975; Bergen et al., 1991, Seglar, 2003).

However, the alfalfa silage in the present study had a final pH of above 4.6 in both treatments. The increased maturity and higher DM content of the alfalfa may have prevented the pH from dropping below 4.6 in either treatment. Maturity and DM are inversely related to packing density (Muck and Holmes, 2000; Johnson et al., 2002). As packing density decreases the pH increases (Johnson et al., 2002). Also, maturity adversely affects silage; as maturity increases, so does pH (Doane et al., 1997).

The trend for TRT alfalfa silage having a lowered the percentage of acetic acid and increased percentage of lactic acid could indicate that TRT shifted the production OA towards lactic acid, which is in line with the findings of others (Filya et al., 2007; Tabacco et al., 2011). Both TRT and CON alfalfa silages did have the desired lactic acid content greater than 60% that is considered a benchmark for quality silage (Seglar, 2003). Despite a greater isobutyric acid concentrations in the TRT, concentrations of all OA except lactic acid and acetic acid (i.e. propionic, butyric, iso-butyric, valeric and isovaleric acids) were minor. This is especially important because butyric acid is a strong antimycotic, decreases voluntary feed intake and increases risk of subacute ketosis at concentrations greater than 0.5% of DM (Kung and Shaver, 2001; Kung, 2010).

Titratable acidity is another method, in addition to pH and OA content, used to measure total acids. In the present study the TRT alfalfa silage had a lower titratable acidity than the CON. This could indicate that the CON had a greater total acid level, however, this is not supported by either the pH or the OA content of the TRT and CON silages. The findings of the present study is contrary to Chen et al. (1994) who reported that a combination of FE and LAB increased both lactic acid and titratable acidity in a grass-legume mixed silage; however, the Chen et al. (1994) study was conducted on grass-legume mixed silage and grasses have a lower acid buffering capacity than legumes do (Greenhill, 1975). The addition of the grass to the silage therefore could have increased the titratable acidity compared to a straight legume silage.

No difference was detected in lactic acid concentrations between TRT and CON for the corn silage, this is consistent with there being no difference detected between treatments for either the pH or rate of pH decline. Both treatments achieved a lactic acid content greater than 60%

of total OA and both achieved a final pH below 4.2, indicating that both treatments resulted in quality silage (Seglar, 2003). Lactic acid bacteria become less active as pH declines and eventually enter dormancy when either WSC are depleted (Bergan et al., 1991) or when pH nears 4.0 (Woolford, 1975; Seglar, 2003). As indicated in Figure 3-2, both treatments dropped below a pH of 4.2 by the second day of ensiling, and were below 4.0 before the seventh day. It is possible that this decrease in pH resulted in dormancy of the LAB in the TRT. Additionally, research suggests that FE are most effective in increasing lactic acid and decreasing pH after the seventh day (Lynch et al., 2015). It is possible then, that the LAB were dormant before the FE were most effective and this could have made detecting a difference related to the TRT more difficult. The TRT may simply have not been able to produce adequate lactic acid to result in a difference before the LAB were rendered dormant. Finally, despite an observed trend towards a difference in valeric acid content, it contributed minimally to total OA content with less than 0.6% in both treatments.

The contrast between the TRT alfalfa silage and TRT corn silage for pH and lactic acid is consistent with the findings of others. Filya et al. (2006) did not observe differences in either pH or lactic acid between corn silages treated with homofermentative LAB and a control. In subsequent research on alfalfa silage, Filya and colleagues (2007) did report that homofermentative LAB increased lactic acid, and decreased pH. This could indicate that homofermentative LAB may be more effective in increasing lactic acid production in legumes compared to corn silages.

Dry matter loss in silage is mainly the result of the initial aerobic phase and, to a lesser extent, the conversion of nutrients, such as WSC, to OA during anaerobic fermentation (Bergen et al., 1991; Seglar 2003; Kung, 2010). Additionally, both increased DM and maturity have been

demonstrated to increase DM loss (Johnson et al., 2002; Muck and Holmes, 2000). Therefore, it is possible that any improvement in DM loss achieved by the TRT was offset by increased maturity and higher DM of the alfalfa used in the current study. Dry matter losses in corn silage were identical for both treatments and no difference was detected for DM loss between TRT and CON, in either alfalfa silage or corn silage. The findings of the current study are in line with previous research, which does not support a consistent improvement, e.g. decrease, in DM loss from homofermentative LAB (Tabacco et al., 2011). Homofermentative LAB may increase DM loss (Hu et al., 2009; Tabacco et al., 2011). Adesogan et al. (2004), however, demonstrated an improvement, e.g. decrease in DM losses when FE were used in the ensiling of warm season grasses. In contrast, Lynch et al. (2015) did not detect a difference in DM loss when FE containing cellulases and xylanases were utilized during ensiling.

Fibrolytic enzymes increase the amount of reducing sugars released from structural CHO such as cellulose and xylan (Columbatto et al., 2003a). During silage fermentation, sugars and other WSC are converted to OA that lower pH and preserve the silage (Bergen et al., 1991). In the present study there was no difference detected between treatments for sugar on d 59 in the alfalfa silage. These findings are contrary to previous research where FE were utilized during ensiling and WSC were increased (Dean et al., 2005; Thomas et al., 2013; Lynch et al., 2015). Water soluble CHO decrease in silages treated with homofermentative LAB (Adesogan et al., 2004; Filya et al., 2007). The decrease in WSC, observed with the use of homofermentative LAB is possibly related to increased fermentation. This is supported by Filya et al. (2007) who also observed that lactic acid increased and WSC decreased to a greater extent in silages treated with homofermentative LAB. It is possible, then, that the

homofermentative LAB in the TRT may have offset any increase in sugar achieved by the FE, in the current study.

As a result of the conversion of structural CHO to reducing sugars (Columbatto et al., 2003a) NDF and ADF are decreased (Dean et al., 2005; Thomas et al., 2011; Sun et al., 2012; Lynch et al., 2015). All fiber fractions, (NDF, ADF and cellulose), were decreased in the TRT as compared to the CON alfalfa silage (and fiber content of both groups was numerically less than the forage on d 0). This decrease in fiber is consistent with the findings of others (Dean et al., 2005; Thomas et al., 2011; Sun et al., 2012; Lynch et al., 2015). In contrast, no difference was detected for NDF, ADF or Cellulose between treatments in the corn silage. The findings of the current study, in both the alfalfa silage and corn silage, were in contrast to the findings of others who reported an increases in WSC (Dean et al., 2005; Thomas et al., 2011; Sun et al., 2012; Lynch et al., 2015). This may indicate that the WSC were fermented by the LAB as fast as they were produced by the FE. This would be especially true in the TRT alfalfa silage, where lactic acid content tended to be greater and pH tended to be lower.

Ensiled forages tend to have a greater CP degradation than dried forages (Kohn and Allen, 1995), which could have decrease livestock production and lactation. Tabacco et al. (2011), observed that CP content of corn silage was increased with the use of homofermentative LAB, whereas CP was not changed in sorghum silage. Additionally, CP content was not changed with the addition of FE to silage (Thomas et al., 2011). In the present study CP was less in the TRT alfalfa, and though this was significant, the difference was only 0.4% units less than the CON and there was no difference detected in the change in CP (percent units) from d0 to d 59. Possibly, this indicates that while the CP content on d 59 was different between treatments, the amount that the CP changed over 59 d of ensiling was not different.

Additionally, there was no detectable differences for both ammonia and the change in ammonia in the alfalfa silage. In contrast, other researchers have observed that the ammonia content has been shown to decrease in silages treated with homofermentative LAB inoculants, where no differences was detected for CP (Hu et al., 2009; Guo et al., 2013). Homofermentative LAB may inhibit the conversion of protein to ammonium salts by rapidly reducing the pH of the silage which causes LAB and yeasts to become dormant as pH nears 4.0, preventing microbial fermentation (Van Soest, 1994; Seglar, 2003). In the current study, neither TRT nor CON alfalfa silages achieved a pH below 4.6. This could indicate that microbial fermentation was not fully inhibited in the alfalfa silage and that is why no difference was detected for ammonia.

The TRT corn silage, though, tended to gain less ammonia (as a percent unit) from d 0 to d 59, which could be considered in line with previous research that have demonstrated decreased ammonia levels when homofermentative LAB inoculants were utilized (Hu et al., 2009; Guo et al., 2013).

In both corn and alfalfa, CP content increased for both treatments on d 59 compared to d 0. Ammonia also increased in both corn and alfalfa silages by d 59. Whereas other plant components, especially NDF and ADF decreased as a result of DM loss and fermentation, in both silages by d 59. This could, in part, explain why the CP increased. In other words CP, as a percentage, increased as the fiber decreased. Overall, ammonia was low and the change percent units of CP was small, which should not substantially affect the quality of the end silage.

The present study was unable to measure differences in yeast cultures between TRT and CON in either corn or alfalfa silage. This is expected because homofermentative LAB are not as efficient as heterofermentative LAB at reducing yeast mainly because lactic acid is not as efficient an antimycotic as acetic acid is (Woolford, 1975; Kung, 2010). This is in part because the yeasts are resistant to acids and may utilize lactic acid as an energy substrate (Muck and Kung, 1997).

Fibrolytic enzymes convert xylan to reducing sugars, which in turn may increase the degradation of fiber especially cellulose (Columbatto, 2003a; Badhan et al., 2014). As xylan is broken down by xylanases, cellulose is exposed to microbial decay and cellulose degradation is improved (Badhan et al., 2014). Although fiber degradation was not tested in experiment I, *IS*DMD was improved with the use of the TRT in the alfalfa. These findings are consistent with those of Columbatto et al. (2003b) and Romero et al. (2015) that indicates that FE increased DM degradation and digestion. Columbatto et al. (2003b) and Romero et al. (2015) also reported that FE increased *in vitro* NDF and ADF digestion of corn silage and bermudagrass baleage, respectively. In addition, similar to findings by both Columbatto et al. (2003b) and Lynch et al. (2015), the majority of the increased *IS*DMD in the TRT alfalfa, was in the first 24 h. The 4 h time point was the only time point in the alfalfa that was increased by TRT, indicating that the majority of the TRT effect may be attributed to an increased early DM degradation. Additionally, whereas the TRT group had greater *IS*DMD over 48 h of incubation, there was no difference detected between treatments for the maximum *IS*DMD over 48 h. This further indicates that the improvement in *IS*DMD in alfalfa silage may be attributed to greater degradation during the early portion of incubation. This could be especially beneficial in high producing dairy cattle where ruminal passage rate is increased. In

contrast, homofermentative LAB inoculants have not been shown to consistently increase *in situ* DM degradation or *in vitro* DM digestion. Some researchers have reported no change in *in situ* DM degradation (Filya et al., 2006) or *in vitro* DM digestion (Queiroz et al., 2012) with the addition of homofermentative LAB to corn silage, whereas others (Jalc et al., 2009) did observe increase *in vitro* digestion of corn silage treated with homofermantative LAB. This may indicate that any increase in *IS*DMD in the present study may be attributed to the addition of the FE to the TRT.

However, no difference was detected between treatments for *IS*DMD in the corn silage. It is possible, that the rate at which the pH declined in the corn silage may have rendered both the LAB and FE inactive before the TRT had much effect on the silage.

Summary

The objective of this experiment was to study how a combination of four homofermentative LAB and four FE affected the ensiling characteristics of alfalfa silage and corn silages at a small scale, laboratory setting.

In this experiment, the treatment improved both lactic acid concentrations and rate of pH decline in alfalfa under challenging conditions, i.e. advanced plant maturity and high DM. No differences between treatments were detected for DM loss and nutrient content of alfalfa silage. Additionally, ruminal *IS*DMD was increased in treated alfalfa silage.

In contrast to the alfalfa silage, no treatment difference was detected in lactic acid for the corn silage. Because lactic acid is the main driver of pH decline, it was not unexpected that there also was no difference detected in the rate of pH decline between treatments. No differences were detected in fiber or in *IS*DMD, either. Both CON and TRT achieved a pH below 4.0 by

the seventh day, and maintained a pH below 4.2 throughout the length of the study in the corn silage. This could indicate that the low pH may have inhibited the LAB, and also may have inhibited the FE in the corn silage.

Overall, the inoculant + enzyme treatment may improve lactic acid and rate of pH decline when applied to a forage that is more challenging to ensile. In addition, the FE portion of the treatment may improve *IS*DMD in the challenged forages. The TRT therefore could prove useful in situations when ensiling conditions are not optimal such as when ensiling forage of advanced maturity, increased DM or low soluble CHO. Further research is needed to test if the treatment has greater impact on forages with increased maturity or if the difference observed in this study were related solely to silage type. Future research should focus on the interaction between the treatment at different moisture contents and the interaction between the treatment at different maturities in both alfalfa silage and corn silage.

Chapter 4: Experiment II

Abstract

A combination silage additive containing homofermentative lactic acid bacteria (LAB) and fibrolytic enzymes (FE) showed an improvement in rumen DM degradation, lactic acid concentration and rate of pH decline, when applied to alfalfa silage in a small scale setting. The present experiment tested the same combination additive when applied at a farm scale to a mixed cool season grass. To test the effectiveness of the inoculant + enzyme additive, a mixed stand of cool season grasses were harvested and the windrows randomly assigned to either the control (CON) or treatment (TRT). The CON was treated with water only and TRT was treated with an equal amount of water plus the combination inoculant/FE additive. Control was baled first. Bales were wrapped with polyvinyl plastic and on d 86, 88, 90, 92, 94, three bales from each group were removed, weighed and core samples obtained for analysis. Initial DM did differ between CON and TRT (P = 0.03; 52.3 v. 58.4 ± 1.9 %, respectively) possibly related to longer drying time for the TRT. Initial pH was not different between CON and TRT. Final pH also was not different between CON and TRT, either (P = 0.45; 5.24 v. 5.36 ± 0.12; respectively). Additionally, DM loss did not differ among CON and TRT (P = 0.32; 24.9 v. 20.9 ± 3.0 %; respectively). Lactic acid concentration was not different between treatments (P = 0.14; 40.89 v. 75.24 ± 19.88 µMol/g DM; respectively). *In situ* organic matter (OM; P = 005), neutral detergent fiber (NDF; P = 0.05) and acid detergent fiber (ADF; P = 0.04) degradation were all improved in the TRT silage. Furthermore, final pH for both CON and TRT were above desired pH of 4.00-4.20, which could indicate that fermentation was in part inhibited by the high initial DM.

