Fall 12-20-2020

Evaluating Lignosulfonates Potential as Legume Hay and Silage Preservatives

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EVALUATING LIGNOSULFONATES POTENTIAL AS LEGUME HAY AND SILAGE PRESERVATIVES

By

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M. Sc. Universidad Nacional Agraria La Molina, 2015

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Animal Science)

The Graduate School

The University of Maine

December 2020

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An Abstract of the Thesis Presented
In Partial Fulfillment of the Requirements for the
Degree of Master of Science
(in Animal Science)
December 2020

The aim was to screen and optimize low-cost lignosulfonates (LST) as legume silage and hay preservatives to decrease losses of DM and nutritive value due to spoilage. In experiment 1, we evaluated the effects of untreated silage (0%), sodium lignosulfonate (NaL) and magnesium lignosulfonate (MgL) applied independently at 0.5, 1, and 1.5 (% w/w, fresh basis) and INO (*Pediococcus pentosaceus* and *Lactobacillus plantarum*; 5 and 4 log cfu/fresh alfalfa g, on high moisture alfalfa (*Medicago sativa* L.) silage nutrient preservation. Data were analyzed as a randomized complete block design (RCBD; 5 blocks) and linear and quadratic polynomial contrasts were used to determine dose rate effects for NaL and MgL and orthogonal contrasts for INO effects. At opening (d 229), both MgL and INO increased DM loss (~13.7 vs 11.3% of DM) due to a lower production of lactic acid (~7.55 and 7.83 vs 9.23% of DM, respectively) which resulted in a higher pH relative to untreated silage (~4.41 and 4.46 vs 4.33; respectively). The high acidification in untreated silage resulted in additives not reducing further the proteolysis that occurred relative to control, measured as NH_3-N (~11% of N). Overall, all additives tested failed to improve the preservation of high moisture alfalfa

silage nutrients. In experiment 2A, we determined the minimum inhibitory (MIC) and minimum fungicidal concentration (MFC) of 4 sodium lignosulfonates [Sappi (NaSP), Sigma-Aldrich (NaAl), Beantown (NaBT), and Spectrum (NaUM)], 1 magnesium lignosulfonate [Sappi (MgSP)], 2 chitosan sources [naive (ChNv) and microparticles (ChMp)], and propionic acid (PRP; positive control) against 3 molds and 1 yeast isolated from spoiled alfalfa hay. Our results showed that both chitosans had the strongest fungicidal activity against all the fungi tested with exception of *M. circinelloides* at both pH 4 and 6. Among lignosulfonates, we found that NaSp was the most antifungal and was further optimized to produce LST. However, none of the lignosulfonates inhibited the molds or yeast at pH 6. Across additives, PRP inhibited all fungal strains at both pH levels. In experiment 2B, we used a factorial combination of three preservatives (LST, ChNv, and PRP) and 5 concentrations (0, 0.25, 0.5, 1, and 2% w/w fresh basis) to determine the effects of their application on the preservation of nutrients in high moisture alfalfa hay. Data were analyzed as a RCBD replicated five times. After 23 d of aerobic storage, LST and PRP prevented DM losses to the same extent with doses as low as 0.25% compared with the untreated hay (~1.61 vs 24.0%). This was explained by reduced mold counts for as low as 1% LST (< 2.0) and as low as 0.5% PRP (< 2.0) compared with untreated hay (6.76 log cfu/fresh g). However, ChNv did not affect DM loss or molds count (~23.2% and 6.59 log cfu/fresh g, respectively). Also, DM digestibility was increased for at least 0.25% LST (71.1) and 1% PRP (71.4) compared with untreated hay (69.3%). As a consequence, both LST and PRP increased total VFA with doses as low as 0.25% compared with the untreated hay (93.6 and 95.1 vs 83.3 mM, respectively). In summary lignosulfonates initially tested did not improve the

preservation of nutrients in high moisture legume silage but an optimized lignosulfonate showed promise as a low-cost preservative for high moisture legume hay.

DEDICATION

To my dear family

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratitude to my committee supervisor, Dr. Romero for his continuous guidance, support and immense knowledge. He has been a mentor and guide for the entirety of this research. To the members of my committee, Drs. Seanna Annis, Anne Lichtenwalner, Brian Perkins, and Denise Skonberg, I thank them for their advice, guidance, and encouragement during the last two years.

My sincere thanks to Saulo Texeira, Bianca Guimaraes and Marjorie Killerby for their deep commitment and exceptional help during all my experiments. To Diana Reyes and Santiago Rivera for their valuable involvement in my first experiment. I would also particularly want to thank Zhengxing Ma for her special guidance about microbiological techniques as well as her support during these two years. To Alexandria Garcia, Rachel Hollandworth, Kianna Dean, Anna Robinson, Cassandra Marshall, and Godloves Mills for always willing to get involved with my experiments.

Finally, I am very grateful to Russ and Sharon deGarybody for their constant support and help during these two years. To Simona Lukasik for being my friendly roommate, and for the memorable time we spent together. Lastly, but by no means less important, I would like to thank my parents Angel and Martha and sisters Yesica and Veronica for their unconditional love and support during all my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADIN	Acid detergent insoluble nitrogen
ADICP	Acid detergent insoluble crude protein
ADV	Additive
BA	Biogenic amines
CFU	Colony forming units
ChNv	Chitosan naïve
ChMp	Chitosan microparticles
CNCPS	Cornell Net Carbohydrate and Protein System
CON	Control
CP	Crude protein
d	Day
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
INO	Microbial inoculant
IVDMD	*In vitro* dry matter digestibility
LAB	Lactic acid bacteria
LST	Optimized lignosulfonate-based product
MgL	Magnesium lignosulfonate
MIC	Minimum inhibitory concentration
NaL	Sodium lignosulfonate

NASS	National Agricultural Statistics Service
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber digestibility
NH_3-N	Ammonia nitrogen
NPN	Non-protein nitrogen
MFC	Minimum fungicidal concentration
PRP	Propionic acid
RCBD	Randomized complete block design
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
TVFA	Total volatile fatty acids
VFA	Volatile fatty acid
WSC	Water soluble carbohydrates

CHAPTER 1

INTRODUCTION

According to NASS (2020a), alfalfa (*Medicago sativa*) is predominantly conserved as hay (117 million Mg per year) and to a lesser extent as haylage (15 million Mg per year) in the U.S. (NASS, 2020a). However, producing hay is challenging in regions with high humidity and frequent rain (Han et al., 2014). Because of this and recent advances in silage technology, total hay production in the U.S. has declined by 1.2 million Mg per year from 2000 to 2019 (NASS, 2004;2020a). In contrast, total silage production increased in the U.S. from 2000 to 2019 by 1.6 million Mg per year (NASS, 2004;2020a). Ensiling is becoming more common, particularly in areas with frequent precipitation (Albretch and Bearchemin, 2003) since this method requires less wilting time compared to hay (Han et al., 2014) and it consequently decreases harvest loss (Mahanna and Chase, 2003).

However, adequate nutrient preservation in legume silages is challenging because of their high buffering capacity, compounded with low sugar concentrations that limit the lactic acid production necessary for a rapid and extensive acidification (Liu et al., 2016). Consequently, microbes such as clostridia and enterobacteria are more likely to cause extensive spoilage in legume silages (Muck and Kung, 2007), due to slow acidification (Pahlow et al., 2003). Furthermore, slow acidification also results in plant enzymes being active for longer, which can result in extensive protein breakdown into non-protein N (**NPN**) until they are finally inactivated by low pH (Heron et al., 1989;Pichard et al., 2006). Therefore, high protein losses can be expected in legume silages and it has been estimated that between 44 - 87% of the forage protein can be

degraded to NPN such as peptides, free amino acids, and amides (Sullivan and Hatfield, 2006).

Due to climate change effects, farmers increasingly have to face unpredictable precipitation patterns (Walker and Vendramini, 2018) that force them to bale hay at moisture levels above those recommended for proper storage (>15-20%), which often results in an increased activity of undesirable microbes during hay storage (Rotz and Shinners, 2007). The subsequent spoilage results in nutrient losses (Turner et al., 2002), potential production of mycotoxins (Raymond et al., 2000), and spontaneous heating as a result of nutrient respiration (Coblentz and Bertram, 2012). Heating in turn will cause the Maillard reaction to occur, increasing the acid detergent insoluble crude protein concentration in hay (ADICP; (Maeda, 1993;Coblentz et al., 1997) which ultimately reduces ruminal protein degradation, microbial protein synthesis, and milk production (Broderick et al., 1993). Evidently, there is a need to improve our understanding of haymaking and ensiling so novel technologies can be developed to improve our efficacy to preserve legume hay and silages, especially when producers are forced to store them under non-ideal conditions.

CHAPTER 2

LITERATURE REVIEW

Forage Protein Composition

Crude protein (CP) analysis is an inadequate methodology to describe protein quality in forages, especially for silage and hay (Cherney, 2000).The chemical and physical properties of proteins affect the degree of susceptibility to hydrolysis by microbial and animal proteases and consequently their degradation rate in the digestive tract of ruminants (Nolan and Dobos, 2005). Thus, a diversity of feed protein fractions exist that vary according to their degradation rate in the rumen and which would enter the rumen degradable (RDP) or undegradable (RUP) pool depending also on their ruminal rate of passage, when applicable. From a pragmatic point of view, techniques used to measure such fractions should be based on intrinsic feed properties, like solubility (Licitra et al., 1996), and not be dependent on laborious microbial and animal techniques, which may not be practical for routine feed analysis (Chrenková et al., 2014). Current animal nutrition models meant to predict requirements, feed utilization, performance, and nutrient excretion (Van Amburgh et al., 2019), depend on uniform procedures to fractionate feed proteins routinely in feed analysis labs (Licitra et al., 1996). The Cornell Net Carbohydrate and Protein System (**CNCPS**) is one of the most used models in dairy, beef, and sheep nutrition. It classifies feed protein fractions according to their ruminal degradation and passage rates to predict RDP supply and RUP flows and estimate animal requirements (Higgs et al., 2015). In the most recent update of CNCPS, Van Amburgh et al. (2015) and Higgs et al. (2015) classified feed protein fractions as follows: Fraction PA1, ammonia-N (**NH_3-N**); PA2, soluble true

proteins (small peptides, aminoacids, globulins, and some albumins) that are rapidly degraded in the rumen; PB1, insoluble true proteins not associated with neutral detergent fiber (**NDF**) that are moderately degradable; PB2, slowly degradable protein, bound in NDF [Neutral detergent insoluble CP (**NDICP**) – acid detergent insoluble CP (**ADICP**)]; and PC (ADICP), which is completely indigestible in the rumen. However, in order to interpret publications preceding the use of the abovementioned system, it is necessary to examine the previous CNCPS classification terminology (Sniffen et al., 1992) which consisted of fraction A (non-protein N, **NPN**); B1 (true soluble protein, rapidly degradable), B2 (Neutral detergent soluble protein; intermediately degradable), and B3 [slowly degradable, bound in NDF (NDICP-ADICP)]; and fraction C (ADICP, indigestible).

The relative proportion of forage protein fractions is affected by plant genetics (Grabber, 2009), field conditions (Mallarino and Wedin, 1990), harvest and conservation methods (Guo et al. 2008), among other factors. It is widely known that ensiling increases the concentration of fraction A several fold due to microbial fermentation (Pichard et al., 2006). For instance, Guo et al. (2008) reported that wilting to 33% DM and subsequently ensiling for 35 d increased fraction A from 15.0 to 68.4%, and decreased fraction B1 from 57.0 to 1.46% but did not affect fraction B2 (~14.1%), fraction B3 (~2.6%) or fraction C (~12.4% of CP) compared with fresh alfalfa. The same authors also found that haymaking (undisclosed DM %) decreased fraction B1 to 3.74%, while it increased fractions B2 from 13.5 to 41.1%, fraction B3 from 1.95 to 15.4%, and fraction A to 28.7% total N, but did not affect fraction C (~11.8% of CP) compared with fresh alfalfa. Likewise, Hristov and Sandev (1998) reported that alfalfa

silage had more NPN, NH_3-N, and free amino acids than alfalfa hay (61.9 vs 20.6, 10.9 vs 0.8, 44.4 vs 5.9% of total N, respectively).

Limited data exists in terms of the amino acid composition of alfalfa and how it is impacted by ensiling or haymaking. The amino acid profile of alfalfa leaf peptides was assessed by Xie et al. (2008) and is presented in (Table 2-1). Guo et al (2008) reported the concentration of isoleucine (1.12 vs. 1.07 and 0.92% of DM, respectively) and aspartic acid (3.12 vs. 2.98 and 2.91) were higher in alfalfa silage relative to hay orfresh alfalfa. Conversely, hay had higher levels of arginine (1.26 vs 0.19 and 0.98% of DM, respectively) and leucine (2.00 vs 1.81 and 1.63% of DM, respectively) among other amino acids relative to silage and fresh alfalfa.

Table 2-1. Amino acid composition of alfalfa leaf peptides (Adapted from Xie et al., 2008)

Amino acid	Amount (g)	Amino acid	Amount (g)
Glutamic acid	11.8	Glycine	4.81
Aspartic acid	8.98	Tyrosine	4.14
Leucine	7.95	Threonine	3.9
Arginine	6.25	Proline	3.82
Lysine	5.99	Serine	3.67
Valine	5.76	Tryptophan	2.88
Alanine	5.51	Histidine	2.61
Phenylalanine	5.39	Methionine	1.63
Isoleucine	4.94	Cysteine	1.53
Total amount of amino acids (g/100 g of alfalfa leaf peptide)			91.56

Plant species also affect the profile of protein fractions. Grabber (2009) reported that fresh alfalfa had the highest proportion of fraction A, followed by fresh birdsfoot trefoil (*Lotus corniculatus*) and red clover (*Trifolium pratense*; 28.8, 24.2, and 18.2% of CP, respectively); only modest differences were reported for fraction B1 across forages evaluated; NDICP was lower in alfalfa and birdsfoot trefoil than in red clover (~10.3 vs 16.6 % of CP, respectively); and ADICP was slightly higher in birdsfoot trefoil vs. the other forages (3.5 vs ~2.9% of CP). Consequently, calculated RUP was highest in red clover, followed by birdsfoot trefoil and alfalfa (32.7, 28.1, and 25.6%, respectively) (Grabber, 2009).

Plant Protein Degradation

Proteolysis results from the activity of proteases that hydrolyze peptide bonds in proteins releasing polypeptides, oligopeptides, and amino acids depending on the specific type of protease activity (Varshavsky, 2001;Pahlow et al., 2003;Ali et al., 2019). In the case of conserved forages, proteolysis is caused not only by plant proteases but also by microbial enzymatic activity (Hao et al., 2019), which ultimately decreases silage (Muck, 1988a) and hay quality (Coblentz et al., 1997) by breaking down plant proteins into NPN of lower nutritional value. Thus, it is critical to describe the role of plant proteases and the forage phyllosphere in the breakdown of proteins after mowing.

Plant Proteases

Proteolysis begins soon after mowing due to the action of plant proteases (aka peptidases, proteinases, proteolytic enzymes) which normally are compartmentalized inside cell vacuoles in the standing crop. These proteases are released into the cytoplasm during wilting, where they promote protein degradation (Cavallarin et al.,

2005). Proteases that cleave the interior region of polypeptide chain are classified as endopeptidases (cysteine, serine, aspartic, glutamic, threonine, and metallo-endopeptidase) and those that cleave at the end of the chain are referred as exopeptidases (aminopeptidase, dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase, and carboxypeptidase; Machado de Castro et al., 2018; Figure 2-1).

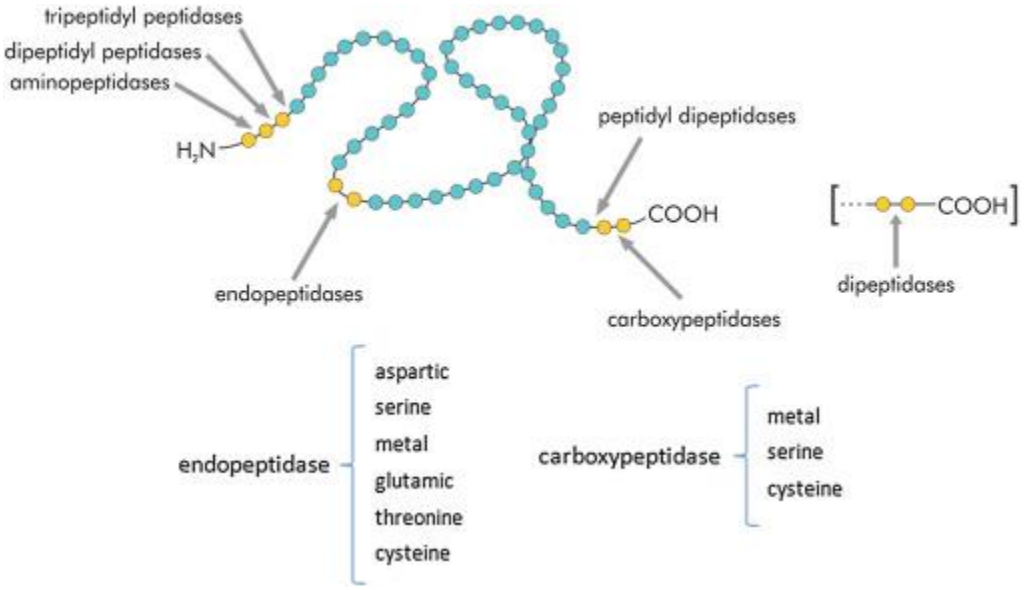

Figure 2-1. Classification of peptidases. Dark gray (blue) circles represent amino acids and light gray (yellow) circles indicate the amino acid sequence that will bind to the peptidase. The arrows point to the cleavage site on the protein substrate. Machado de Castro et al. (2018).

As with any other enzyme class, temperature and pH greatly affect the activity of plant proteases and consequently the extent of protein breakdown (Purich, 2010). In an experiment evaluating crude enzyme extracts from alfalfa leaves using artificial substrates (Tao et al., 2012), it was found that serine and metallo-endopeptidase activity was high between pH 3-5 while aspartic and cysteine peptidase were active between 6-8. In the case of dipeptidase, dipeptidyl-peptidase, and tripeptidyl-peptidase activity was high across a wide pH range (3-9) while aminopeptidase was more active

between 6-9 and carboxypeptidase between 4-6. Raising the temperature from 20 to 40°C increased the activity of both endo and exopeptidases in the same study. Tao et al. (2012) argued that alfalfa is especially susceptible to protein losses during ensiling due to the overall pH optimum of its proteases being on average lower than the ones reported for other major crops such as corn (Feller et al., 1977) and ryegrass (Heron et al., 1989).This is especially problematic if we consider that legume silages tend to acidify less than other forage crops, as mentioned earlier in this review. It is also important to mention the synergistic role of each of the proteases in breaking down plant protein. Guo et al. (2011) found in fermented green alfalfa extract that aspartic and cysteine peptidases mainly degrade protein into oligopeptides, while serine and metallo-peptidases contribute to the degradation of peptides into free amino acids. Novel preservatives could be developed to inhibit key enzymes in the proteolysis process and allow for an integral preservation of legume proteins.

Legume phyllosphere

Few studies have evaluated the phyllosphere of forage legumes and its role in in nutrient breakdown during wilting is poorly understood. McGarvey et al. (2013) described that the epiphytic bacterial community (on the plant surface) of wilted alfalfa foliage consisted mostly of an unknown *Enterobacteriaceae* (25), *Erwinia amylovora* (21.3), and *Enterobacter* sp. (16.7); and to a lesser extent of *Pseudomonas oryzihabitants* (8.8) and *Lactococcus garvieae* (4.8%), among other minor taxa. Furthermore, Zheng et al. (2017) reported that the epiphytic population of direct-cut alfalfa was mostly composed of the *Pantoea* (67.2), and *Enterobacter* (18.5), and *Buchnera* (5%) genera, all members of the *Enterobacteriaceae* family. Clearly, a

significant proportion of the epiphytic community in alfalfa and other forage crops (Romero et al., 2018) is dominated by enterobacteria that have the potential of causing spoilage if rapid acidification and anaerobiosis is not achieved during silage making (Pahlow et al., 2003) or if the material is not rapidly and adequately dried in the case of hay (Weinberg et al., 2007). The abovementioned studies demonstrate, in the case of silage, that if ensiled forages are produced adequately the relative abundance of all taxa related to enterobacteria diminishes much more rapidly relative to silos ensiled under non-ideal conditions. A more rapid decline of undesirable enterobacteria during ensiling results in silos with less DM losses (Bolsen et al., 1996) and proteolysis (Davies et al., 1998).