Introduction

Over the past 40 years, silage has become a popular method of forage preservation that is used in dairies and in finishing rations of beef and lambs (Brassley, 1996; Burke et al., 2007). During ensiling, water soluble carbohydrates (WSC) are converted to organic acids (OA; Bergen et al., 1991, Seglar, 2003). Lactic acid is one of the OA most responsible for the pH decline of the silage, although it is not considered a strong antimycotic (Woolford, 1975). To increase OA production, the addition of inoculants containing LAB is the one of the most common methods used (Filya et al., 2007). These inoculants may contain homo- and/or heterofermentative LAB. Silages treated with homofermentative LAB decrease pH to a greater extent and produce greater amounts of lactic acid than those treated with heterofermentative LAB, although, compared to untreated silages the results are not consistent (Filya et al., 2006, 2007; Tabacco et al., 2011). Additionally, homofermentative LAB have not shown consistent improvement in nutrient retention, DM loss, rumen degradation or aerobic stability (Muck and Kung, 1997; Filya et al., 2006, 2007).

Fibrolytic enzymes increase lactic acid production and lower pH, especially in forages high in structural CHO (Sun et al., 2012). The use of FE has been shown to increase WSC, while decreasing NDF, ADF and cellulose (Thomas et al., 2013; Lynch et al., 2015). Additionally, FE have also been reported to improve digestibility of structural carbohydrates (CHO), especially NDF in the first 24 h of incubation (Columbatto et al., 2003a; Romero et al., 2015).

The objective of this research was to evaluate the effects of a silage treatment, which contained four homofermentative LAB in combination with four FE a mixed cool season grass haylage in a farm scale setting.

Materials and Methods

Silage preparation: On July 7th, 2014 a 20 acre plot of a mixed cool season grasses [14% oats (*A. sativa*), 26% orchardgrass (*D. glomerata*), 44% mountain brome (*B. marginatus* Nees ex Steudel), 11% Italian rye (*L. multiflorum*), and 5% opportunistic plants, mainly downy brome (*B. tectorum*) and triticale (*Triticale heaploide* Lart.)], was harvested at the University of Idaho Dairy Center (Moscow, Idaho, 83844). Field survey to quantify composition was done by dividing the field into quarters. Two trained personal then randomly selected three 1^2 m survey sites per person, per field quarter to survey and the results averaged and reported as an average vegetation composition of the field. Grass was mowed starting at 06:00 h. Baling began at 08:00 h. Individual windrows were randomly assigned to either TRT or CON. The CON was treated with only water, dispensed from a sprayer mounted on the back of an all-terrain vehicle (ATV) driven directly in front of the baler. The other half was treated with the same volume of water plus a commercial inoculant, Sil-All 4x4, (Lallemand Animal Nutrition, North America, Milwaukee, WI) containing four species of homofermentative LAB (*L. plantarum, L. acidophilus, P. acidilactici* and *P. pentosaceus*) in combination with four FE (cellulase, xylanase, β-glucanase and α-amylase), at the manufacturers recommended application of 2×10^5 CFU (LAB)/g of silage. The CON was baled first, and then a separate bale was run with no treatment to clean out the baler (this bale was discarded) and then the TRT bales were baled. A total of 17 bales for each TRT were prepared (n =5, 3 bales per n, average weight of bales = 718.9 kg, average total weight of n = 2156.8 kg), and loaded onto a flatbed truck immediately after baling, weighed and transported approximately 1 km to the wrapping and storage site. Bales were wrapped with polyvinyl plastic, in a tube, by treatment with bale 1 and 17 of each treatment used as cap bales. Bales were than ensiled for an average

of 90 d. Starting on October 1st, 2014, three bales from each treatment were removed, weighed and sampled for further analysis. The remaining silage was then resealed and capped with the end bale- one of the two selected as cap bales and not on trial. Sampling occurred every 2 d starting on d 86 (d 86 88, 90, 92 and 94) for logistical purposes, and reported as d 90 ± 4.

On the day of sampling, samples were collected from 3 bales using forage sampler (Penn State; 3.5 cm diameter, 40 cm long; Nasco, Ft. Atkinson, WI.) and portable drill. Approximately 2 kg of sample from each treatment were collected. Samples were then placed in a labelled sealable bag and placed in a cooler for transportation to the laboratory. Samples were analyzed on the day of sampling for DM, pH, titratable acidity, LAB cultures and yeast-mold cultures. Sub-samples were also frozen for further analysis, including fiber analysis, nutrient analysis and *in situ* rumen degradation.

Dry Matter and pH: The pH of each treatment was determined using an Oakton pH 11 series pH/mV/0C meter (Cole-Parmer, Court Vernon Hills, IL). Samples were prepared in a similar manner as in experiment I: 50 grams of haylage was mixed with 450 mL of distilled water and blended for one minute on liquefy setting in a Rival 6 speed mixer (Jarden Corp., New York, NY) and the slurry was then filtered through 2 layers of cheese cloth. Then 70 mL of the filtered fluid was removed and placed into a clean beaker for titratable acidity, which was measured as in the procedure described in experiment I.

Dry matter was determined as per procedure described in experiment I. Briefly, 40 g of samples were dried in an oven set at 60°C for 48 hours and then reweighed. Additionally, 105° C DM were conducted as per AOAC (1999).

Microbial analysis: Lactic acid bacteria were plated for each sampling day (d 90 ± 4). Samples were prepared as described in experiment I: 25 grams of silage were added to 225 mL of sterile physiological saline and serial dilutions in physiological saline were made from the filtered liquid. The serial dilutions were than plated on MRS agar (Difco Laboratories, Detroit, MI). The plates were incubated at 35°C for 72 h and only plates between 30 and 300 CFU were counted.

Yeast-mold cultures were also plated as described in experiment I. Samples were prepared as per LAB cultures then 1 ml of diluted sample were applied to 3M petri-film yeast-mold plates (3M, St. Paul, MN) and incubated at room temperature for 5 days. Only plates with 1-400 CFU were counted.

Aerobic stability: Aerobic stability was determined using a modified version of procedure described in Honig (1990). Two kilograms of silage was placed in a Styrofoam container and a Track-It Temperature Logger (Monarch Instruments, Amherst, NH) was placed in the geometric center. Temperature recordings were obtained every 10 minutes until temperature had increased to 2° C above ambient temperature, samples were stored at a constant 20° C indoors.

Organic acids: Volatile fatty acids were determined using GC. Samples were prepared by mixing 100 grams of silage with 400 ml of water, as per experiment I. Gas chromatography was conducted on a Hewlitt-Packard 7890 GC using an Agilent DB-FFAP column (Hewlitt-Packard, Avondale, PA) for VFA, loaded in the front inlet. Settings for GC were: initial temperature of oven was 100°C, run temperatures were set at 150°C for 9.25 minutes, then 175°C for 1.50 minutes, then 225°C for 1 minute at a constant pressure of 3.00 psi with a 1:10 split. Hydrogen was used as the carrier gas.

Lactic acid was analyzed using anion chromatography on a Dionex ED 40 detector and a GP40 pump (Thermo Scientific, Sunnyville, CA), using a modified version, as described in experiment I, of the procedure recommended by Dionex corporation for analysis of OA (Dionex, Thermo Scientific).

Fiber and ash analysis: Samples were prepared for fiber and ash analysis as described experiment I. Fiber analysis (NDF, ADF and cellulose) was performed as described by Van Soest et al. (1991) and ash was analyzed as described in AOAC (1999).

In situ: All animal trials were performed in accordance to procedures approved by the University of Idaho's Institutional Animal Care and Use Committee (Protocol # 2014-2).

Two half-sister Holstein primiparous dairy cows from the University of Idaho Dairy Center were selected and cannulated by Dr. S. Parish at the College of Veterinary Medicine, Washington State University (Pullman, WA, 99164). While on trial, the cows were fed a total mixed ration containing a mixed cool-season grass hay, alfalfa hay, alfalfa silage, triticale silage, canola, dry distiller's grain, corn and barley.

Silage samples were prepared as in experiment I; 4.5 grams of ground samples were weighed into Dacron bags (50 µM pores, 10 x 20 cm) and double sealed. These were then loaded into large mesh bags and introduced into the rumen of one of two cannulated animals. Each mesh bag had samples from both treatments for all five days of sampling so that treatment and time point were evenly balanced between animals. Samples were incubated at 0, 12, 24 and 48 h, rinsed and dried as described by Stern et al. (1997). Neutral detergent fiber and ADF degradation was performed on dried, post *in situ* sample bags using a method modified from Van Soest et al. (1991). Neutral detergent fiber degradation was performed by adding 15 mL

of NDF solution per sample bag to a large beaker, and brought to a boil, with a pan of ice placed over the mouth of the beaker. Samples and NDF solution was boiled for 75 min and stirred every 3-5 min. After 75 min the NDF solution was drained off, and the sample bags rinsed with boiling water for 5 min. This was repeated 3 times for a total of 4 rinses, sample bags were then soaked in acetone for 3 min, air dried for 1 h then oven dried overnight at 105°C.

Acid detergent fiber was determined by boiling post *in situ* sample bags in 15 mL ADF solution per bag for 1 h, in a large beaker with a pan of ice over the mouth. Samples were stirred every 3-5 min. Solution was then drained off and samples rinsed with boiling water for 5 min, while stirring. This rinse was repeated 3 times for a total of 4 rinses, then samples were soaked in acetone for 3 min, air dried for 1 h and oven dried at 105°C overnight.

Organic Matter degradation was determined by combusting the post *IS*ADF degradation samples in a muffle furnace at 600°C for 6 h (AOAC, 1999).

Nutrient Analysis: Frozen samples, from d 0 and each d of sampling, were shipped to Dairyland Laboratories (Arcadia, WI) and analyzed for crude protein (CP), ammonia (as part of CP; by method described by AOAC, 1999), and WSC (by method described in Derias, 1961). Randomly selected *in situ* bags (3 per treatment) from time point 24 and 48 h was also sent for CP analysis to Dairyland Laboratories.

Statistical analysis: Statistical analyses for experiment II was conducted using SAS, version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA), day was not used as a variable. Chemical analysis, VFA, microbial cultures, fiber and nutrient analysis were analyzed using a T-test (PROC TTest). All data were checked for outliers by a studentized T-Test and any with

a value greater than three were considered as possible outliers. *In situ* results were analyzed by a MIXED model (PROC MIXED) with repeated measures over time and the means adjusted for their respective covariant, i.e. NDF degradation was adjusted for the sample NDF. Repeated measurements were subjected to a COV and any with a COV greater than 15% were repeated for verification.

Rate of degradation over 48 h for DM, OM, NDF, ADF and hemicellulose degradation was analyzed using a nonlinear regression (PROC NLMIXED, SAS 9.4), with the model $\mu = m \times (1\text{-}\exp^{(-B \times h)})$, where μ is the mean of the treatment, -B is the slope of the line (rate of increase) and m is the maximum value predicted by the nonlinear regression. Significance was set at $P \leq 0.05$ and trend at $P \leq 0.10$.

Results

The chemical, microbial and nutrient analyses for the grass silage prior to ensiling (d 0) are shown in Table 4-1. Dry matter was greater in the TRT than for the CON ($P = 0.03$; 58.4 v. 52.3% SEM 1.9%; respectively). No difference was detected in NDF, ADF, hemicellulose, cellulose, pH, ash, yeast cultures or titratable acidity between treatments ($P \geq 0.13$). Lactic acid bacteria cultures tended to be greater in the TRT than CON ($P = 0.08$; 7.3 v. 6.8 ± 0.2 $\log_{10}$ CFU/g; respectively).

Dry matter on d 90 (Table 4-2) was greater in the TRT than CON silage ($P = 0.006$; 53.1 vs. 43.8 ± 2.1%; respectively), although no difference was detected in the percent unit change in DM from d 0 to d 90 between treatments ($P = 0.24$). No difference was detected between treatments for DM loss ($P = 0.32$). There were no differences detected for either yeast or LAB cultures between treatments ($P \geq 0.39$). No difference was detected between treatments

for aerobic stability (P= 0.23). Figure 4-2 represents the readings for aerobic stability for each monitor on the each d of sampling.

Fiber content and nutrients of the grass silage on d 90 are shown in Table 4-3. No difference was detected between treatments for NDF (P = 0.12). In addition, no difference was detected in the difference in NDF (d 0 to d 90; percent units) between treatments (P = 0.15). Acid detergent fiber was lower in the TRT than the CON silage (P = 0.02; 40.9 vs. 41.9 ± 0.3%, respectively) although no difference was detected in the difference in ADF (d 0 to d 90, percent units) between TRT and CON (P = 0.17). No difference was detected in cellulose between treatments (P = 0.14). In contrast, the difference in cellulose from d 0 to d 90 was greater in the TRT than the CON silage (P = 0.007, 0.6 vs. -0.4 ± 0.2% units; respectively). This may indicate that the percent of cellulose decreased in the CON and increased in the TRT silage. Hemicellulose (calculated) was greater in the TRT than CON silage (P = 0.007; 21.8 vs. 19.8 ± 0.5%; respectively) whereas the difference in hemicellulose (d 0 to d 90) was less in the TRT than the CON (P = 0.03, -3.2 vs. -4.6 ± 0.5% units; respectively). No difference was detected in CP between treatments (P = 0.15). In contrast, the change in CP (d 0 to d 90) was less in the TRT than in the CON (P = 0.01, 1.6 vs. 3.0 ± 0.3% units; respectively). Ammonia was less in the TRT (P = 0.03, 1.1 vs. 1.4 ± 0.1%; respectively) and the change in ammonia (d 0 to d 90) was also less in the TRT than the CON (P = 0.04, 1.1 vs. 1.3 ± 0.1% units); respectively. Water soluble CHO tended to be greater in the TRT than the CON (P = 0.06, 6.0 vs. 4.3 ± 0.5%; respectively) although no difference was detected in the change in WSC (d0 to d 90) between treatments (P = 0.64).

Organic acid concentration as a percent of total OA is shown in Table 4-4. No differences were detected in any of the OA between treatments ($P \geq 0.22$). No difference was detected between treatments for lactic acid (Figure 4-3) or lactic acid to acetic acid ratio ($P \geq 0.14$).