Using an alternative approach developed to assess endophytic bacterial communities (within plant tissue), Pini et al. (2012) found that in the stem and leaves of alfalfa the most abundant taxa were alphaproteobacteria (50%), followed by Sphingobacteria (12%), and betaproteobacteria (10%). Within the alphaproteobacteria class, they reported that *Methylobacteriaceae* (40%) and *Sphingomonadaceae* (50%), and to a lesser extent *Rhizobiaceae* (5%) and *Aurantimonadaceae* (2%) were the most abundant families. However, the role of foliage endophytic bacteria in spoilage of conserved forages remains to be elucidated. For wilted alfalfa (60% moisture), Guo et al. (2020) reported that the dominant genus were *Xanthomonas* (50.2) and *Cyanobacteria* (23.85), while *Pantoea* (4.78), *Pseudomonas* (4.60), *Sphingomonas* (3.26), and *Nethylobacterium* (2.93%) were present in a lower proportion. Unfortunately, to the best of our knowledge, no assessment of the fungal community in the phyllosphere of legumes has been conducted. More research needs to be conducted to

improve our understanding of how the bacterial and fungal communities transition from the moment of mowing across wilting an into the storage period for both silage and hay production in order to develop novel strategies that can mitigate nutrient losses caused by microbial spoilage.

Protein Degradation across key stages of Conserved Forage Production

At each of the stages in silage and hay-making, protein is degraded to different extents (Rooke and Hatfield, 2003; Rotz and Shiners, 2007). Thus, it is crucial to understand how spoilage proceeds across these critical steps in order to develop solutions that can prevent loss of protein quality during the production of conserved forages.

Wilting

In order to preserve forages, it is necessary to wilt them for haymaking (<20% of moisture) and in some cases for ensiling (50-65% moisture) (Fahey et al., 1994). Most of the DM losses in this process result from the loss of leaves (the most nutritious plant organ) during the harvesting process, especially for low-moisture legume hay [< 10-15% DM; (Fahey et al., 1994)]. Most standing legumes have between 17-30% DM (Albrecht and Muck, 1991) and need to be ensiled between 30-50% DM (Albretch and Bearchemin, 2003) to prevent effluent losses and the growth of clostridia and enterobacteria due to their low sugar concentration and high buffering capacity that pose a significant barrier to rapid acidification (Muck et al., 2003;Kung et al., 2018). In the case of hay, it is especially important to dry hay below to 40% moisture as rapidly as possible to prevent nutrient losses due to plant tissue respiration rate and proteolysis (Greenhill, 1959;Brady, 1965) as well as to microbial degradation (Fahey et al., 1994). Ideally, moisture concentration should be decreased to below 20% within 3-5 d in order

to prevent significant nutrient losses during wilting (Rees, 1982;Coblentz et al., 1996). For large hay bales, decreasing moisture concentration further to 15-10% is required to avoid spoilage during storage (Collins et al., 2017).

In the case of forage legumes, wilting takes longer since they have a slower drying rate relative to grasses due to the latter having a higher surface area to dry weight ratio (Rotz, 1995). Consequently, moisture concentrations that allow for plant proteolytic activity last longer in legumes, making them more susceptible to proteolytic losses during wilting (Rooke and Hatfield, 2003). For instance, protease activity was reduced from 30 to 20 units/h/g of DM during wilting of first cut alfalfa from 20 to 40% of DM, and it was further reduced to 15 units/h/g when alfalfa was wilted to 60% DM (Papadopoulos and McKersie, 1983). The same authors measured proteolysis using soluble NPN and reported that wilting periods of 6 and 24 h increased soluble NPN compared with the initial concentration in alfalfa (16.4 and 25.2 vs. 8.5 % of N, respectively). Moreover, it was also reported that the second cut of alfalfa is less susceptible to proteolysis during a wilting period of 24 h compared to the first cut, in terms of soluble NPN (10.2 vs 16.7 %of N, respectively). This may be related to the higher digestibility observed for first cut alfalfa relative to later cuts (Palmonari et al., 2014).

Rainfall during wilting is another critical factor that will prolong wilting time and results in nutrients being leached (Rotz et al., 1993). For instance, Tao et al. (2017) reported that when alfalfa was wilted for 6.6 h and then exposed to rainfall for 1 and 3 h it had less CP relative to alfalfa wilted for 5.2 and 8.5 h without rainfall before ensiling (19.4 and 19.0 vs. 22.0 and 21.4% of DM, respectively). Notably, they also reported that

3 h rain-damaged alfalfa had higher mold counts compared with alfalfa wilted for 8.5 without rainfall (6.19 vs. 5.14 log cfu/g, respectively).

During Ensiling

Before active fermentation can begin, oxygen trapped in silos promotes biochemical processes that result in the oxidation of nutrients (McAllister and Hristov, 2000). The amount of residual O_2 depends on silo design, crop structure and chop length, and silo density (Rooke and Hatfield, 2003). When the silo is well sealed, the residual O_2 is rapidly consumed by lingering plant respiration (Rooke and Hatfield, 2003). Oxygen presence is undesirable because it maintains plant tissue metabolism and obligate and facultative aerobic organisms such as molds, yeasts, and certain bacteria (Pahlow et al., 2003). These undesirable activities halt once all the oxygen is consumed or when acidification is enough to inhibit their metabolism (Dunière et al., 2013).

Once anaerobic conditions are achieved, silage bacteria –mainly LAB- ferment WSC, converting them to organic acids (mainly lactic acid) and decreasing the pH. A fast initial acidification from 6.0 to a range of 3.8 - 5 (Musa and Mustafa, 2020) is a key factor to inhibit the growth of undesirable microorganisms such as enterobacteria and clostridia (Pahlow et al., 2003) and inactivate plant proteases (Kung, 2010). This fermentation phase can lasts from one week to more than a month (Musa and Mustafa, 2020). Lactic acid is the most abundant organic acid in silages and is ≈10-12 times more acidic than acetic, propionic, and butyric acids (Kung et al., 2018). The second most abundant organic acid in silages is acetic acid, which has strong antifungal properties and is preferred for silages that struggle with aerobic stability, such as whole-crop corn silage and high moisture corn grain silage (Kung et al., 2018). The ratio of

lactic to acetic acid (**L:A**) ranges from 1 to 6 and is mostly determined by the relative proportions of obligate homofermentative and facultatively and obligate heterofermentative LAB. Kung et al. (2018) suggested that good silages should have L:A values between 2.5 to 3, with legumes silages ideally being on the higher end of this range.

As mentioned earlier, legume silages usually have a higher final pH compared with corn silage (4.3-5.0 vs 3.7-4.0, respectively) (Kung et al., 2018). Low silage pH values promote the growth of acid-tolerant lactic acid bacteria (**LAB**) such as *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactobacillus buchneri* (Holzer et al., 2003) and inhibit the growth of enterobacteria and bacilli when below 4.5-5.0 (Muck, 2010). However, clostridia can grow at lower pH values than enterobacteria and bacilli (Muck, 2010). Muck et al. (2003) reported that clostridia are inhibited if pH drops at or below 4 within 3 d of ensiling. Although low pH is the most important factor to inhibit these microorganisms, it is important to consider other factors such as DM concentration (Ávila and Carvalho, 2020). For instance, the critical pH that inhibits clostridia growth varies with the plant DM concentration (Figure 2-2; Leibensperger and Pitt, 1987), with less acidification needed at higher DM values.

In general, it is considered that enterobacteria and Clostridium are the most important proteolytic microorganisms in silage (Ávila and Carvalho, 2020). Lactic acid can actually be converted to butyric acid, hydrogen and CO_2 by clostridia with ideal growth conditions (Driehuis and Oude Elferink, 2000). Also, *Clostridium* species can ferment sugars directly to butyric acid (McDonald et al., 1991). Some clostridial species such as *Clostridium sporogenes* and *Clostridium bifermentans* are considered highly

proteolytic while others such as *Clostridium tyrobutyricum* and *Clostridium butyricum* are weakly proteolytic (Driehuis and Oude Elferink, 2000). Proteolytic clostridia are of special concern because they ferment amino acids (Rooke and Hatfield, 2003) releasing ammonia, amines, and butyric acid among other major organic acids (Pahlow et al., 2003). Moreover, clostridia produce biogenic amines such as cadaverine, glucosamine, histamine, putrescine, and tyramine in silage (Queiroz et al., 2018).

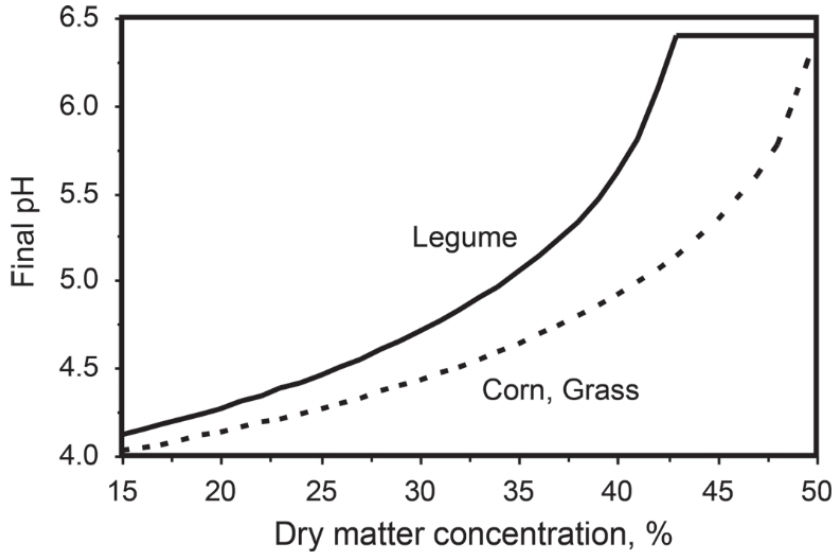

Figure 2-2. The pH below which growth of Clostridium tyrobutyricum is inhibited. Adapted based on equations of Leibensperger and Pitt (1987). Taken from Driehuis et al. (2018).

In particular, *C. tyrobutyricum* is an acid tolerant species that can increase silage pH and promote growth of less acid-tolerant clostridia and other microorganisms (Driehuis and Oude Elferink, 2000). If silages reach a pH below 4 the growth of clostridia can be inhibited but legumes and to a lesser extent grasses have difficulties in reaching to that pH fast enough to prevent clostridial growth (Muck, 2010), as discussed in previous sections.

Enterobacteria species can also degrade proteins and decarboxylate and deaminate aminoacids (Pahlow et al., 2003), which releases NH_3-N (Kaiser et al., 2004) and biogenic amines (Driehuis and Oude Elferink, 2000). Among the enterobacteria species, *Escherichia coli* O157:H7 is of special concern due to its pathogenicity (Driehuis et al., 2018) and has been detected as part of the epiphytic community in some forage crops that were harvested soon after manure application and at low mowing heights (Dunière et al., 2013). For instance, when liquid dairy manure was applied close to 50 d before harvest of alfalfa, the fresh forage had 2.3 log cfu /g of *E. coli* (Ogunade et al., 2016). Furthermore, Ogunade et al. (2016) reported that the addition of 5 log cfu/g of E. coli before ensiling alfalfa numerically increased NH_3-N concentration from 0.34 to 0.42 % of DM in plastic bag silos after 100 d of ensiling. However, the addition of the same dose of *E. coli* with 6 log cfu/g of *L. plantarum* or *L. buchneri* to alfalfa before ensiling caused a reduction of *E. coli* killing time (100 vs. ~16 d, respectively) since the pH was lower than 5 at d 16 (Ogunade et al., 2016). In contrast, when *E. coli* was added to corn silage, it was eliminated only after 3 d of ensiling due to low pH (<4) with or without addition of LAB (Ogunade et al., 2017). Enterobacteria are also involved in the degradation of nitrate during silage fermentation by using it as an electron acceptor in place of oxygen, and reducing it to nitrite and ultimately to ammonium (Spoelstra, 1987). They are also capable of reducing nitrite to nitrous oxide (Bleakley and Tiedje, 1982). Both nitrite and nitric oxide are considered effective inhibitors of clostridia but producing them uses protons which may lead to higher pH values (Driehuis and Oude Elferink, 2000). However, nitrate poisoning is seldom a problem with forage legumes (Undersander et al., 1999), and nitrate levels

that are considered to be safe to feed range from 4 to 1760 mg/kg in fresh alfalfa or alfalfa hay (Crowley, 1985).

Hay Storage

Significant microbial metabolism of nutrients occurs when hay is stored above 20 and 15% moisture in small and large bales, respectively (Coblentz et al., 1996;Collins et al., 2017). As a result microbial of spoilage, fiber concentration and DM losses increase and protein digestibility and energy density decreases (Coblentz and Bertram, 2012). During hay storage, there are two peak temperatures, the first is related with to respiration of plant cells and microflora associated with hay at baling time (Roberts, 1995) and occurs during the first 4 d of storage (Figure 2-3). For instance, temperature increased from 30 to 50°C during the first days of storage in high moisture alfalfa hay (30.6%) (Coblentz et al., 1994). This increase in temperature allows the growth of thermophilic microorganisms (Duchaine et al., 1995) such as *Saccharopolyspora rectivirgula* and *Thermoactinomyces vulgaris* (Pepys et al., 1963). The second peak temperature is related to the respiration of bacteria, fungi, and yeast in hay, as shown in Figure 2-3 (Rotz and Muck, 1994). For instance, Coblentz et al. (1994) reported that the maximum temperature for small square bales of high moisture alfalfa (31.1%) was 54.9°C. If the temperature is high enough (> 60°C; Van Soest, 1982), the Maillard reaction occurs rapidly and forms ADICP, which is indigestible. Also, high moisture conditions during storage can result in the production of mycotoxins, which can affect animal performance and health (Jovaisiene et al., 2016). Eventually, temperature decreases over time because bale heating evaporates enough moisture to limit microbial activity (Collins et al., 2017). According to a recent meta-analysis conducted

by Killerby et al. (2020a), legumes seem to be more susceptible to spoilage during storage than grasses, most likely due to their higher nutritive value.

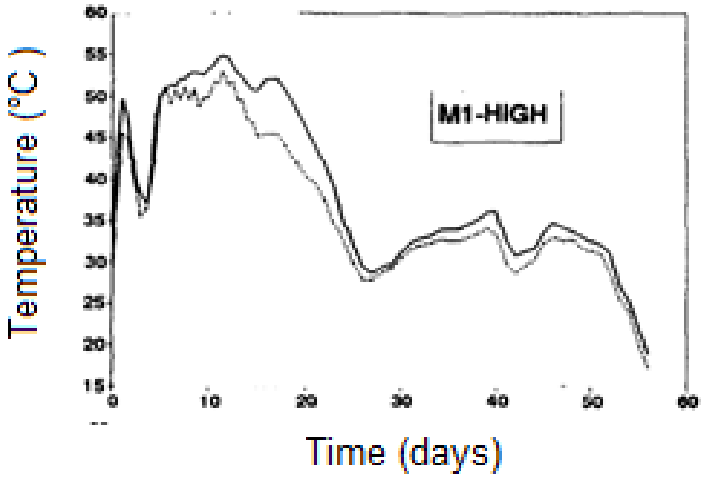

Figure 2-3. Temperature vs time curve of laboratory (dashed lines) and conventional bales (solid lines) at high density and high moisture (30.6%) (Taken from Coblentz et al., 1994).

Silage Feeding

During the feed-out phase, as oxygen enters through the silo face, yeast, molds and aerobic bacteria growth resume (Pahlow et al., 2003;Wilkinson and Davies, 2013). When yeast and acetic acid bacteria raise both the pH (≥ 4.5) and temperature (40°C) of aerobically challenged silage, the growth of undesirable microorganisms is facilitated, such as bacilli (Muck, 2010), *L. monocytogenes* (Driehuis et al., 2018), clostridia (Borreani and Tabacco, 2008), and molds, which complete the silage deterioration (Borreani et al., 2018).

In general, aerobic stability is not a problem in legume silages (Pahlow et al., 2002) because of their high concentration of ammonia (10-15% of total N) and acetic acid (2-3% DM) which have antimicrobial activity (Kung et al., 2018). Alfalfa silage is known to have a higher aerobic stability compared with corn silage (Muck and O'kiely, 1992). For instance, Tabacco et al. (2009) reported that corn silage after 90 d of ensiling

had 1.23 acetic acid (% of DM), 5.5 NH_3-N (% of N) and 39 h of aerobic stability. Conversely, Ke et al. (2015) reported that 60-d alfalfa silage had 2.64 acetic acid (% of DM), 11.6 NH_3-N (% of N) and 338 h aerobic stability. Also, Wambacq et al. (2013) reported that red clover silage had 2.21 acetic acid (% of corrected DM; Dulphy and Demarquilly 1981), 14.5 NH_3-N (% of N) and 296 h of aerobic stability after 90 d of ensiling. Therefore, legume silages do not have issues in terms of aerobic stability relative to other forage crops that are inherently aerobically unstable (e.g. corn).

Factors affecting protein degradation

The extent of proteolysis in silage is influenced by factors such as forage species (Papadopoulos and McKersie, 1983), DM concentration of the forage at ensiling (Muck et al., 2003), pH (McKersie, 1981), and temperature (Muck, 1988a). Next, we will review each of them in detail.

Crop

Silage. Legumes are known for having higher levels of proteolysis relative to grasses, because they have a higher buffering capacity and lower sugar concentration (Kung et al., 2018). For instance, alfalfa ensiled for 170 d produced more NH_3-N than corn and sorghum ensiled for 90 d (9.10 vs 5.5 and 4.6% of total N, respectively) at comparable DM concentrations (34.3, 34.9, 40.2%, respectively) (Colombari et al., 2001;Tabacco et al., 2009). However, studies have also shown differences among legume species. For example, alfalfa ensiled at 25.3% DM presented more NH_3-N concentration than birdsfoot trefoil, sainfoin (Onobrychis viciifolia Scop.), cicer milkvetch (Astragalus cicer L.) or red clover ensiled at ~ 22.2% DM (6.5 vs 3.6, 3.2, 6.1, 3.8 % of total N, respectively) after 35 d of ensiling (Albrecht and Muck, 1991). The same study

reported a high inverse correlation (r^2=0.75) between tannin and protein degradation. Tannins reduce the extent and rate of proteolysis in silage by forming complexes with forage proteins (at pH 3.5-7.5; Barry and McNabb, 1999) that are possible due to the presence of multiple phenolic hydroxyl groups in tannins which facilitate protein binding (Reed, 1995). Moreover, it is widely accepted that condensed tannin-protein complexes not only can escape from ruminal degradation but they can be degraded in the lower gastrointestinal tract for the most part (Cortés et al., 2009).For instance, the addition of purified condensed tannins to soybean meal, reduced in vitro ruminal protein degradation by 16-55% but the ruminally undegraded protein which was incubated afterwards with HCl/pepsin increased by 18- 412% (Cortés et al., 2009)**.**

In the case of red clover (*Trifolium pratense*), its higher resistance to proteolysis relative to alfalfa is explained by the presence of polyphenol oxidase (**PPO**) and *o*-diphenol PPO substrates (Sullivan et al., 2006). PPOs are enzymes catalyzing both hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones in the presence of molecular oxygen (Matheis and Whitaker, 1984). These enzymes are stored in the chloroplast in two forms: active (5-10%) and inactive (95-90%) (Lee, 2014). PPO can be activated by the presence of diphenol substrates but this activation is prevented in healthy red clover because these substrates are stored in vacuoles (Mayer, 2006). However, plant cell damage can activate latent PPO (Lee et al., 2009) by mixing these enzymes with their diphenol susbtrates. When PPO is active, this enzyme transforms diphenols to quinones which can react with protein forming protein-bound phenols (**PBP**). This results in red clover losing only 7-40% of its protein during ensiling, whereas alfalfa loses between 44-87% (Jones et al., 1995). At the ruminal

level, Grabber et al. (2009) reported that PPO effects increase RUP in ensiled red clover compared to alfalfa (5.4 vs 3.2% DM, respectively).

Hay. During the hay making process, predominant losses of leaves during harvest results in a decrease in N concentration relative to the standing crop. These losses have been consistently higher in legumes relative to grasses as reported by Michalet-Doreau and Ould-Bah (1992; -0.54 vs. -0.08) and Jarrige et al. (1981; -0.42 vs. 0.09% of DM; respectively). Furthermore, N *in situ* ruminal degradability can decrease by 2.5% during hay making, especially for forage crops with high initial N degradability and when harvesting conditions are poor (e.g. rain damage; Michalet-Doreau and Ould-Bah, 1992).