The results of the *IS* ruminal degradation trials can be shown in Table 4-5, means were adjusted for their respective covariant. No difference was detected for *in situ* DM degradation (*IS*DMD) between treatments ($P = 0.71$). Although there was a time effect ($P < 0.0001$) no interaction was detected ($P = 0.67$). *In situ* NDF degradation (*IS*NDFD) was greater in the TRT than the CON (Figure 4-4; $P = 0.05$, 33.0 vs. 30.8 ± 2.1%; respectively). Although there was a time effect ($P < 0.0001$), no interaction was detected for *IS*NDFD ($P = 0.32$). *In situ* NDF degradation also tended to be greater at 24 h of incubation in the TRT than the CON ($P = 0.06$; 33.9% vs. 30.2 ± 2.4; respectively). *In situ* ADF degradation (*IS*ADFD) was greater for the TRT compared to the CON (Figure 4-5; $P = 0.04$; 27.7 vs. 21.1 ± 2.3%). Although there was a time effect ($P <0.0001$) no interaction was detected in *IS*ADFD ($P = 0.12$). *In situ* ADF degradation was greater in the TRT than CON silage at 12 h of incubation ($P = 0.02$; 20.6 vs. 9.2 ± 3.6%; respectively) and tended to be greater in the TRT than the CON at 24 h ($P = 0.07$; 30.9 vs. 21.8 ± 3.6%; respectively) and 48 h ($P = 0.08$; 34.1 vs. 25.6 ± 3.4%; respectively). No difference was detected for *in situ* CP degradation (*IS*CPD) between treatments ($P = 0.29$). Furthermore, no time effect or interaction was detected for *IS*CPD ($P \geq 0.18$). In contrast, *in situ* OMD (*IS*OMD) was greater in the TRT than the CON (Figure 4-6; $P = 0.0005$; 48.6 vs. 46.7 ± 0.7%; respectively). Although there was a time effect ($P < 0.0001$) no interaction was detected for *IS*OMD ($P = 0.29$). No difference was detected for *IS* hemicellulose degradation (*IS*HCD) between TRT and CON ($P = 0.87$), although there was a time affect ($P < 0.0001$) and there was an interaction between treatment and time ($P = 0.04$).

In situ HCD tended to be greater at 36 h for the TRT than the CON ($P = 0.06$, 66.9 vs. 49.9 ± 6.2%; respectively).

The parameters for the rate of *IS*DMD over 48 h are in Table 4-6 and Figure 4-7 presents the contrast in rate of *IS*DMD between TRT and CON. No difference was detected for the rate or lines (treatments) of *IS*DMD between treatments ($P \geq 0.18$). Maximum *IS*DMD over 48 h tended to be greater in the TRT than the CON silage ($P = 0.07$; 56.0 vs. 54.6 ± 0.5%; respectively).

The parameters for analysis of the rate of *IS*NDFD over 48 h are in Table 4-6 and Figure 4-8 presents the contrast in rates of *IS*NDFD between TRT and CON. No difference was detected for rate of *IS*NDFD between TRT and CON ($P = 0.74$). In contrast maximum *IS*NDFD over 48 h tended to be greater in the TRT than CON ($P = 0.07$; 43.8 vs. 39.2 ± 2.0%; respectively) and the lines were different ($P = 0.01$).

The parameters for *IS*ADFD over 48 h are in Table 4-6 and Figure 4-9 presents the contrast in rates of *IS*ADFD between TRT and CON silages. No difference was detected in the rate, maximum over 48 h or lines of *IS*ADFD between TRT and CON ($P \geq 0.15$).

The parameters for the rate of *IS*HCD over 48 h are in Table 4-6 and Figure 4-10 presents the contrast for the rate of *IS*HCD between TRT and CON silages. No differences were detected in rate, maximum or lines of *IS*HCD between treatments ($P \geq 0.13$).

The parameters for the rate of *IS*OMD over 48 h are in Table 4-6 and Figure 4-11 presents the contrast for the rates of *IS*OMD between TRT and CON silages. No difference was detected in the rate of *IS*OMD between treatments ($P = 0.27$). Maximum *IS*OMD over 48 h was greater

in the TRT than the CON silage (P = 0.02; 53.7 vs. 51.3%, SEM 0.8%), and the lines also tended to be different between TRT and CON (P = 0.06).

Discussion

Initial DM can adversely impact packing density, OA production and pH decline (Garcia et al., 1989; Muck and Holmes, 2000; Johnson et al., 2002). The difference observed for initial DM resulted probably from the weather conditions at the time of ensiling and delay in baling the TRT. The TRT group was baled after the CON group and therefore, had longer to dry (approximately 3 h). Additionally, temperatures increased from 16°C at the start of baling to over 32°C at the time of finish. The tendency for LAB cultures to be greater in the TRT group was expected because samples were collected after the TRT was added but prior to baling and the additive contained LAB.

Despite the DM of the TRT remaining greater after ensiling, no difference was detected for the change in DM between treatments. As with experiment I, there was no difference detected for DM loss during ensiling between the treatments.

Although homofermentative LAB decrease pH and increase lactic acid production (Filya et al., 2007), there are many other factors that can impact pH decline. Maturity and DM are inversely related to packing density (Johnson et al., 2002; Muck and Holmes, 2010). A low packing density in turn has been shown to decrease OA content and inhibit pH decline (Johnson et al., 2002; Zheng et al., 2011). In addition, increased DM, and advanced maturity has been demonstrated to lower OA production and increase pH (Garcia et al., 1989; Johnson et al., 2002; Hu et al., 2009; Kung 2010; Hao et al., 2015). It should be noted that both the TRT and CON grasses used in the present study were mature as evidence by DM (> 50% DM

at harvest) and increased NDF and ADF (> 66% NDF and > 42% ADF), and because of experimental conditions the two factors could not be studied separately. The combination of increased maturity and DM could have impeded the production of OA and, thus, increased pH. In addition, the TRT silage had greater DM content both before and after ensiling. Further, this could explain why no difference was detected for pH between treatments and why pH remained greater than 5.0 for both TRT and CON. It should be noted that both silages achieved a greater than 4:1 lactic acid to acetic acid ratio indicating that with or without TRT, lactic acid was the predominant OA produced. This could indicate that homofermentative LAB were the dominant strains of LAB present. Also, the LAB cultures, after the TRT was added only tended to be greater in the TRT silage compared to the CON, which may indicate that natural LAB were already present in sufficient numbers to ensure adequate lactic acid content. Finally, both TRT and CON achieved the greater than 60% lactic acid content that is considered desirable (Seglar, 2003) despite the challenges presented as a result of increased DM and advanced maturity. Due to experimental conditions it was not possible separate the effect of DM and maturity, however, because TRT DM was greater, this could indicate that DM was the limiting factor in the TRT haylage.

The use of FE has been shown to reduce fiber components such as xylan and cellulose, by converting these structures into reducing sugars (Columbatto et al., 2003a). The present study is not consistent with other researchers where FE decreased NDF content of silages (Dean et al., 2005; Sun et al., 2012; Thomas et al., 2013). Additionally findings of the present experiment are contrary to the findings in the experiment I were NDF was observed to be lower in the TRT alfalfa silage. In contrast, in experiment I, no difference was detected for NDF between treatments in the corn silage, which is consistent with the findings of the

present study. In experiment I the lack of detectable difference in NDF, however, may have been the result of the low silage pH inactivating the FE. That is not the case here, because both TRT and CON had pH greater than 5.0. Despite not observing a detectable difference for NDF, it should be noted that both CON and TRT decreased in NDF from d 0 to d 90. The lack of detectable difference for NDF was unexpected, since the silage additive contained xylanase, cellulases and β-glucanase, which would be expected to reduce NDF, ADF and hemicellulose content. Xylan is a CHO associated with hemicellulose (Van Soest, 1994) and therefore it would be expected that hemicellulose would decrease in the TRT silage. Furthermore, cellulases have been observed to also decrease hemicellulose (Van Soest, 1994). Hemicellulose did decrease in both TRT and CON, however, CON decreased to a greater extent than did TRT. Hemicellulose was calculated by subtracting ADF from NDF. Van Soest (1994) cautions that this may lead to an overestimation of hemicellulose in grasses. Since the method was applied to both TRT and CON, however, it is unlikely this contributed to the difference that was observed in the present study. Under certain circumstances, such as in the presence of acetic acid, hemicellulose that is typically insoluble when bound to lignin will become more soluble (Van Soest, 1994). The CON had a greater moisture content (i.e. lower DM content) and acetic acid content of the CON haylage was 14% of total OA. This may have resulted in greater amount of hemicellulose solubilized and lost in the CON compared to the TRT. This is likely the result of greater moisture content and not a greater acetic acid, because no difference was detected between treatments for acetic acid content.

Acid detergent fiber was lower in the TRT, which is similar to the findings in the TRT alfalfa silage in experiment I, and to research of others who also reported FE decreased ADF content (Dean et al., 2005; Sun et al., 2012; Thomas et al., 2013). The decrease in ADF, paired with

the increase in cellulose, could indicates that other portions of the cell wall are being decreased in comparison to cellulose, in the TRT. This seems unlikely since nothing in the TRT should have affected the lignin. The silage did contain cellulase and β-glucanase and both would have potentially hydrolyzed cellulose. The change in cellulose was small, though, less than one unit percent difference in both TRT and CON silages and may not be biologically relevant.

The degradation of CP is a concern during ensiling. Silages have been demonstrated to increase CP degradation compared to dry forages (Kohn and Allen, 1995). During the ensiling process both CON and TRT silages had an increases in CP, although, the increase was less in TRT compared to CON silage (Table 4-3). The TRT silage also had less of an increase in percent units of ammonia. This could indicate that the trend towards increased WSC observed in the TRT haylage may have supplied the microbes with adequate energy and carbon sources that it decreased deamination of amino acids. These findings are consistent with Hu et al. (2009), where CP was unchanged, however, ammonia levels were reduced, with the use of homofermentative LAB. Guo et al. (2013), in contrast, reported an increase in ammonia in inoculated grass silages from the first cut. Subsequent cuttings were found to have a decreased ammonia content with the use of inoculants. This could also could indicate that as the days grew longer, and photosynthesis increased, WSC increased and there was less of a need for deamination of amino acids in the later cuttings.

Additionally, ruminal bacteria can use NPN as a source of nitrogen for amino acid synthesis, though feeding excessive CP can result in increased nitrogen excretion in the waste (Van Soest, 1994). Excreted nitrogen can have detrimental effects on the environment and is considered to contribute to climate change (Smith et al., 2007). The decreased amount of

ammonia in the TRT therefore may prove beneficial to the environment, especially if the silage is part of a TMR with elevated CP, although ammonia content was less than 1.5% in both CON and TRT.

Lactic acid and other OA are produced by the fermentation of WSC by LAB (Bergen et al., 1991; Seglar 2003). Therefore, the addition of homofermentative LAB may lower WSC (Adesogan et al., 2004; Filya et al., 2007). Other researchers did not detect differences in WSC between silage inoculated with homofermentative LAB and control silage (Hu et al., 2009). This is counterintuitive, because an increase in LAB would be expected to result in increased fermentation and decreased WSC. Columbatto et al. (2003a) demonstrated that cellulases and xylanases increased reducing sugars when added to pure cellulose and xylan mixtures. The trend towards increased WSC observed in the TRT haylage is consistent with the findings of other researchers who reported that WSC are either increased or tend to increase in silages treated with FE (Dean et al., 2005; Thomas et al., 2013; Lynch et al., 2015). In contrast, no difference was detected for the change in WSC (d 0 to d 90; percent units) between treatments. This suggest that the addition of the LAB in the TRT did not result in increased fermentation of WSC fermentation. This is likely a result of the negative effect of maturity and DM on the LAB in the TRT. Furthermore, this is supported by the observation that no difference was detected in any of the OA between treatments. It is possible that because ADF of the TRT silage did decrease, at least in part the trend towards increased WSC in the TRT silage was derived from enzymatic action on the ADF. If this were the cause, however, it would be expected that cellulose would have also decreased, which is not the case with the present study. Additionally, this could indicate that DM and maturity were the factors that caused fermentation to cease.

The addition of FE has been shown to increase fiber degradation and digestion of silages (Columbatto et al., 2003b; Lync et al., 2015; Romero et al., 2015), with the majority of the improvement generally occurring during the first 24 h of digestion or degradation. Thomas et al. (2013) demonstrated that the kinetics of *IS* DMD, NDFD, and ADFD were improved with the utilization of FE. The improvement in fiber degradation may be a result of increased cellulose degradation (Badhan et al., 2014). Increased rumen cellulose degradation in forages, when xylanases and cellulases are used, is attributed to a decrease in xylan (Badhan et al., 2014). Xylan is a carbohydrate associated with hemicellulose, which forms a complex with cellulose, rendering it less digestible (Van Soest, 1994; Badhan et al., 2014). The increase in *IS*OMD of the TRT silage, is likely the result of increased fiber degradation, because both *IS*NDFD and *IS*ADFD were increased when no difference was detected for *IS*CPD between treatments. Additionally, at 24 h, both the *IS*NDFD and *IS*ADFD tended to be increased, whereas *IS*OMD was greater in the TRT than the CON silage (Table 4-5). This possibly indicates that the increased fiber and OM degradations may be the result of increased *IS*ADFD. Although, not tested, cellulose is the most degradable portion of the ADF fraction (Van Soest, 1994); therefore the increased *IS*ADFD could be the result of increased cellulose degradation. As Columbatto et al. (2003b) observed the majority of increase in the present study for fiber and OM degradation of the TRT haylage occurred by 24 h. Although in both the *IS*ADFD and *IS*OMD the 48 h time point also tended to be greater in the TRT silage. Increased *IS*NDFD and *IS*ADFD is important, because the cell wall is typically less than 50% degradable in the rumen (Hatfield et al., 1999). Therefore, even a small increase in cell wall degradation can result in improved performance in both dairy and beef cattle and also, improve sustainability by reducing manure output (Hatfield et al., 1999).

Aerobic yeasts are the main source of silage spoilage (Seglar, 2003; Kung 2010). Yeasts in turn may produce mycotoxins that adversely impact livestock health and lactation (Skladanka et al., 2013; Santos et al., 2014). The findings of the present study are similar to those reported by Dean et al. (2005) and Thomas et al. (2013) who also noted that no differences were detected in yeast cultures with the addition of FE. The present study, however, is contrary to a report by Lynch et al. (2015) who observed increased yeast counts with the addition of FE to silage. This may be as a result of increased WSC which provided more fuel and thus better survival and increased reproduction, for yeasts. Although, overall the findings of the present study with regards to yeast cultures, is not unexpected because lactic acid is not considered a strong antimycotic agent (Woolford, 1975; Ranjit and Kung, 2010). Additionally, many of the yeasts that contribute to silage spoilage are not only acid tolerant, but also may utilize lactic acid as an energy source (Muck and Kung, 1997). Finally, although lactic acid increases pH decline (Kung, 2010), it is not as effective at increasing aerobic stability. A meta-study of homofermentative LAB inoculants demonstrated that they improved aerobic stability in only 30% of studies (Muck and Kung, 1997). Although, no difference was detected for aerobic stability between TRT and CON silages, it should be noted that numerically, for each sample, the aerobic stability was greater in the TRT than the CON (see Figure 4-2).

Summary

The objective of this experiment was to examine the impact of four homofermentative LAB combined with four FE on the ensiling characteristics of a mixed cool-season grass haylage at a farm scale.