Plant maturity

In alfalfa hay, CP decreases from 22 to 16.2% DM as maturity increases from early bud to early flower (Yari et al., 2012). Furthermore, these authors reported that as maturity increases, the fraction B2 decreases from 30.1 to 26.9% of CP while RUP increases from 5.8 to 7.7% of CP. Consequently, *in situ* ruminal degradability of CP at 12 h decreases from 13.4 to 8.5% DM as maturity increases in alfalfa hay (Yari et al., 2012). In addition, Yu et al. (2003) reported that in fresh alfalfa as maturity increases from early bud to early bloom, fraction A, B2, and B3 decreases from 50.2 to 41.9, 9.7 to 2.9, and 37.0 to 19.2% of CP, but fraction B1 and C increases from 0.0 to 18.6 and 3.0 to 17.4% of CP, respectively.

Temperature

Although a forage producer cannot exert control over environmental temperature at harvest and storage, several management decisions will influence the extent of plant and microbial aerobic respiration that occurs from harvest to feeding and thus the degree of spoilage heating affecting the nutritional value of the stored forage. For instance, conserved forage temperatures above 60°C during storage will increase ADICP dramatically (Van Soest, 1982). Also, it is important to note that plant proteases are inactivated with temperatures above 40°C, as reported for red clover and alfalfa (Jones et al., 1995).

Silage. Wilting extensively (>60% DM) can compound heat damage issues since this decreases the specific heat capacity of silages and higher porosity that can sustain longer periods of aerobic activity (Garcia et al., 1989). Furthermore, these authors also reported an interaction of DM and temperature on 21-d alfalfa silage ADICP levels. When temperature was increased from 38 to 65°C, ADICP increased to a greater extent at 62% DM (1.44 vs. 2.31) relative to 46 (1.25 vs. 1.99% of DM, respectively). Furthermore, the same temperature increase decreased NPN at 46% DM, 2.06 vs 1.99% of DM at 38 to 65°C, respectively but it increased NPN at 62% DM from 1.49 vs 1.53% DM at 38 to 65°C during ensiling. In the case of high moisture silages (direct cut to 30% DM) higher temperatures can compound issues with clostridial fermentation. For instance, Gibson et al. (1958) reported that grass silages stored at 30 and 40°C were more likely to suffer clostridal activity than those stored at 22°C. This is because clostridia have higher temperature optima than LAB (McDonald et al., 1991).

Hay. In high-moisture hay (> 15-20%), plant and microbial respiration during storage results in heat production that raises ADICP levels (Coblentz et al., 2000). For instance, Coblentz et al. (1996) observed that the ADICP concentration of alfalfa hay baled at 29.7% moisture was 3.12% of CP after baling but then increased to 3.32, 5.36, 5.6, and 6.5% of CP after 4, 11, 22 and 60 d of storage in which the mean internal hay temperature reached 44.9, 49.5, 46.1, and 28.7°C, respectively. Furthermore, Broderick et al. (1993) reported that when alfalfa hay was heated for ~47 min at 100 - 110°C, ADICP was increased from 4.6 to 15.3% of CP. When the heated hay was fed to dairy cows in the same study, the estimated net ruminal CP escape was higher compared with the control (50 vs 29% of CP, respectively). Moreover, Coblentz et al. (2010) reported that when large round bales of alfalfa and orchardgrass at moisture concentrations ranging from 26.7 to 46.6 were stored, pre-storage ADICP was 5.6 % of CP and after storage it ranged from 5.9 to 21.4% of CP, and the maximum internal bale temperature ranged from 54.4 to 77.2°C.

Silage pH

As previously discussed, fast acidification is crucial to halt the activity of plant proteases and the protein degradation caused by undesirable microbes such as clostridia and enterobacteria. However, in legume silages the higher buffering capacity (Table 2-2) alongside low initial sugar concentrations delays and reduces the extent of acidification relative to other forage crops. Forage buffering capacity is mostly correlated with its anion concentration (organic acids, orthophosphates, sulfates, nitrates, and chlorides) and to a lesser extent with CP concentration. The main organic acids found in legumes are malic, citric, quinic, malonic and glyceric acids (Doelle et al., 2009).

Table 2-2. Buffering capacities (mEq/kg DM) for selected forage crops (compared from various sources by Coblentz, 2015).

Crop/Species	Range
Corn silage	149-225
Timothy	188-342
Fall Oat (headed)	300-349
Orchardgrass	247-424
Red Clover[1]	552-639
Fall Oat (Boot)	360-371
Italian Ryegrass	265-589
Alfalfa (mid-bloom)	313-482
Perennial Ryegrass	257-558
Alfalfa (1/10 bloom)	367-508
Alfalfa	390-570
White Clover[2]	373-562

[1](King et al., 2012)
[2](Dewhurst et al., 2003)

In general, proteolysis is reduced by quickly achieving a low pH, and by maintaining anaerobic conditions (Collins et al., 2017). It is important to mention that high temperature can increase pH in corn and wheat silages (40% DM) due to a decrease in lactic acid (Weinberg et al., 2001). This in turn can result in an increased proteolysis (in direct cut to 30% DM silages) since clostridia have higher temperature optima than LAB (McDonald et al., 1991). However, the addition of homofermentative LAB may rapidly lower silage pH as a result of an increase of lactic acid during the

fermentation (Chen et al., 2019). These authors reported that the addition of homofermentative inoculant on high moisture alfalfa silage increased lactic acid (5.21 vs 3.53% DM), decreased both silage pH (5.07 vs 5.58) and proteolysis, measured as NH_3-N (12.3 vs 15.8 % of N, respectively), compared with the control after 45 d of ensiling.

Effects of Additives on Forage Protein Preservation

Considerable research has been conducted to reduce proteolysis in conserved forages using additives because protein is an expensive component of ruminant diets (Tremblay et al., 2001). Sullivan and Hatfield (2006) estimated that $100 million are added to supplementation expenses each year in the US to compensate the loss of protein in legume silages.

Silage

Organic acids. Formic acid and formaldehyde are effective antimicrobials and reduce proteolysis during the ensiling process (Kung et al., 2003b). Nagel and Broderick (1992) reported that when formic acid was applied at 2.8% DM to alfalfa silage, NPN, ammonia, and total free amino acids were reduced compared to the control (29.1, 1.2, and 14.4 vs. 43.1, 6.4, and 31.2% total N, respectively). Pahlow et al. (2002) evaluated the addition of formic acid or a homofermentative inoculant (Ecosyl; 6 log cfu/ g fresh) on mixed silage composed of alfalfa, red clover, lotus (*Lotus corniculatus*), and galega (*Galega orientalis*) ensiled at 25% DM for 90 d. formic acid had the lowest concentration of NH_3-N followed by the inoculant and the control (4±1, 9±4, and 14±4% N, respectively). Similarly, Guo et al. (2008) reported that when formic acid, formaldehyde (0.54 and 0.3% fresh weight, respectively) or a mixture of formic acid (0.27 % fresh weight) with formaldehyde (0.15% fresh weight) were applied on high moisture alfalfa

silage (76.8%), the lowest concentration of fraction A was observed in the treatment mixture followed by formic acid, formaldehyde, and control (43.4, 50.7, 57.2, and 68.4% of CP, respectively). Furthermore, the mixture treatment had higher concentration of fraction B3, which is an important contributor to RUP, than formic acid or formaldehyde (21.6 vs 14.0 and 3.4% of CP, respectively) (Guo et al., 2008). These results show that organic acids are efficient on reducing proteolysis. However, in recent times, these acids have been gradually substituted by biological additives because the acids are unsafe to handle and apply, and they corrode equipment (Yitbarek and Tamir, 2014).

Acid salts. Acid salts are an alternative to acids that do not cause equipment corrosion and are safer to handle (Kung et al., 2003b). However, their results are not as consistent as with acids (Kung et al., 2003b). Application of ammonium tetraformate, which is a buffered form of formic acid, on alfalfa silage decreased NH_3-N concentration relative to control (3.4 vs 4.1% of N, respectively) (Broderick et al., 2007). Conversely, Cussen et al. (1995) reported that when sodium formate was added to a perennial ryegrass and white clover mixture silage (40:60, respectively), sodium formate did not decrease NH_3-N compared to the control (~7.50% of N, respectively) but formic acid did (4.14). Wen et al. (2017) reported that when formic acid, potassium diformate, sodium diacetate, and calcium propionate were applied at 0.4, 0.55, 0.7, and 1% fresh weight, respectively to alfalfa ensiled; the treatments decreased DM loss compared with the control (8.9, 9.55, 10.0, and 10.6 vs 12.7%, respectively). The same authors reported that butyric acid and clostridia counts were decreased by all the treatments. Among all the acid salts tested, potassium diformate was the most similar to formic acid but a higher dose was necessary to match its effects (Wen et al., 2017).

Preservative Salts. Sodium benzoate, potassium sorbate, and other salt-based preservatives are also quite effective at inhibiting undesirable microorganisms in silages (Kung et al., 2003b). For instance, Knicky and Spörndly (2011) evaluated the effect of a sodium benzoate, potassium sorbate, and sodium nitrite mixture (200, 100, and 50 g/kg of fresh matter, respectively) added to mixtures of mostly red clover or alfalfa mixed with grass and ensiled for at least 90 d. This preservative effectively decreased NH_3-N (17.78 to 5.17% of N), butyric acid (5.5 to 0.04% DM), and clostridia counts (4.5 to 1.9 log cfu/g, respectively) compared with the control. Similarly, König et al. (2017) evaluated the addition of a sodium nitrite-hexamine mixture or formic acid on mixed silage composed of *Lupinus albus* and *Triticum aestivum* (2:1, respectively) ensiled for 100 d. The sodium nitrite-hexamine mixture had less NH_3-N (3.7 vs 24.1% N), butyric (0.05 vs 4.3% DM) and clostridia counts (3.67 vs 5.66 log gene copies/g) compared with formic acid or the control In general, the sodium nitrite-hexamine mixture was the most effective additive in inhibiting clostridia activity during ensiling and for decreasing NH_3-N concentration (König et al., 2017).