In this experiment both silages had terminal pH of greater than 5.0, indicating that fermentation and OA production may have been inhibited in both control and treated silages. This could be the result of both advanced maturity and low moisture content, as well as decreased packing density. Despite these challenges, there are indications that the treatment did improve the quality and rumen degradability of the silage. Treated silage gained less ammonia and had lower ADF, while tending to have greater WSC compared to the control. Additionally, the maximum *IS*OMD was increased in the treatment silage, which also demonstrated a trend towards an increase in maximum *IS*NDFD and *IS*DMD. Furthermore, *IS*OMD, *IS*NDFD and *IS*ADFD, were all improved in the treated silage. The increases in *IS* degradation could possibly be a result of the removal of xylan and the breakdown of cellulose by the FE in the treatment. These findings indicate that even under challenging conditions the treatment used had a positive impact on the silage.

Further research is needed using less mature, lower DM grasses, with a larger number of samples. Although voluntary feed intake was not a parameter examined in this study it is important to animal performance. Therefore, the impact of the treatment on voluntary feed intake of silages and productive performance should be included in future research to determine if the increased fiber and OM degradation translates into improved performance.

Chapter 5: Summary and Implications

The objective of this research was to evaluate the effects of a commercially available silage treatment, Sil-All 4x4, which contains four homofermentative LAB in combination with four FE on: 1) alfalfa and corn silage in a small scale, laboratory setting and 2) a mixed cool season grass haylage in a farm scale setting.

Lactic acid concentrations were increased in the TRT alfalfa silage. The increase in lactic acid concentrations in the TRT alfalfa silage was probably responsible for the increased rate of pH decline and the trend towards a lower pH. No difference, though, was detected for pH and rate of pH decline between TRT and CON corn silage that was possibly the result of a rapid rate of pH decline in both treatments. When pH is less than 4.0, LAB become inhibited and because both TRT and CON corn silage had a pH that declined rapidly, the LAB in the additive may have been dormant before they could have much effect on the silage. The OA content in the cool season grass haylage (experiment II) was probably decreased related to maturity of the grass and increased DM of the grass. This also likely contributed to the observation that both CON and TRT grass haylages had a pH greater than 5.0.

The gain in ammonia was less in the TRT grass haylage and tended to be less in the TRT corn silage. Additionally, no difference was detected for CP in the corn silage or grass haylage. Finally, the TRT grass haylage had a smaller change in CP content. These results could indicate that the TRT may have prevented some degradation of CP. This is likely the result of the LAB in the TRT, because there is little evidence that FE impact CP content. Whereas homofermentative LAB have been found to decrease ammonia in silage.

Fibrolytic enzymes have been observed to decrease NDF, ADF and cellulose levels whereas increasing WSC levels. Although NDF and ADF were lower in the TRT alfalfa silage, no

difference was detected for sugar. The TRT corn silage, also showed numerically lower NDF, ADF and cellulose, although it was not significant. This could indicate that, as with the LAB, the rapid pH decline may have inhibited the FE before they effected the corn silage. The TRT grass haylage also was observed to have less ADF and tended to have greater WSC. It is possible that the increase in WSC is related to the decrease in ADF as a result of the addition of the FE.

Fibrolytic enzymes have also been demonstrated to increase DM and fiber degradation. This may be the result of the breakdown of xylan, which exposes cellulose and renders it more degradable in the rumen. The present studies observed similar findings in the TRT alfalfa silage and in the TRT grass haylage. *In situ* fiber degradation and *IS*OMD over was improved in the TRT grass haylage. Additionally, maximum *IS*OMD over 48 h was greater and the maximum *IS*DMD, *IS*NDFD over 48 h tended to be greater. The TRT alfalfa silage was observed to have greater *IS*DMD. As in Columbatto et al. (2003b), the majority of the increase in rumen degradation was observed in the first 24 h, especially in the TRT alfalfa silage. The increase in *IS*OMD and *IS*NDFD in the grass haylage could be attributed to increased *IS*ADFD. Because cellulose is the most degradable portion of ADF, it is probable that the increase in *IS*ADFD is possibly related to an increased cellulose degradation.

Overall, the TRT may increase lactic acid concentrations, especially in challenging forages. It may also prevent CP degradation to a degree. The FE appear to decrease fiber content, especially ADF and there is evidence that they may also improve WSC. Finally, the FE may increase DM, OM and fiber degradation in the rumen, most notably during the first 24 h. This could be especially beneficial to producers in a high performance setting by reducing the amount of feed needed to support livestock. The cell wall is generally less than 50%

digestible, so even a small increase in fiber degradation may have huge benefits for the producer in terms of increased production and lowered feed costs. Additionally increased fiber digestion results in a lowered environmental impact, especially manure output.

Future research should be conducted to further examine the impact that the additive has on different plant species, at different DM and, maturities. Additionally, research should be conducted on voluntary feed intake, animal performance, and manure output. Finally, research should be conducted to study if the lower ammonia observed in the grass silage effects nitrogen levels in the blood, milk, urine and feces.

Tables: Experiment I

Table 3-1: Chemical analyses of forage (alfalfa and whole plant corn) after treatment application, prior to packaging in mini-silos (d 0; n = 3).

	Alfalfa			Corn		
	Control	Treatment	SEM	Control	Treatment	SEM
Chemistry						
DM (%)	42.0	42.1	1.2	33.0	33.7	0.7
pH	5.98	5.96	0.01	5.85	5.85	0.02
NDF (%)	42.2	43.2	0.9	51.2	45.6	1.1
ADF (%)	29.2	30.2	0.7	28.5	24.9	2.4
Cellulose (%)	20.0	20.7	0.7	23.7	20.4	2.4
CP (%)	13.1	12.9	0.2	9.5	9.0	0.2
NH_4 (%)	0.1	0.1	0.01	0.1	0.2	0.1
Starch (%)	NT	NT	NT	27.0	27.8	2.2
Sugar (%)	7.4	7.1	0.4	NT	NT	NT
Ash (%)	12.0	12.7	0.6	5.1	4.8	0.1

NT indicates test not conducted for that silage.

Table 3-2: Rate of pH decline, d 1-13 in alfalfa and d 1-17 for corn silages. Parameters provided through a non- linear regression analysis of means of pH on d 1, 2, 3, and 13 for alfalfa and d 1, 2, 3, 7 and 17 for corn silage, until pH reached asymptote of both silages as measured in mini-silos.

	Control	Treatment	SEM
Alfalfa			
Slope (B1, rate)	-1.17	-2.22	0.32
Asymptote	4.89	4.90	0.03
Intercept (B0)	3.66	11.14	3.52
Corn			
Slope (B1, rate)	-1.09	-1.05	0.22
Asymptote	3.97	3.94	0.02
Intercept (B0)	1.10	0.99	0.27

Table 3-3: Chemical and microbial analyses of alfalfa and corn silage. Measured in bucket silos, d 59 (n = 5).

	Alfalfa				Corn			
Item	Control	Treatment	SEM	*P*	Control	Treatment	SEM	*P*
DM (%)	39.1	39.1	0.8	0.96	32.2	32.4	0.8	0.88
DM loss (%)[a]	3.3	3.9	0.7	0.53	6.6	6.5	1.0	0.98
pH	4.69	4.66	0.02	0.07	3.91	3.91	0.01	0.92
Tit Acid[b]	328.5	310.9	5.1	0.02	528.7	526.4	10.8	0.89
Cultures[c]								
Yeast	2.8	2.9	0.1	0.94	7.4	7.3	0.1	0.53
LAB	8.1	8.1	0.1	0.79	7.6	7.6	0.1	0.94
Nutrient (%DM)[d]								
CP	13.4	13.0	0.1	0.001	9.5	9.3	0.1	0.11
Ammonia	1.1	1.1	0.03	0.96	0.4	0.3	0.01	0.14
Starch	NT	NT	NT	NT	27.2	28.0	0.9	0.58
Sugar	1.7	1.6	0.1	0.73	NT	NT	NT	NT
Ash	15.1	15.1	0.3	0.92	5.7	5.5	0.1	0.27
Nut diff (% unit)[e]								
CP	0.4	0.01	0.16	0.14	0.1	0.3	0.1	0.25
Ammonia	1.0	1.0	0.03	0.71	0.4	0.3	0.01	0.07
Starch	NT	NT	NT	NT	0.8	-0.1	1.5	0.68
Sugar	-5.8	-5.6	0.3	0.56	NT	NT	NT	NT

[a] Dry matter (DM) loss (%) indicates the difference between initial DM weight and final DM weight

[b] Titratable Acidity (Tit Acid) is mmol/g DM of 1N NaOH needed to raise 70 mL of a 1:10 silage solution, to a pH of 6.8.

[c] Cultures are reported as the $\log_{10}$ of the number of CFU/g of DM for yeast cultures and lactic acid bacteria (LAB) cultures of the silage on d 59.

[d] Nutrient content, crude protein (CP), ammonia, starch, sugar and ash, are reported %DM as per laboratory analysis conducted by Dairyland laboratories (Arcadia, WI).

[e] Nutrient difference (Nut Diff) indicates the difference between initial nutrient content of pre-ensiled forage and nutrient content of ensiled forage, in % units.

NT indicates test not conducted on that silage.

Table 3-4: Organic acid (OA) concentration in alfalfa silage and corn silage on d 59 as measured in bucket silos (n = 5) in µmol acid/g DM and percent of total OA.

	Alfalfa				Corn			
	Control	Treatment	SEM	*P*	Control	Treatment	SEM	*P*
OA (µmol/g DM)								
Acetic	353.5	284.2	37.6	0.23	119.2	131.0	3.2	0.03
Propionic	1.0	0.9	0.4	0.63	0.6	0.2	0.02	0.92
Isobutyric	1.1	1.9	0.2	0.03	ND	ND	-----	-----
Butyric	0.1	0.2	0.2	0.62	ND	ND	-----	-----
Iso-valeric	0.7	0.9	0.2	0.59	0.6	0.7	0.2	0.51
Valeric	0.7	0.1	0.1	0.006	6.6	7.9	0.5	0.09
Lactic	1680	2368	184	0.02	1117	1585	212	0.14
OA (%Tot OA)								
Acetic	17.63	11.35	2.03	0.06	12.31	8.93	2.34	0.34
Propionic	0.06	0.04	0.02	0.42	0.05	0.04	0.02	0.68
Isobutyric	0.05	0.08	0.01	0.14	ND	ND	-----	-----
Butyric	0.01	0.01	0.01	0.70	ND	ND	-----	-----
Isovaleric	0.02	0.01	0.01	0.32	0.01	0.01	0.003	0.19
Valeric	0.05	0.01	0.01	0.001	0.59	0.37	0.04	0.005
Lactic	82.2	88.5	2.0	0.06	86.8	90.6	2.5	0.34

ND indicates that the level of that acid was not detectable.

Table 3-5: *In situ* dry matter (DM) degradation. Measured in a ruminally cannulated multiparous crossbred cow during the periparturent period, days in milk -4 to + 4. Alfalfa silage was incubated from d -4 through d -2 of calving and corn silage from d +2 to d +4 of calving. Samples were incubated at 4, 12, 24 and 48 h (n = 5/time point).

	Time (h)				*P* - value		
	4	12	24	48	Treatment	Time	Treatment × Time
Alfalfa (%DM)					0.02	<0.0001	0.49
Control	44.2*	52.0	64.8	74.9			
Treatment	46.7*	52.8	66.3	75.2			
SEM	0.8	0.8	0.8	0.8			
Corn (%DM)					0.71	<0.0001	0.29
Control	34.7	40.5	46.2	66.0			
Treatment	35.2	41.9	45.6	63.6			
SEM	1.0	1.1	1.0	1.0			

*indicates a significant different ($P < 0.05$) between treatments for that time point.

Table 3-6: Parameter estimates of *in situ* dry matter degradation (DMD) over 48 h, for alfalfa and corn silage. Analyzed using a non-linear regression of the means of DMD at 4, 12, 24 and 48 h for control and treatment, alfalfa silage by PROC NLMIXED (SAS 9.4).

	Control	Treatment	SEM
Alfalfa			
Maximum(M)	67.5	66.8	1.4
Rate(B)	0.21	0.25	0.03
Corn			
Maximum(M)	56.0	53.5	1.8
Rate(B)	0.18	0.22	0.03

Tables: Experiment 2

Table 4-1: Chemical, microbial and nutrient analyses of mixed cool-season grass, including dry matter (DM), pH, titratable acidity (Tit Acid), neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and ash. Measurements taken after treatment application, prior to ensiling, d 0 (n = 5).

	Control	Treatment	SEM	*P* -Value
DM (%)	52.3	58.4	1.9	0.03
pH	6.10	6.05	0.04	0.42
Tit Acid[a]	18.4	22.5	1.9	0.13
Cultures [b]				
LAB	6.8	7.3	0.2	0.08
Yeast	5.9	6.1	0.2	0.58
Fiber (%DM)				
NDF	66.1	66.2	0.4	0.84
ADF	41.7	41.2	1.3	0.77
Cellulose	34.0	32.6	3.0	0.67
Hemicellulose	24.4	25.0	1.6	0.76
Ash (%DM)	6.9	7.1	0.1	0.26
CP (%DM)[*]	9.2	9.8	----	-----
Ammonia (%DM)[*]	0.1	0.1	----	-----
WSC (%DM)[*]	8.9	10.2	----	-----

[a] Titratable Acidity (Tit Acid) is mmol/g DM of 1N NaOH needed to raise 70 mL of a 1:10 silage solution, to a pH of 6.8.

[b] Cultures are reported as the log10 of the number of CFU/g for yeast cultures and lactic acid bacteria (LAB) cultures, of the silage on d 0

[*]n=1, no SEM

Table 4-2: Chemical and microbial analyses, including dry mater (DM; %), DM change (% unit), microbial analysis (Log_{10} CFU/g) and aerobic stability (h) of mixed cool-season grass haylage on d 90 ± 4 (n = 5).

	Control	Treatment	SEM	*P*-Value
DM (%)	43.8	53.1	2.1	0.006
DM Change (% unit)[a]	8.5	5.3	2.1	0.24
DM loss (%)[b]	24.9	20.9	3.1	0.32
pH	5.2	5.3	0.1	0.45
Tit Acid[c]	104.6	80.6	13.2	0.18
Cultures[d]				
LAB	7.2	7.2	0.1	0.65
Yeast	3.2	3.0	0.3	0.39
Aerobic Stability (h)[e]	124.5	209.5	54.1	0.23

[a] DM change is the difference in DM on d 0 and on d 90

[b] Dry matter (DM) loss (%) indicates the difference between initial DM weight and final DM weight

[c] Titratable Acidity (Tit Acid) is mmol/g DM of 1N NaOH needed to raise 70 mL of a 1:10 silage solution, to a pH of 6.8.

[d] Cultures are reported as the log_{10} of the number of CFU/g of DM of yeast cultures and lactic acid bacteria (LAB) cultures of the silage on d 90

[e] Aerobic stability is reported as the number of hours needed for the temperature of the silage, when exposed to air, to raise 2°C above basal temperature.

Table 4-3: Fiber (% DM), Nutrient content (% of DM) and nutrient difference (% unit), of mixed cool-season grass haylage, d 90 ± 4 (n = 5). Fiber analyses includes neutral detergent fiber (NDF), acid detergent fiber (ADF) and cellulose. Nutrient content includes crude protein (CP), ammonia, and water soluble carbohydrates (WSC).

	Control	Treatment	SEM	*P*-Value
NDF (% DM)	61.7	62.7	0.5	0.12
ADF (% DM)	41.9	40.9	0.3	0.02
Hemicellulose (% DM)	19.8	21.8	0.5	0.007
Cellulose (% DM)	33.6	33.2	0.2	0.14
Ash (% DM)	7.9	8.1	0.3	0.74
Nutrient[a] (%DM)				
CP	12.0	11.4	0.3	0.15
Ammonia	1.4	1.1	0.1	0.03
WSC	4.3	6.0	0.5	0.06
Nutrient Difference[b] (% Unit)				
NDF	-4.4	-3.5	0.5	0.15
ADF	0.2	-0.3	0.3	0.17
Hemicellulose	-4.6	-3.2	0.5	0.03
Cellulose	-0.4	0.6	0.2	0.007
CP	2.8	1.6	0.3	0.01
Ammonia	1.3	1.1	0.1	0.04
WSC	-4.6	-4.3	0.5	0.64

[a] Nutrient content determined by Dairyland Laboratories (Arcadia, WI).

[b] Nutrient difference is the difference between final nutrient content of haylage and initial nutrient content of mixed cool-season grass.

Table 4-4: Organic acid (OA) content of mixed cool-season grass haylage, as a percent of total OA (mol/g DM), d 90 ± 4 (n = 5).

Organic Acid (% Total OA):	Control	Treatment	SEM	*P*- Value
Lactic (LA)	79.3 (40.9)	87.2 (75.2)	6.9(19.9)	0.32
Acetic (AA)	14.6 (6.5)	9.9 (6.7)	4.4 (0.9)	0.38
Propionic	0.5 (0.2)	0.3 (0.2)	0.1 (0.1)	0.22
Isoubutyric	0.3 (0.1)	0.02(0.01)	0.3 (0.1)	0.27
Butyric	4.7 (2.0)	2.2 (1.5)	1.9 (0.5)	0.23
Isovaleric	0.08(0.03)	0.03(0.02)	0.05(0.01)	0.37
Valeric	0.4 (0.2)	0.3 (0.2)	0.2 (0.04)	0.69
LA:AA ratio	7.2	11.4	3.1	0.25

Table 4-5: *In situ* (*IS*) Dry matter degradation (DMD; %,), neutral detergent fiber degradation (NDFD; %), acid detergent fiber degradation (ADFD; %), crude protein degradation (CPD; %) organic matter degradation (OMD; %) and hemicellulose degradation (HCD; %) of mixed cool-season grass haylage, measured in two ruminally cannulated primiparous Holstein cows, at 12, 24, 36 and 48 h incubation (n = 5/time point/animal), for both treatments of grass haylage, d 90 ± 4. Except CPD was measured at 24 and 48 h (n = 3/time point/animal).

	Time (h)				*P*- value		
	12	24	36	48	Treatment	Time	Treatment × time
*IS*DMD					0.71	<0.0001	0.67
Control	43.1	51.3	54.5	55.0			
Treatment	42.4	51.9	55.5	55.4			
SEM	1.5	1.5	1.5	1.5			
*IS*NDFD					0.05	<0.0001	0.32
Control	20.3	30.2‡	36.0	36.8			
Treatment	19.6	33.9‡	38.7	39.5			
SEM	2.4	2.4	2.4	2.4			
*IS*ADFD					0.04	<0.0001	0.12
Control	9.2*	21.8‡	27.8	25.6‡			
Treatment	20.6*	30.9‡	25.4	34.0‡			
SEM	3.6	3.6	3.6	3.4			
*IS*CPD					0.29	0.73	0.18
Control		46.4		48.7			
Treatment		46.6		42.9			
SEM		2.4		2.8			
*IS*OMD					0.005	<0.0001	0.29
Control	38.7	46.8*	50.1‡	51.4‡			
Treatment	38.6	49.6*	52.6‡	53.8‡			
SEM	2.0	2.0	2.0	2.0			
*IS*HCD					0.87	<0.0001	0.04
Control	40.2	44.7	49.9‡	57.0			
Treatment	24.8	42.9	66.9‡	52.7			
SEM	6.7	6.3	6.2	6.0			

* indicates a difference ($P < 0.05$) between treatment for that time point

‡ indicates a tendency ($p < 0.10$) towards difference between treatments for that time point

Means are adjusted for DM, NDF, ADF, CP, Hemicellulose and OM respectively

Table 4-6: Parameter estimates of *in situ* (*IS*) degradation over 48 h. Analyzed using a non-linear regression of the means of *IS* dry matter degradation (*IS*DMD), *IS* neutral detergent fiber degradation (*IS*NDFD), *IS* acid detergent fiber degradation (*IS*ADFD), *IS* hemicellulose degradation (*IS*HCD), and *IS* organic matter degradation (*IS*OMD) of mixed cool-season grass haylage at 12, 24, 36 and 48 h for control and treatment.

	Control	Treatment	SEM
*IS*DMD			
Maximum(M)	594.6	56.0	0.5
Rate (B)	0.13	0.12	0.01
*IS*NDFD			
Maximum (M)	39.2	43.8	2.0
Rate (B)	0.06	0.06	0.01
*IS*ADFD			
Maximum (M)	33.4	30.0	5.5
Rate (B)	0.04	0.09	0.03
*IS*HCD			
Maximum (M)	52.8	66.7	8.8
Rate (B)	0.10	0.05	0.03
*IS*OMD			
Maximum (M)	51.3	53.7	0.8
Rate (B)	0.11	0.11	0.01

Figures: Experiment 1

Figure 3-1: Rate of pH decline of alfalfa silage for control (solid line with squares) and treatment (dashed line with circles), d 1 -13(n = 3). Parameters and contrasts provided by a non-linear analysis of means of pH as measured on d 1, 2, 3 and 13.

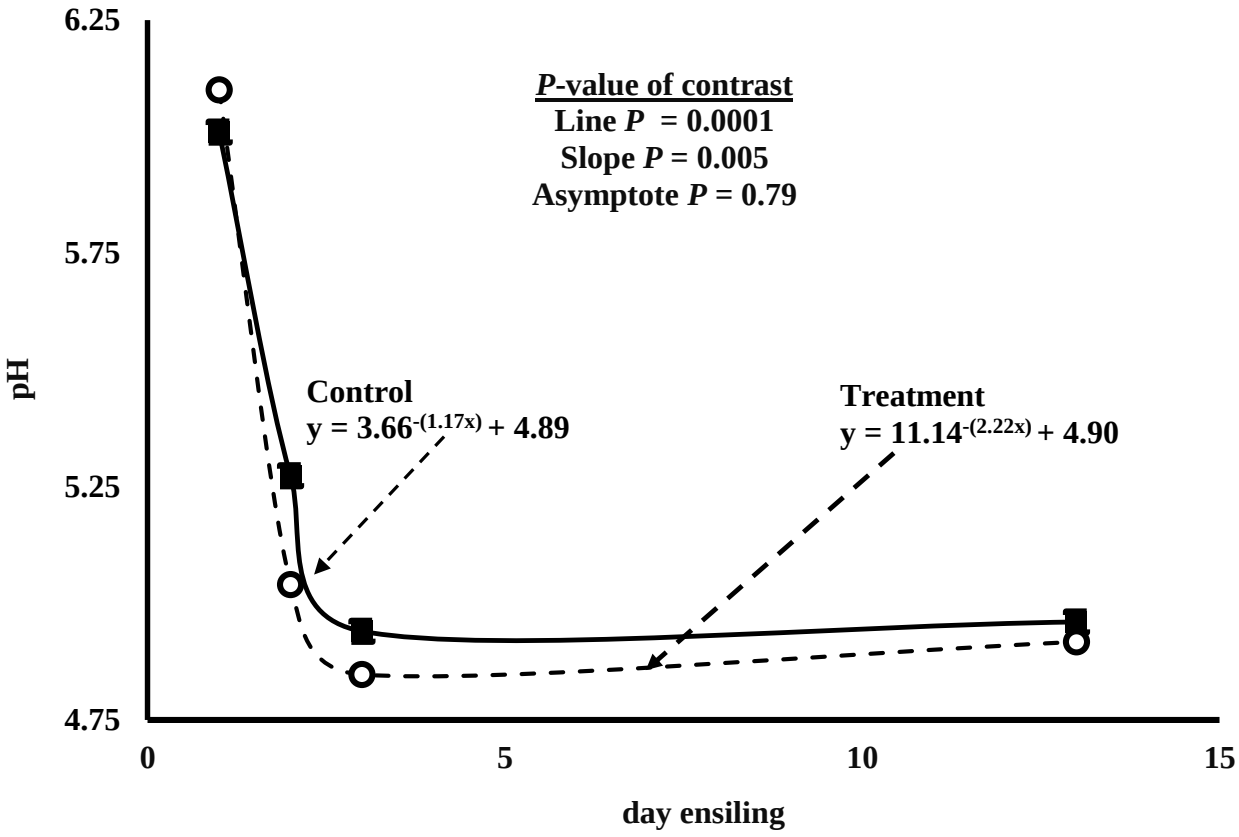


Figure 3-2: Rate of pH decline in corn silage for control (solid line with squares) and treatment (dashed line with circle), d 1-17 (n = 3). Parameters, and contrasts, provided by a non-linear regression analysis of means of pH on d 1, 2, 3, 7 and 17.

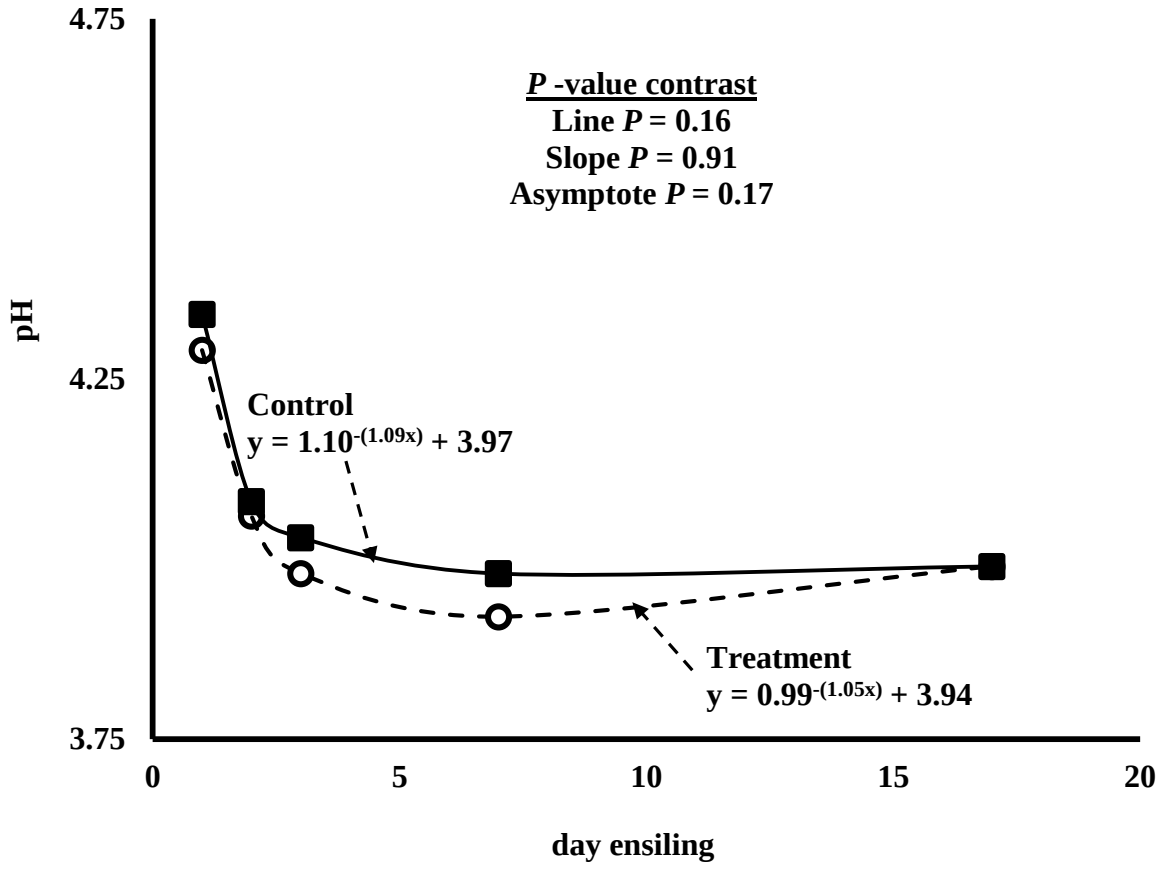
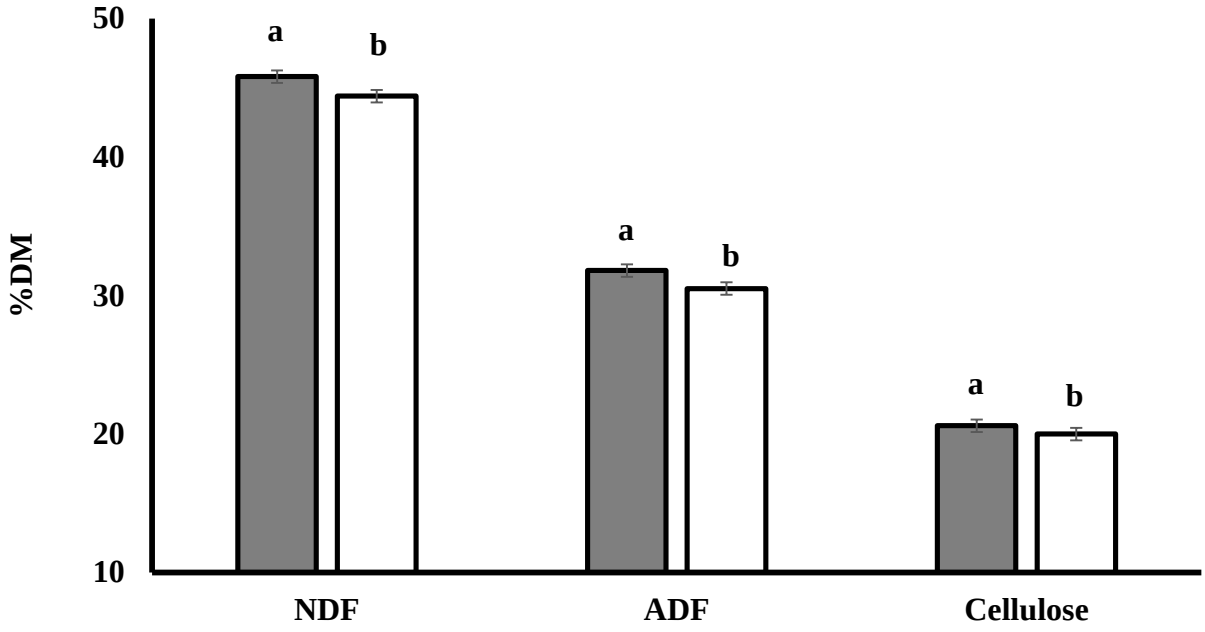
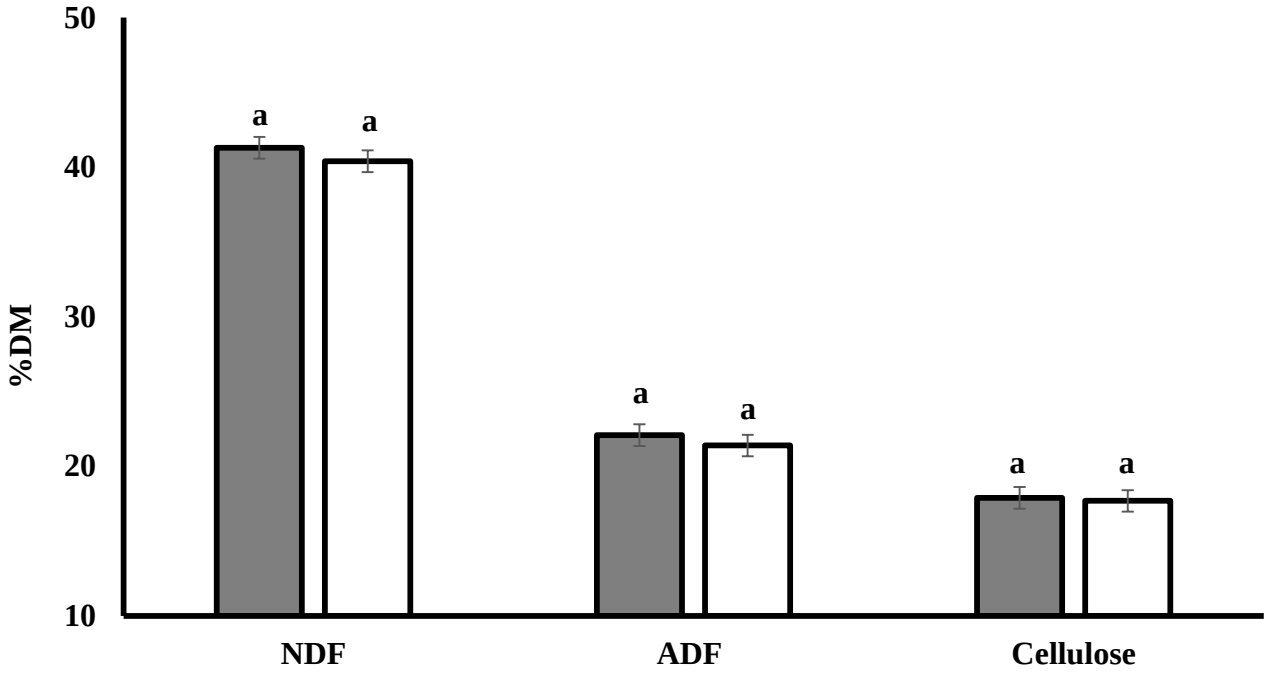