Sugars. Molasses, sugar, whey, citrus pulp, and potatoes, among others can be added to legume silages to increase the supply of rapidly fermentable substrate for LAB (Yitbarek and Tamir, 2014). Molasses has been extensively tested in forage crops low in soluble carbohydrates such as legumes and tropical grasses (Henderson, 1993). For instance, Hashemzadeh -Cigari et al. (2011) reported that when wilted and fresh alfalfa were treated with 5 and 10% (DM basis, w/w) molasses before ensiling, the highest dose of molasses produced less NH_3-N than the control (32.9 vs 36 % N in fresh alfalfa, and 20.5 vs 21.4% N in wilted alfalfa). Conversely, the lowest dose of molasses

produced more NH_3-N than the control in wilted alfalfa (22.4 vs 21.4% N, respectively) but it decreased NH_3-N in fresh alfalfa (34.0 vs 36.0 % N, respectively). Similarly, when dextrose (2% fresh basis, w/w) was added to alfalfa ensiled at 33, 43, and 54% DM for 60 d, the effectiveness of sugar addition on NPN fractions was influenced by the DM of the silage (Jones et al., 1992). The added dextrose was more effective in reducing NPN (57.5 vs 59.0), ammonia (5.5 vs 6.4) and free amino acids (37.6 vs 39.8%) compared with the control at 33% DM silage; but failed to do so at 54%DM (Jones et al., 1992).

Commercial tannins. Tannins have been added to halt protein breakdown during ensiling and decrease RDP, especially in legumes that do not synthesize tannins, like alfalfa (Mueller-Harvey, 2006)., especially in legumes that do not synthesize tannins, like alfalfa (Mueller-Harvey, 2006). Tabacco et al. (2006) evaluated the effects of chestnut (*Castanea sativa* L.) tannin applied at three doses (2, 4, and 6% on DM basis) to alfalfa ensiled for 120 d. As tannin application rates increased, NH_3-N concentration decreased compared with the control (11.4, 10.0, and 9.6 vs 12.8 % of total N, respectively). Furthermore, soluble protein was also decreased (82.1, 77.6, and 74.7 vs 84.2%, respectively). Similar results were reported by Colombini et al. (2009) when alfalfa silage with or without chestnut hydrolysable tannins applied at 4.6% DM (w/w) were added to the diet of 50 lactating Holstein cows. The effective rumen protein degradability was reduced when tannin was applied relative to control (82.0 vs. 77.3% at a ruminal rate of passage of 6%/h and 85.8 vs. 82.3% at 3%/h, respectively).

Bacterial Inoculants. In the case of legume silages, homofermentative LAB are conceptually more desirable than heterofermentative LAB because the former group decrease the silage pH more rapidly than the latter (McGarvey et al., 2013). However,

conflicting reports precluded a clear identification of LAB inoculant benefits in legume silages, most likely due to differences in species and strains, and divergent ensiling conditions across studies. For instance, Whiter and Kung (2001) reported that when *Lactobacillus plantarum* (LP; 5 log cfu/forage) was applied as liquid or dry inoculant to alfalfa ensiled at 30 or 54% DM, liquid and dry inoculant produced less ammonia concentration compared with the control (0.066 and 0.084 vs 0.126% DM, respectively) in alfalfa ensiled at 54% DM. However, there was no difference for alfalfa ensiled at 30% DM after 45 d of ensiling. Furthermore, Contreras-Govea et al. (2011) reported no benefits of adding 4 different inoculants consisting of a wide array of homofermentative and facultative heterofermentative LAB on NPN and NH_3-N concentrations of alfalfa silage (39.5% DM). Oliveira et al. (2017) conducted a meta-analysis to examine the effects of homofermentative and facultative heterofermentative LAB on a wide variety of ensiled forage crops and reported that these types of inoculants decrease silage pH (-0.26), mold counts (-2.06 log cfu/g as fed), and ethanol (-0.32% DM) and increase DM (+0.38%) but do not affect NDF, LAB counts, or acetic acid in the case of alfalfa silages. No specific effect of these inoculants on the DM recovery of legumes was presented in that meta-analysis but grasses benefited (+2.77%) while sugarcane values were actually reduced (-2.39%). Similarly, no specific results on NH_3-N were presented for legumes but overall, a reduction of 1.31% of N was reported across studies. Recently, Blajman et al. (2020) conducted a meta-analysis to assess the effect of homofermentative LAB on alfalfa silage and reported that the inoculum increased lactic acid and CP (+4.9% of DM, +0.53% of DM) and decreased NH_3-N, pH, acetic, and

butyric acid (-4.53% of N and -0.04, -0.25% of DM, -0.55% of DM; respectively) compared with untreated.

Hay

Organic and buffered organic acids. Propionic acid-based products are mainly used to inhibit fungal growth and prevent spoilage during storage, especially for high-moisture hay (Coblentz et al., 2013a). Killerby et al. (2020a) conducted a meta-analysis of 50 articles to examine the effects of propionic acid, buffered organic acids, and other organic acids (defined as a variety of proprietary mixtures that included or not propionic acid, acetic acid, and others) on the preservation of hay. The effect size was calculated as standardized mean differences. The authors reported that propionic acid, buffered organic acids, and other organic acids decreased DM loss (-5.44, -5.93, and -0.59) and visual moldiness on legumes (-58.8, -7.32, and -40.33, respectively), relative to untreated hay. Buffered organic acids were less effective at reducing moldiness but more effective at reducing bale heating compared to propionic acid (-9.88 vs -3.40, respectively) (Killerby et al., 2020a).

Microbial inoculants. A recent meta-analysis of 21 articles conducted by Killerby et al. (2020b) examined the effects of microbial inoculants (mostly LAB) on the preservation of hay. The effect size was calculated as standardized mean differences. The authors reported that microbial inoculants did not affect DM losses, visual moldiness, maximum temperature, heat degree-days, or IVDMD compared with the untreated legume or grass hay. However, microbial inoculants decreased sugars (-

1.10), NDF (-4.68), and ADIN (-1.07) compared with untreated legume hay (Killerby et al., 2020b).

Lignosulfonates. Lignosulfonates are defined as amorphous branched polymers of lignin (EFSA, 2015) and they contain mainly sulfonic groups and few phenolic hydroxyl groups on their surfaces, and carboxyl groups mainly located in the core of the lignosulfonates molecules and aggregates (Figure 2-4; Yan et al., 2010). It has been reported that certain lignosulfonates not only inhibit the growth of fungi (Jha and Kumar, 2018), and bacteria (Dong et al., 2011), but also have antiproteolytic properties (Petit et al., 1999;Wang et al., 2009;Reyes et al., 2020). Lignosulfonates have been commercially used to increase ruminal protein bypass of legume meals by up to 173% with no negative effects on performance (Petit et al., 1999). Windschitl and Stern (1988) reported that when calcium lignosulfonate was applied to soybean meal, this lignosulfonate reduced ruminal protein degradation compared with the control (53.7 vs 70.6%, respectively). Furthermore, Mansfield and Stern (1994) reported that when lignosulfonate was added to soybean meal, dietary N ruminally digested decreased compared with the control (37 vs 43 % of N intake, respectively). Only Reyes et al. (2020) have evaluated the effects of sodium lignosulfonate on hay protein breakdown and ruminal fermentation. They reported that sodium lignosulfonate reduced hay NH_3-N with doses as low as 0.5% (0.071) and in vitro ruminal NH_3-N with doses as low as 3% (49.6) relative to the control (0.249 % DM and 58.2 mg/dL, respectively). More studies are needed to evaluate the potential of lignosulfonates to preserve protein quality during storage and prevent extensive ruminal degradation of proteins, which can be a major issue in legumes that do not produce tannins.

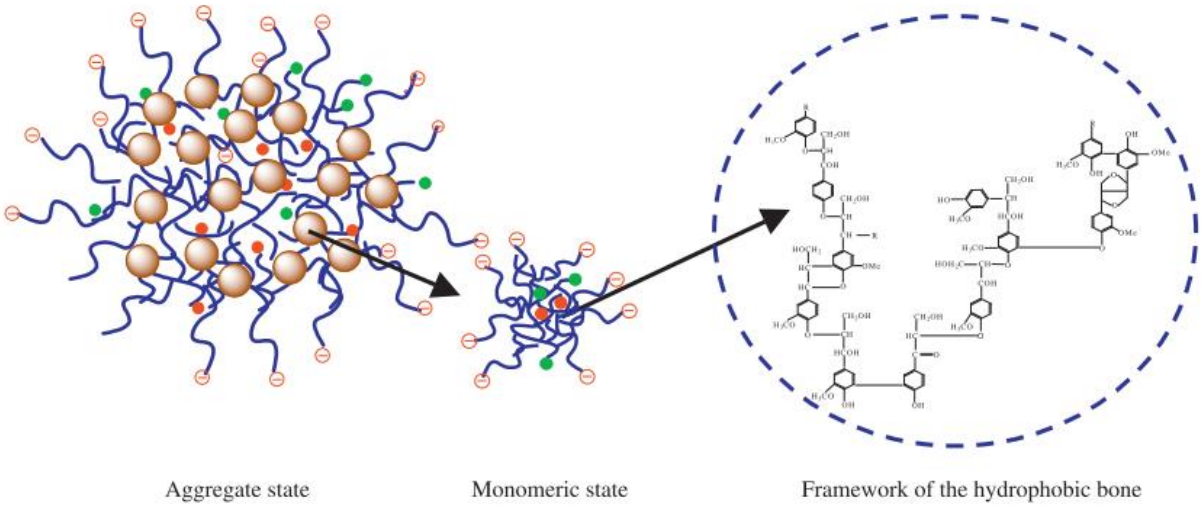

Figure 2-4. The schematic structures of purified and commercial sodium lignosulfonate aggregate. Sulfonic groups are represented by white circles with a red line in the middle, phenolic hydroxyl group by green circles, carboxyl group by red circles, and hydrophobic chain by blue line (Yan et al., 2010)

Consequences of Protein Spoilage on Animal Performance and Health

In general, high-moisture silages have high concentrations of ammonia (>15% total N) and soluble N (> 60% total N) (Kung et al., 2018). High ammonia levels can result in an excess of RDP which can have negative consequences on milk and reproductive performance (Kung, 2010). Ammonia is transported in two ways according to ruminal pH: as NH_3 when ruminal fluid pH is above 7, and as NH_4 at physiological pH of 6.5 or lower (Abdoun et al., 2006). According to Reynal and Broderick (2005), maximum microbial protein synthesis requires at least 11.8 mg of NH_3-N/dL of rumen fluid of and a RDP of 12-13% (DM basis). A concentration of NH_3-N ≥ 2 mg/dL in blood indicates excess NPN exposure with clinical signs of poisoning occurring > 80 mg/dL of rumen fluid (Thompson, 2015). High ammonia concentrations can have negative effects on reproduction of dairy cows (Jorritsma et al., 2003), which include hampering of the

cleavage and formation of blastocyst when oocytes in antral follicles are exposed to high levels (Sinclair et al., 2000). Urea also can have a similar effect as ammonia during the formation of the fertilized embryo (Jorritsma et al., 2003). Gustafsson and Carlsson (1993) reported that when a group of 29 dairy herds were fed with silage containing more than their requirements of energy (17%) and protein (6 to 15%), the interval to the last service was lengthened by 2.2 d for each percentage unit of increased NH_3-N concentration in the silage, The authors speculated that the increased ammonia levels decreased the palatability of the silage or had an adverse effect on the rumen microflora, which ultimately decreased the energy balance and fertility (Gustafsson and Carlsson, 1993).