Figure 3-3: Neutral detergent fiber (NDF), acid detergent fiber (ADF) and cellulose for both control (gray bar) and treatments (open bar), of Alfalfa silage (% DM), as measured in bucket silos (n = 5), on d 59 *.



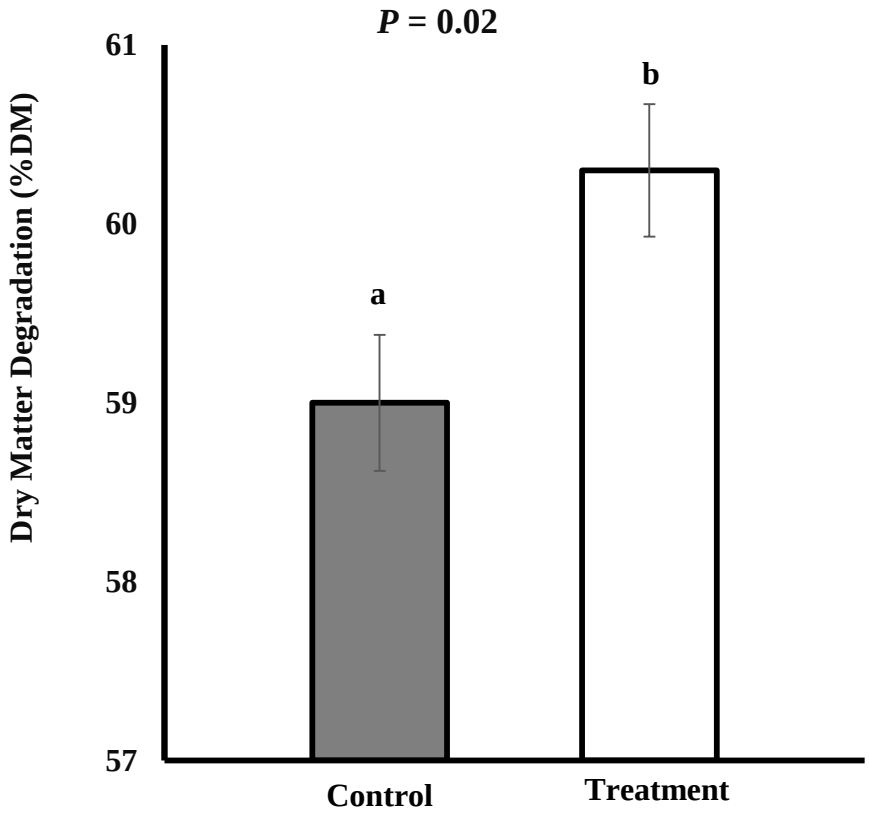
*Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 3-4: Neutral detergent fiber (NDF), acid detergent fiber (ADF) and cellulose, of control (gray bar) and treatment (open bar), of corn silage (%DM), as measured in bucket silos (n = 5), on d 59.*



*Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 3-5: *In situ* dry matter degradation of alfalfa silage for control (gray bar) and treatment (open bar), over 48 h (n = 5/time point) incubation in a ruminally cannulated multiparous crossbred cow.*



*Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 3-6: Rate of *in situ* dry matter degradation, alfalfa silage for control (solid line with squares) and treatment (dashed line with circles), measured over 48 h (n = 5/time point) incubation in a ruminally cannulated multiparous crossbred cow.

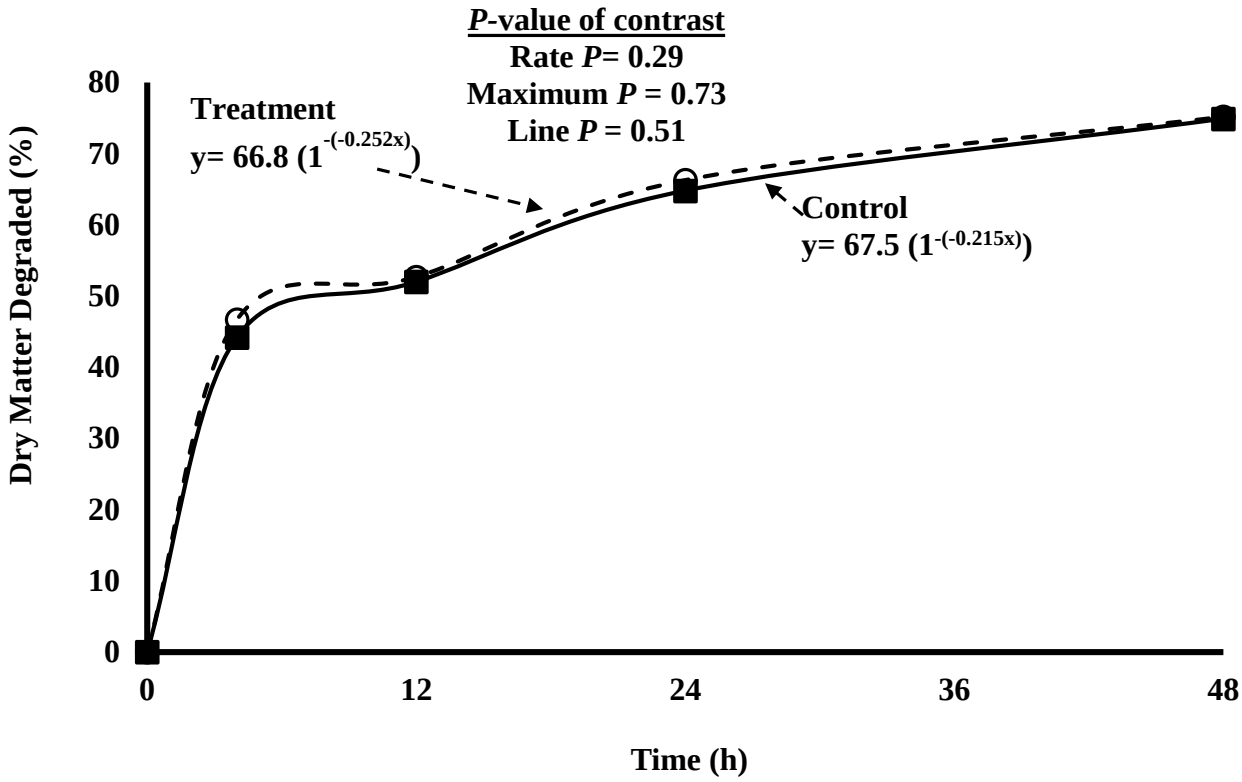
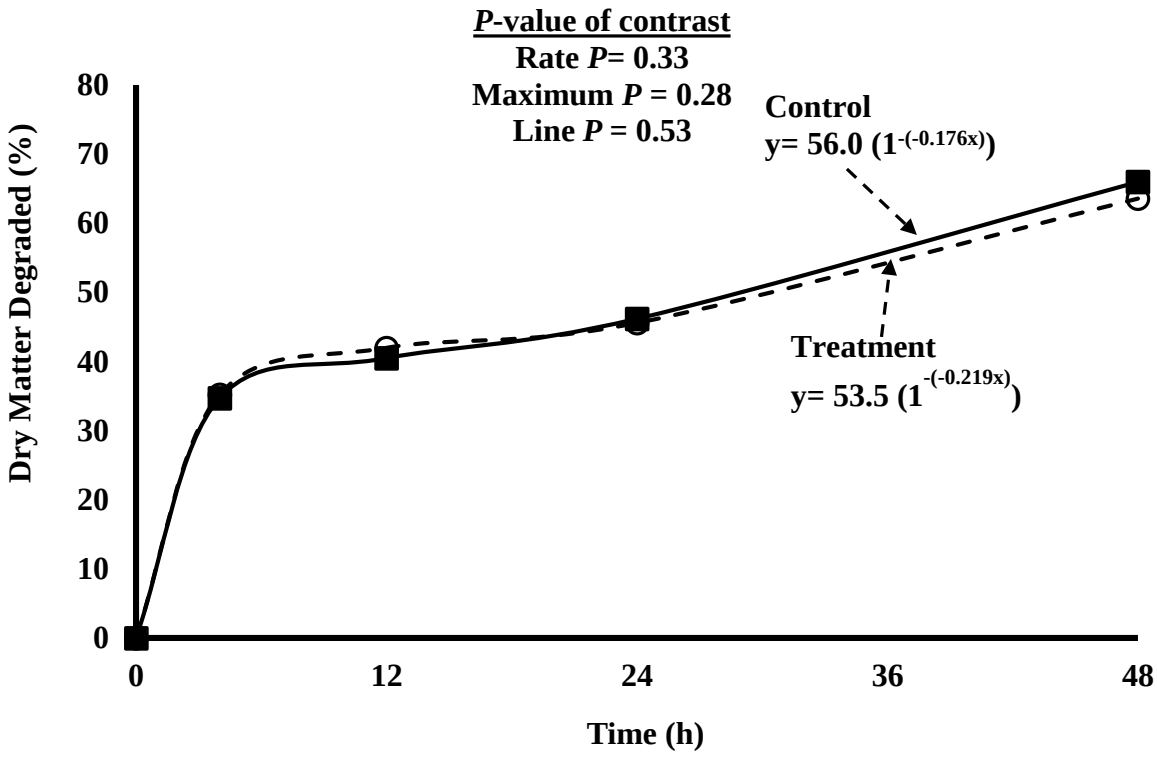


Figure 3-7: Rate of *in situ* dry matter degradation, corn silage for control (solid line with squares) and treatment (dashed line with circles), measured over 48 h (n = 5/time point) incubation in a ruminally cannulated multiparous Angus mixed cow.



Figures: Experiment 2

Figure 4-1: Dry matter loss (%) of mixed cool-season grass haylage for control (gray bar) and treatment (open bar) on d 90 ± 4 (n = 5).

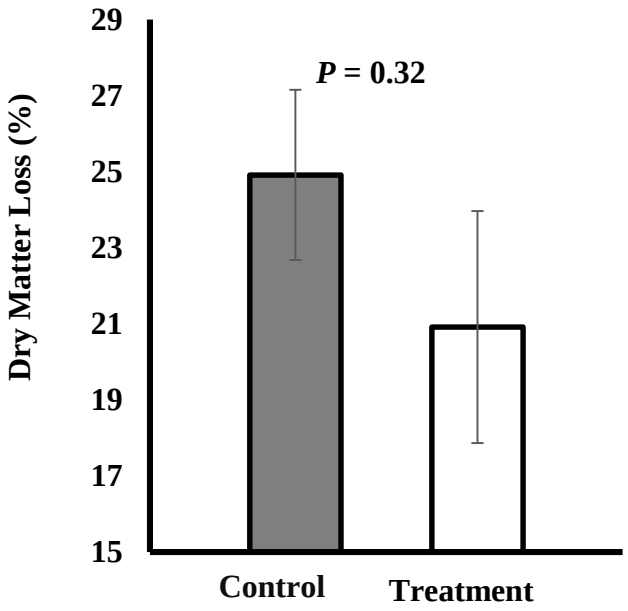


Figure 4-2: Aerobic stability readings of mixed cool-season grass haylage for each day of sampling (n =1 per time point). Aerobic stability is measured in h to reach 2°C above baseline, for d86, 88, 90, 92, 94 for control (solid line with squares) and treatment (dashed line with circles).