Biogenic amines (**BA**) can affect the intake and digestibility of ruminants. High levels of BA are frequently observed in silages prepared from high-protein forages such as alfalfa, clover, and certain grass species (Mlejnkova et al., 2016). Certain biogenic amines can cause detrimental effects to feed intake and animal health (Driehuis et al., 2018). The main biogenic amines found in silage are putrescine, cadaverine, and tyramine (Dunière et al., 2013). When cows were fed 100 g/d of putrescine through silage, this BA caused depression in both feed intake and milk yield compared with the control (Lingaas and Tveit, 1992). Moreover, putrescine was considered the most sensitive indicator of the extent of putrefaction in silage after analyzing other BA such as cadaverine, spermidine, and histamine (Krízek, 1993). A positive relationship (r^2=0.898) was reported between the concentration of putrescine and degree of proteolysis (Krízek, 1993). Phuntsok et al. (1998) also reported that increasing the concentration of putrescine and cadaverine causes a decrease of reticular contractions (from 1.41 to

1.28 n°/min), intake (from 8.18 to 6.07 kg DMI/d), ruminal DM digestibility (from 48.5 to 43.61%), ruminal outflow (from 4.25 to 3.41 kg/d), ruminal total volatile fatty acids (119.91 to 111.08 m*M*), and total tract DM digestibility (67.14 to 66.75%).

Summary

Conserving the quality of the protein fraction during the production of hay and silage is essential to reduce supplementation costs and increase the profitability of livestock operations considering that protein is the most expensive component of herbivore's diets. This is especially significant for legume forages, since they typically have the highest concentration of protein among all forages. The proteolysis process, which starts right after mowing, is initially carried out by plant proteases which are gradually inactivated as moisture decreases during wilting. In the case of hay, plant proteases are fully inactivated when moisture levels are reduced below 40%. Aerobic microbial spoilage also starts after mowing but its role is more relevant during hay storage if bale is stored with moisture levels above 20%. At that moisture level, fungal species are especially active in oxidizing nutrients while releasing metabolic heat which will increase the ADICP fraction. In the case of legume silages, wilting to 45-65% moisture yields the best results in terms of nutrient preservation. Equally important is to reach anaerobic conditions as quickly as possible so acidity and lack of oxygen stops plant and microbial respiration. These conditions are harder to achieve in legumes relative to grasses due to their high buffering capacity and low sugar concentration. Consequently, protein fermentation can significantly decrease protein quality and result in the production of excessive ammonia levels which will reduce protein utilization in the animal. Several preservatives and inoculants are available to mitigate nutrient losses in

both hay and silage, with organic acids being the most effective preservatives. Unfortunately, the use of organic acids is expensive, hazardous, and corrosive to farm machinery. Lactic acid bacteria inoculants are a viable option for the preservation of legume silages but are completely ineffective in the case of hay. Further research is needed to develop next generation of conserved forage preservatives that are inexpensive and safe to use by farmers.

CHAPTER 3

EFFECT OF LIGNOSULFONATES ON THE DRY MATTER LOSS, NUTRITIONAL COMPOSITION, AND MICROBIAL COUNTS OF HIGH MOISTURE ALFALFA SILAGE

Introduction

In the US, silage production has risen from 117 Mg per year in 2000 to 148 in 2019 at the expense of hay production, which declined from 139 to 117 in the same period (NASS, 2004;2020a). One of the reasons behind this change in preference is that relative to haymaking, ensiling requires a much shorter wilting time (Han et al., 2014). This reduces the risk of rain damage in areas with high precipitation during the harvest season (Albretch and Bearchemin, 2003). However, legumes are the hardest forage to ensile because of their high buffering capacity, which is compounded by low sugar concentrations that limit the lactic acid production necessary for a rapid and extensive acidification (Liu et al., 2016). Due to these limitations, legume silages are especially susceptible to nutrient losses during storage, ranging from 5 to 21% of DM loss depending on ensiling conditions (Borreani et al., 2018).

Since legume silages have high concentrations of CP relative to other forages，the preservation of protein quality is of special concern (Dewhurst et al., 2009). It is estimated that approximately 44-87% of alfalfa protein can be degraded to NPN during ensiling (Albrecht and Muck, 1991). For alfalfa, this decrease in protein quality represents losses of $100 million per year in the US alone (Sullivan and Hatfield, 2006). Nutrient losses can be especially high when producers are forced to ensile legumes below 30% DM, because conditions are favorable for spoilage microbes (e.g. clostridia)

and effluent losses. Thus, high moisture alfalfa silage presents a formidable challenge for nutrient preservation but also a great platform to test novel silage preservatives.

Lignosulfonates are byproducts of the papermaking process and approximately 1 million tons are produced each year (Gosselink et al., 2004). For several decades, they have been largely used as pelleted feed binders (Corey et al., 2014) and to protect legume seed proteins from microbial degradation in the rumen (Petit et al., 1999). In particular, several studies report lignosulfonates ability to increase RUP of legume meals with a subsequent improvement in protein utilization (Windschitl and Stern, 1988;Mansfield and Stern, 1994;Petit et al., 1999), most likely due to their capacity to bind and precipitate proteins (Cerbulis, 1978). Recently, Reyes et al. (2020) observed that when sodium lignosulfonate (Sappi North America., Skowhegan, ME) was added to high-moisture alfalfa hay, there was a decrease of both hay and ruminal NH_3-N concentration. However, there are no studies that have been conducted to evaluate the effects of lignosulfonates in silages.

Currently, homofermentative and facultative heterofermentative lactic acid bacteria inoculants are preferentially used as silage additives for legumes (Oliveira et al., 2017) in the US due to the hazardousness and cost of chemical preservatives such as formic acid (Drouin et al., 2019). In the meta-analysis conducted by Oliveira et al. (2017), it was reported that inoculation with homofermentative and facultative heterofermentative LAB to forages in the dataset resulted in an increased production of lactic acid with a subsequent decrease in pH that improved DM recovery and depressed mold and clostridia counts and NH_3N relative to untreated silage. When the alfalfa data subset was analyzed in the same meta-analysis, benefits identified were limited to

decreases in pH, mold counts, and ethanol concentration. Consequently, there is a need to develop novel alternatives that can preserve legume silage nutrients, especially when ensiled under non-ideal conditions that favor spoilage. The objective of this study was to evaluate the effect of sodium and magnesium lignosulfonate (NaL and MgL, respectively) at different rates and a homofermentative LAB inoculant on high moisture alfalfa silage. We hypothesized that lignosulfonates can improve the preservation of silage nutrients due to their antiproteolytic and antimicrobial properties and the inoculant by an extensive pH decrease that inhibits the activity of plant enzymes and spoilage microbes.

Materials and Methods

Substrate, Additives, and Design

An established 13-acre stand of alfalfa (*Medicago sativa*, Pioneer 54QR04) located in Exeter, Maine, was fertilized based on soil test results and recommendations for alfalfa production in Maine (Hoskins, 1997). Five plots were randomly located within the experimental site when the alfalfa was at the bud stage. On August 17, 2018, third-cut alfalfa was mowed with a BCS 725 sickle bar mower (Portland, OR) to 7.6-cm stubble height and subsequently chopped to 1.3 cm-theoretical length using a New Holland 900 Forage Harvester (New Holland, PA) when the DM concentration was 21.5%. Treatments were randomly assigned to one of the 8 forage piles generated from each plot, for a total of 40 piles (5 blocks). Treatments applied were sodium (**NaL**) or magnesium (**MgL**) lignosulfonate (Table 3-1; Sappi North America.; Skowhegan, ME) at rates of 0.5, 1, and 1.5% (w/w, fresh basis); sterile water for the untreated control [0.1% v/w, fresh basis; **CON**]; and a microbial inoculant (**INO**) solution (0.1% v/w, fresh basis).

Inoculation resulted in theoretical final application rates of log 4.0 and 4.95 cfu/ g of fresh alfalfa of *Lactobacillus plantarum* and *Pediococcus pentosaceus*, respectively. Both bacteria are classified as facultative heterofermentative LAB species (Pahlow et al., 2003). Treated alfalfa (~0.239 kg on a fresh basis) was packed into 0.29-L mini-silos using an automated mini-silo pneumatic press and sealed using a rubber lid with a water valve (~180 kg of DM/m^3; Stokes and Chen, 1994). Mini-silos were stored at 25 °C for 229 d, and weights were recorded individually at d 0 and 229 to determine DM recovery.

Sampling Procedure

At d 0 and 229, samples (200 g, fresh basis) were taken from each individual replicate to determine nutritional value, fermentation profile, and the bacterial and fungal population via standard plating technique. For d 0, samples (200 g) were obtained immediately after treatment application.

Nutritional analysis. From samples taken at d 0 and 229, subsamples were processed for the determination of DM concentration by drying at 60°C until constant weight in a forced–air oven. Dried samples were ground to pass a 1 mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA). Ground samples were analyzed for ash (600°C in a muffle furnace for 8 h; AOAC, 2000). Concentration of NDF (Van Soest et al., 1991) and ADF (AOAC, 2000) were measured sequentially using an ANKOM 200 Fiber Analyzer (ANKOM, Macedon, NY). Heat-stable-α-amylase was used for the NDF assay, but sodium sulfite was not used. Hemicellulose (NDF minus ADF) was calculated. Silage N concentration was determined using the total Kjeldahl digestion procedure. Digested samples were analyzed colorimetrically using

the sodium salicylate-nitroprusside method (Baethgen and Alley, 1989). Crude protein was calculated by multiplying N concentration by 6.25 (Church, 1993).