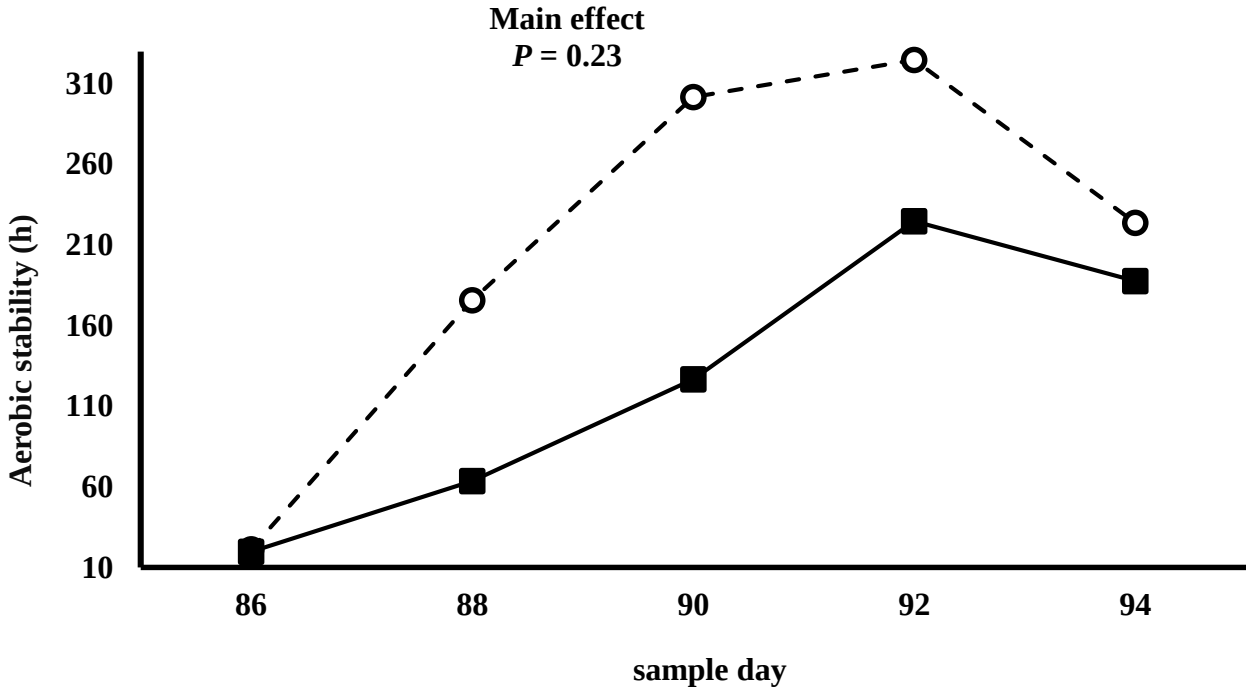


Figure 4-3: Lactic acid concentration (µmol/g DM) of mixed cool-season grass haylage for control (gray bar) and treatment (open bar) on d 90 ± 4 (n = 5).

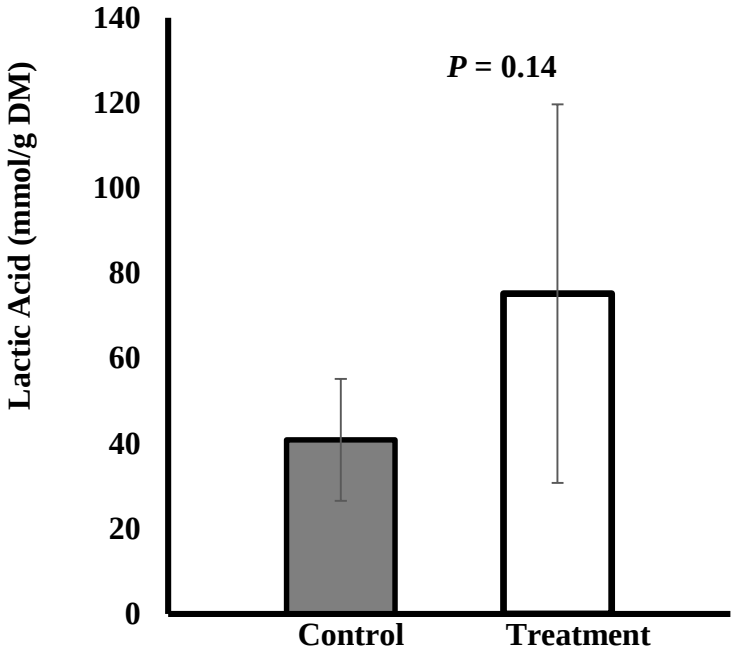
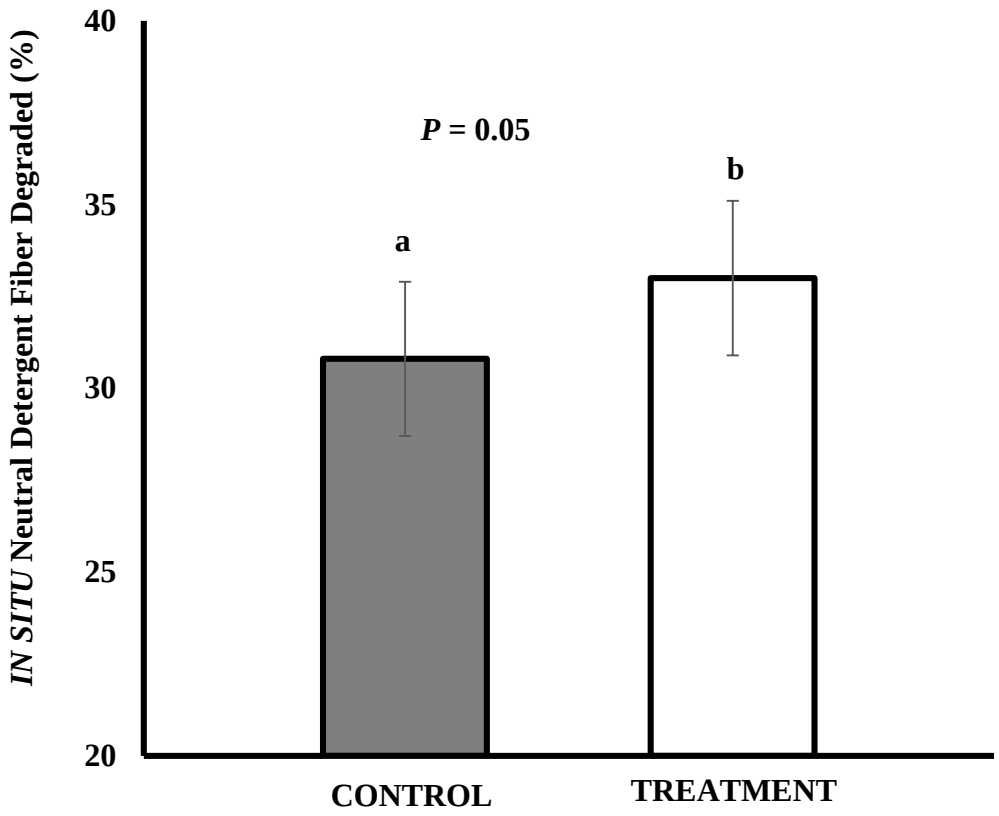
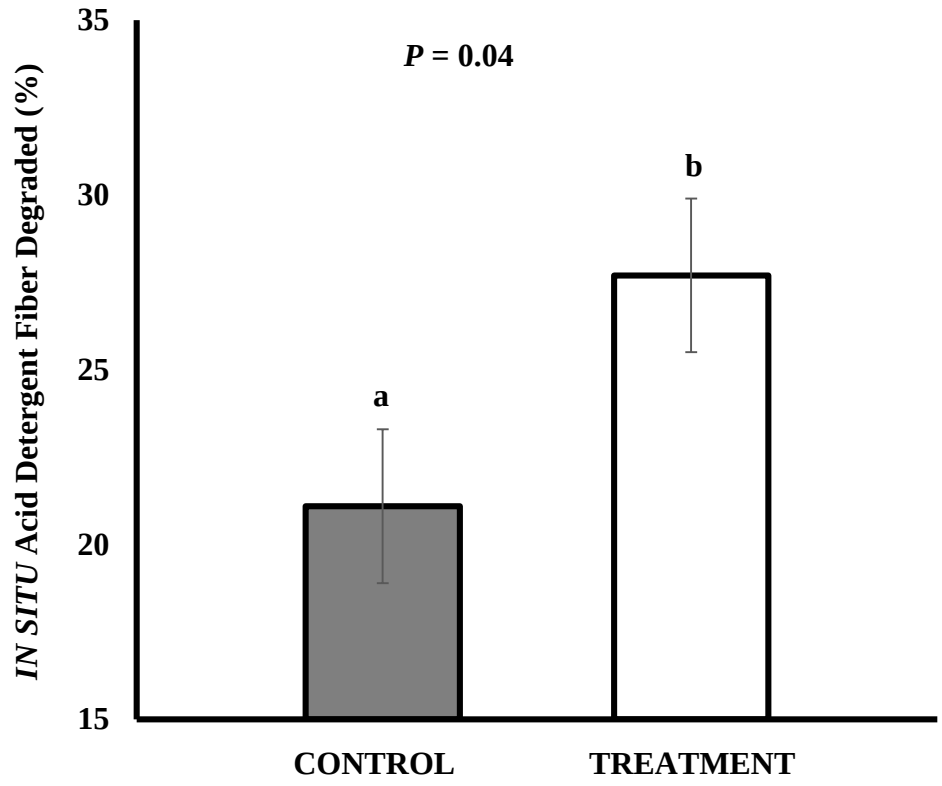


Figure 4-4: *In situ* neutral detergent fiber (NDF) degradation (percent degraded) of mixed cool-season grass haylage of control (gray bar) and treatment (open bar) on d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).*



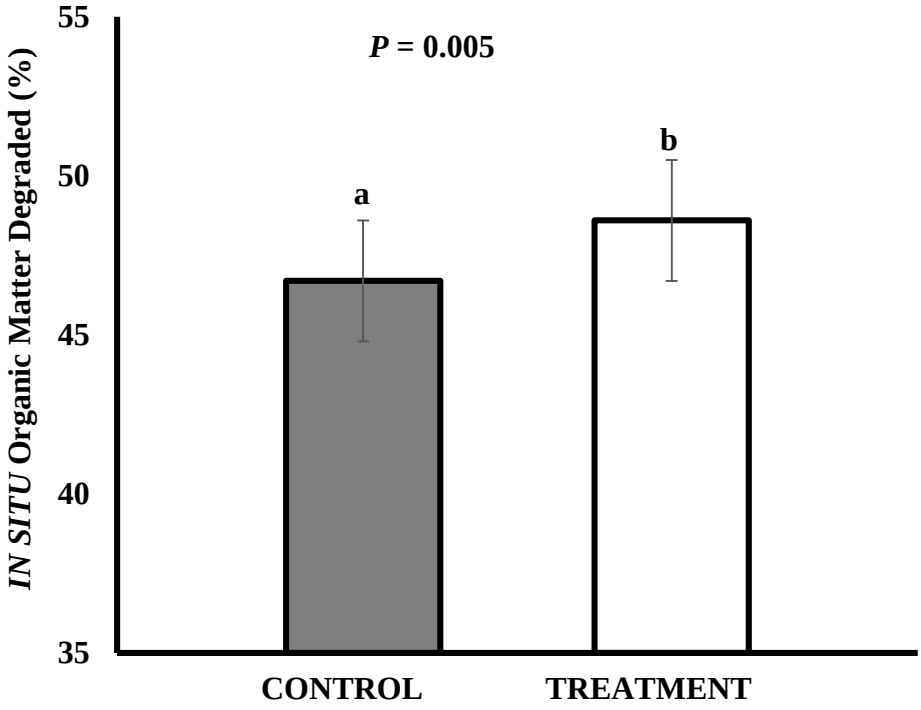
*Means adjusted for the covariant of NDF. Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 4-5: *In situ* acid detergent fiber (ADF) degradation (percent degraded) of mixed cool-season grass haylage of control (gray bar) and treatment (open bar) on d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).*



*Means adjusted for the covariant ADF. Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 4-6: *In situ* organic matter (OM) degradation (percent degraded) of mixed cool-season grass haylage of control (gray bar) and treatment (open bar) on d 90 ± 4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).*



*Means adjusted for the covariant OM. Bars with different superscript letters indicate significant difference ($P < 0.05$).

Figure 4-7: Rate of *in situ* dry matter degradation of mixed cool-season grass haylage for control (solid line with squares) and treatment (dashed line with circles) on d 90 ± 4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).

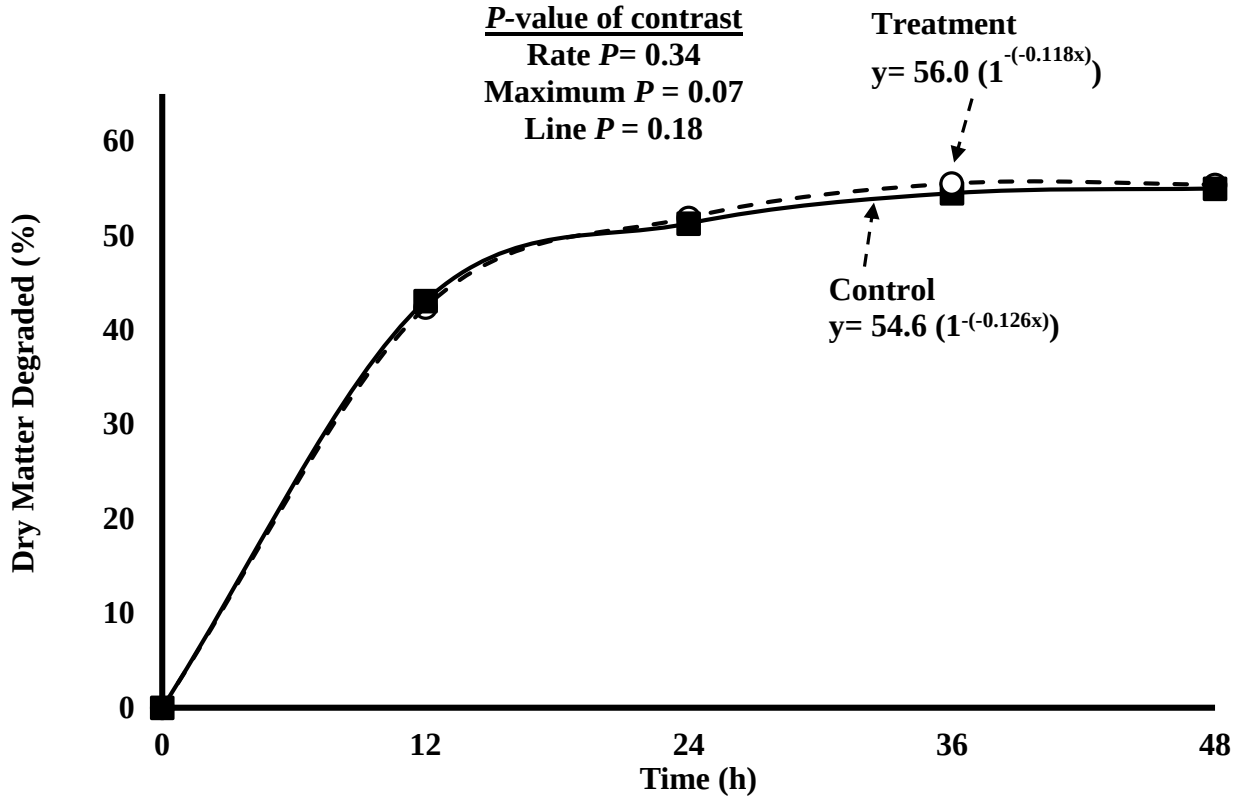


Figure 4-8: Rate of *in situ* neutral detergent fiber degradation of mixed cool-season grass haylage for control (solid line with squares) and treatment (dashed line with circles) on d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).

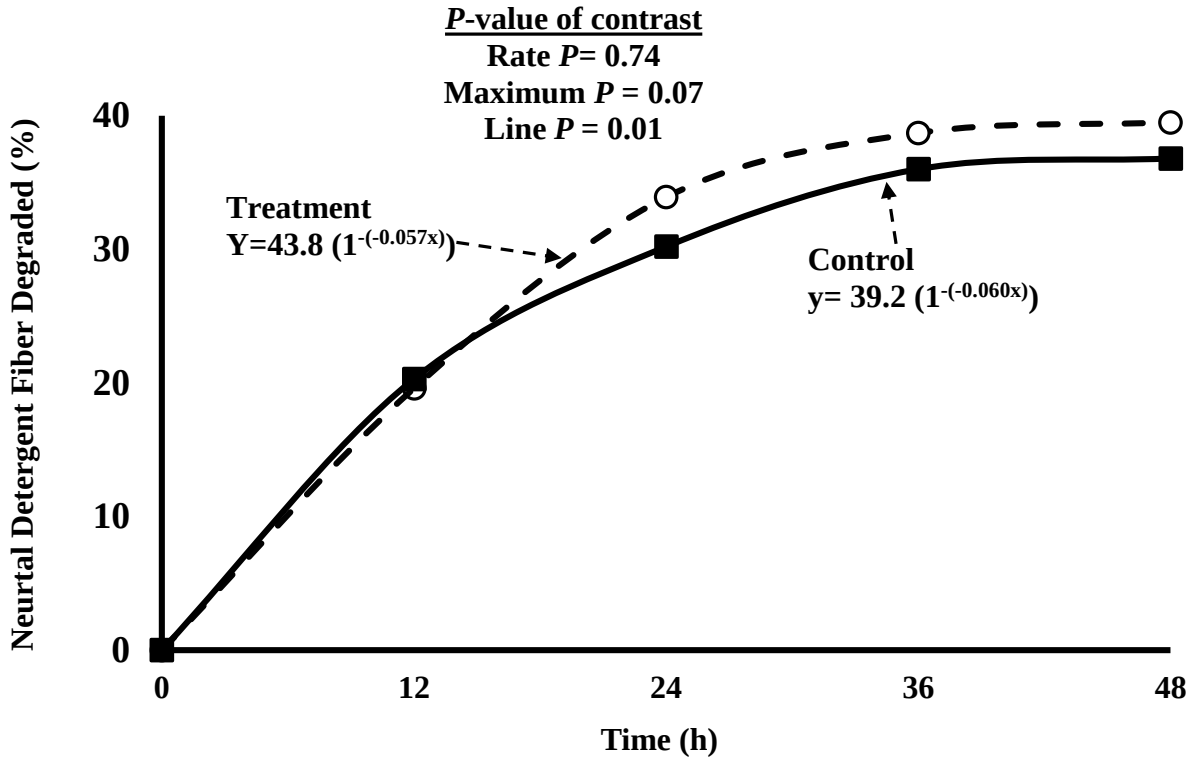


Figure 4-9: Rate of *in situ* acid detergent fiber degradation of mixed cool-season grass haylage for control (solid line with squares) and treatment (dashed line with circles), d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).

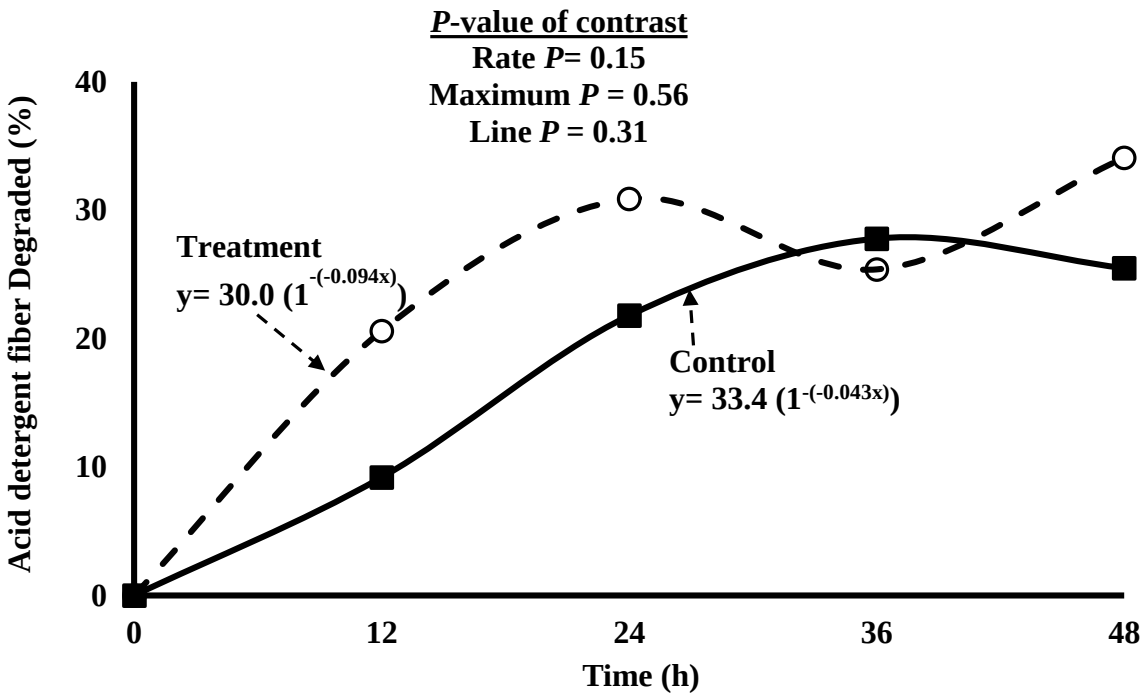


Figure 4-10: Rate of *in situ* Hemicellulose degradation of mixed cool-season grass haylage for control (solid line with squares) and treatment (dashed line with circles) on d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).

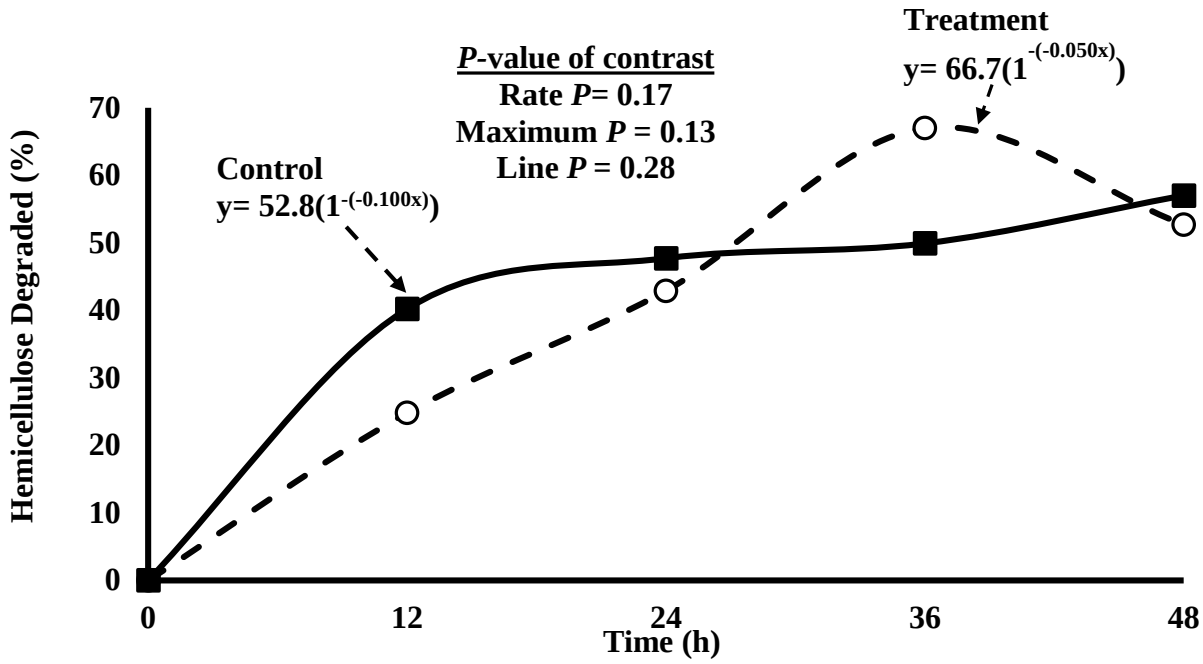
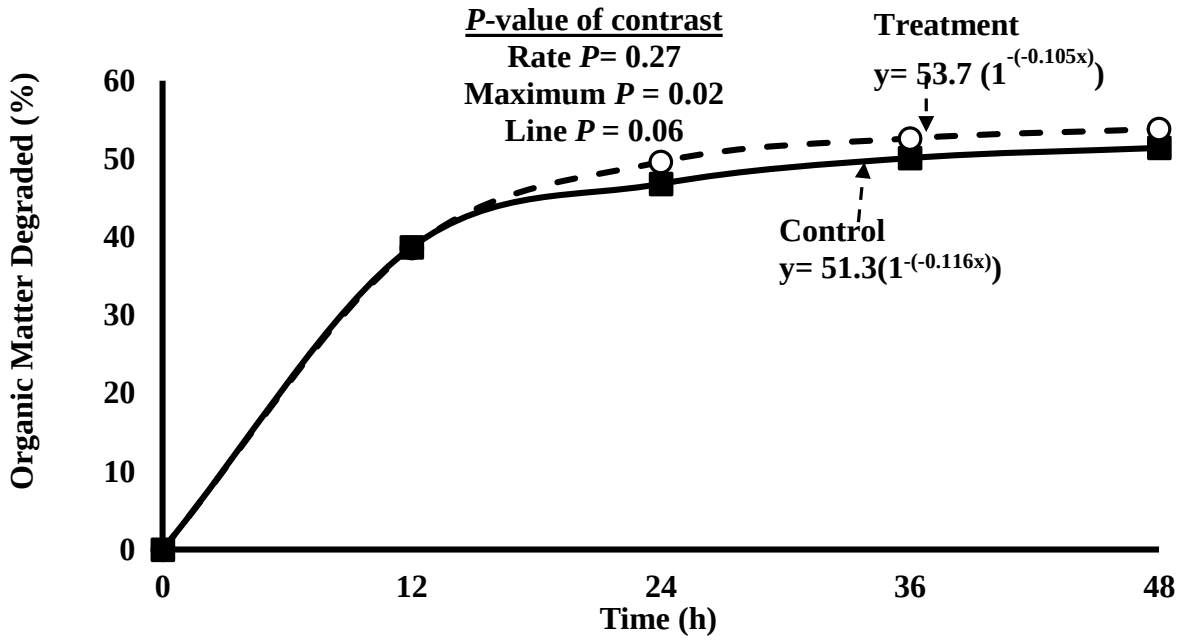


Figure 4-11: Rate of *in situ* organic matter degradation of mixed cool-season grass haylage for control (solid line with squares) and treatment (dashed line with circles), d 90 ±4, measured in two ruminally cannulated primiparous Holstein cows over 48 h (n = 5/time point).



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Appendix A: IACUC Approval

From: Institutional Animal Care and Use Committee (iacuc@uidaho.edu)
Sent: Monday, October 20, 2014 12:12 PM
To: Rezamand, Pedram (rezamand@uidaho.edu)
Subject: IACUC Approval of 2014-2 Amendment

University of Idaho
Institutional Animal Care and Use Committee

Date: Monday, October 20, 2014

To: Pedram Rezamand

From: University of Idaho
Institutional Animal Care and Use Committee

Re: Protocol 2014-2
Studying the effects of Sil-Al^{4x4}, an inoculant used to aid in ensiling on ruminal digestibility of silage

Your requested amendment to the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Monday, October 20, 2014.

This protocol was originally submitted for review on: Monday, January 27, 2014
The original approval date for this protocol is: Tuesday, February 18, 2014
This approval will remain in effect until: Tuesday, October 20, 2015
The protocol may be continued by annual updates until: Saturday, February 18, 2017

Comments

This amendment increased the number of animals from 1 to 2 and added surgical procedures.

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Appendix B: OSP Approval

This message was sent with high importance.

Rezamand, Pedram:

Your proposal entitled "The Effects of Combination of Lactic Acid-producing Bacteria and Hydrolytic Enzyme Inoculants on Ensiling Characteristics of Mixed Cool Season Grasses" has been signed by the Office of Sponsored Programs. If this is a manual submission, you may now submit your proposal to the Sponsor unless this is a commodity commission or other type of proposal that should go to your college for submission as part of a package. Please contact your college if you have question on the method of submission. If this is an electronic submission through a sponsor site (e.g. Grants.gov, etc.), your proposal will be submitted by OSP. If there are any documents that require signature, you may pick them up from Morrill Hall Room 103. If you have any questions, please contact Bilderback, Ann-Marie (Email: abilderback@uidaho.edu, Phone: 208-885-5154).

Customer service is very important to us. Please click on the link below to complete a short survey on your pre-award experience
https://docs.google.com/forms/d/1EqgS2aAPVTSbI8Z8vrQUyY7cIJz7JZUXBeVDbR_llAY/viewform

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