Water extracts were prepared by mixing 25 g of fresh alfalfa from subsamples with 225 mL of 0.1% sterile peptone water in a 400C Stomacher blender for 3 min (Seward Ltd., Worthing, UK). The solution was filtered through 2 layers of sterilized cheesecloth and the pH of the fluid was measured with a calibrated Φ34 Beckman pH meter (Beckman, Brea, CA) fitted with an Accumet Universal pH electrode with an integrated temperature sensor (ThermoFisher Sci., Waltham, MA). Afterward, a portion of the extract was acidified to pH 2 with 50% H_2SO_4 and frozen (-30°C) until further analysis. Thawed samples were centrifuged at 8,000 × g for 20 min at 4°C and the supernatants was analyzed for lactic, acetic, butyric, and propionic acids, and 1,2-propanediol and ethanol concentrations using an Agilent High Performance Liquid Chromatograph 1200 series system fitted with an Agilent Hi-Plex H column (Agilent Technologies, Santa Clara, Ca) coupled to an Agilent refractive index detector (Siegfried et al., 1984). Ammonia-N concentration was measured from the acidified samples using an adaptation of the procedure outlined by Weatherburn (1967). Water soluble carbohydrates were measured using the protocol outlined by Dubois et al. (1956) using sucrose as the standard as described by Hall (2003).

Lactic acid bacteria, yeast and mold populations*.* An aliquot was taken immediately after filtering with sterilized cheesecloth and used for enumeration of bacterial and fungal populations. Serial (10-fold) dilutions of the water extracts were done in 0.1% sterile peptone water and pour-plated in de Man, Rogosa and Sharpe agar (BD Difco, Franklin Lakes, NJ) for LAB and in Malt Extract agar (BD Difco, Franklin

Lakes, NJ) for yeast and mold counts. Plates were incubated for 48 h at 37°C for LAB and for 72 to 120 h at 25°C for yeast and molds.

In vitro ruminal digestibility and fermentation. All the treatments were evaluated with a 24-h in vitro ruminal digestibility assay using alfalfa silage as the substrate, as described by Hall (2015), using 50 mL borosilicate glass tubes (Pyrex 8422; Corning NY) with phenolic screw caps fitted with a rubber liner. The ruminal fluid was representatively collected by aspiration 3 h after feeding (1200 h) from 3 lactating, ruminally cannulated Holstein cows consuming a ration consisting of grass haylage (2.8 kg), corn silage (9.5 kg), and concentrate (12.2 kg, DM basis). The ruminal fluid collection protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maine. Ruminal fluid was filtered through 2 layers of cheesecloth and flushed with CO_2, and 26 mL of medium containing rumen fluid inoculum and Goering (1970) medium were added to each tube and the suspension was incubated for 24 h at 39°C. The fermentations were terminated by placing tubes at 5°C. Tubes were centrifuged at 900 × g for 20 min at 4°C and filtered through pre-weighed F57 ANKOM bags (ANKOM, Macedon, NY). Filtrate samples were analyzed for pH as previously described, acidified to pH 2 with 50% H_2SO_4 and centrifuged at 8,000 × g for 20 min at 4°C. The supernatant was frozen (-20°C) and subsequently analyzed for concentration of VFA (Muck, 1988b) using the same HPLC as described before but fitted with a diode-array detector. Ammonia-N concentration was measured as described previously. Residues contained in ANKOM bags were analyzed for NDF as previously described. True DMD and NDFD were calculated from the residue and original sample weight and their DM and NDF concentrations.

Statistical Analysis

Data on nutritional value and microbial population were analyzed separately for d 0 and 229 using the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). The main effects of the treatments and block (n= 5) were included in the model. When differences were significant, means were separated using orthogonal polynomial contrasts to examine linear or quadratic effects of NaL or MgL. Specific orthogonal contrasts were used to compared CON, NaL or MgL with INO. Data were tested for normality using the Shapiro-Wilk test and significance was declared at $P \leq 0.05$.

Results

Before Ensiling (0 d)

We found no INO effect ($P \geq 0.074$) on any nutritional composition nor microbiological measures (Table 3-2). Increasing NaL and MgL from 0 to 1.5% increased DM (21.9 to 22.7 and 23.2 ± 0.12%, respectively; L) and ash (8.78 to 10.6 and 9.10 ± 0.08% DM, respectively; L). In the case of CP, increasing MgL linearly decreased CP (22.4 to 20.8; L) but NaL did not affect it (~22.0 ± 0.39% DM). A minor cubic response was observed on NH_3-N concentration due to NaL and MgL applications. Both NaL and MgL did not have an effect on WSC (~6.18 ± 0.21), NDF (~42.9 ± 0.84), hemicellulose (~ 10.7± 0.27), ADF (~32.2 ± 0.68% DM) concentrations, and LAB (~7.03 ± 0.09 log cfu/g fresh alfalfa) counts. However, increasing NaL dose from 0 to 1.5% decreased mold counts linearly (4.48 to 3.62 ± 0.14 log cfu/ g fresh alfalfa) and increased silage pH (5.97 to 6.24 ± 0.039). Application of MgL had a minor cubic effect on yeast counts but did not affect silage pH (~6.01).

Silo Opening (229 d)

Nutritional Composition. The effects of NaL and MgL at different doses and INO on the nutritional composition of ensiled alfalfa are shown in Table 3-3. At opening, increasing NaL from 0 to 1.5% linearly increased DM (18.9 to 19.9 ± 0.19%) and ash (9.7 to 10.9 ± 0.15% of DM) and decreased CP (21.0 to 19.9 ± 0.30% of DM), following the trends reported at d 0. Increasing the application rate of NaL did not affect WSC (~0.70 ± 0.650% of DM), hemicellulose (~8.76 ± 0.325% of DM) or ADF (~33.8 ± 0.59% DM). In the case of MgL, as doses increased to 1.5%, the concentrations of DM and ash linearly increased to 19.6% and 10.2 % of DM and decreased for CP to 20.4 % DM and ADF to 32.8% DM. Quadratic effects of MgL were observed on WSC and cubic effect on hemicellulose concentrations (Table 3-3). Neither NaL nor MgL had an effect on NH_3-N (~10.9 ± 0.54% of N), or NDF (~42.5 ± 0.59) values. Compared to untreated silage ($P \leq 0.018$), INO increased NDF (42.7 vs. 44.5% of DM) and hemicellulose (8.55 vs. 9.75% of DM), and decreased WSC (0.81 vs. 0.45% of DM), but did not have effect on DM (~18.9%), ash (~9.64% of DM), CP (~21.1% of DM), ADF (~34.4% DM), and NH_3-N (~11.6% of N).

Silage Fermentation. We observed that increasing the MgL dose from 0 to 1.5% linearly increased DM losses (11.3 to 14.1 ± 0.67%) and pH (4.33 to 4.45 ± 0.018) and quadratically decreased lactic acid concentration (9.23 to 7.15 ± 0.235 % DM) and the L:A ratio (2.30 to 1.67 ± 0.065) but did not affect acetic acid (~4.22 % of DM; Table 2-4). Similarly, INO increased DM losses (13.7%) and pH (4.46) and decreased lactic acid (7.83% of DM) and the L:A ratio (1.89; $P \leq 0.008$) but did not affect acetic acid (~4.10% of DM) concentration relative to untreated silage. Increasing the application rate of NaL to 1.5% linearly increased pH to 4.56 but the DM losses were not increased as with the

latter treatments (~11.8%). However, lactic acid and the L:A ratio decreased [7.58% DM (Q) and 1.71 (L), respectively) and acetic acid increased (4.47% DM; L). No additive had an effect on ethanol concentration (~0.53% of DM). Propionic acid, butyric acid, and 1,2-propanediol were not detectable in this study for any treatment (<0.014% of DM).

Microbial Population. The effects of NaL and MgL at different doses and INO on microbial counts are shown in Table 2-4. At opening, increasing the MgL dose from 0 to 1.5% linearly increased LAB counts (6.42 to 7.06 ± 0.136 log cfu/g fresh alfalfa). Similarly, as NaL application rate increased to 1.5%, LAB counts were quadratically increased to 6.90 log cfu/g fresh alfalfa. The INO also increased LAB counts to 6.98 log cfu/g fresh alfalfa, when compared to untreated silage (P = 0.003). Across all treatments, yeasts and mold counts were below the detection level at silo opening (< 2 log cfu/g of fresh alfalfa).

In vitro Ruminal Digestibility. The effects of NaL and MgL at different doses and INO on 24-h ruminal in vitro dry matter digestibility (**DMD**), neutral detergent fiber digestibility (**NDFD**), and fermentation measurements are shown in Table 2-5. Treatments did not affect ruminal pH (~7.08 ± 0.057), DMD (~71.0 ± 0.82%), NDFD (~32.2±1.85 % of DM), NH_3-N (~60.2 ± 1.32 mg/dL), and isobutyric concentrations (~2.39 ± 0.114m*M*). The application of NaL quadratically decreased total VFA concentration (**TVFA**, 97.1 to 86.8 ± 0.893 m*M*) and linearly decreased acetic (53.1 to 52.0 ± 0.39), propionic (22.0 to 20.7 ± 0.27), butyric (11.9 to 11.0 ± 0.13), and isovaleric acids (4.37 to 3.97 ± 0.093 m*M*) but increased the A:P ratio (2.41 to 2.52 ± 0.025; L). Sodium lignosulfonate did not affect valeric acid concentrations (~3.10 m*M*). In the case of MgL, as doses increased to 1.5%, there was a decrease in the concentration of TVFA

to 91.5 (m*M*, L), acetic acid to 52.3 (m*M*, Q), propionic to 20.7 (L), butyric to 11.6 (L), isovaleric to 4.02 (L) and the A:P ratio to 2.53 (L). The INO increased the A:P ratio (to 2.48; $P < 0.03$) but did not affect total VFA, acetic acid, propionic, butyric, isobutyric, isovaleric, and valeric concentrations (~97.3, ~53.5, ~21.9, ~12.0, ~2.41, ~4.34, and 3.21 m*M*, respectively).

Discussion

Before ensiling (0 d)

Concentrations of ash, CP, and NDF were closer to the values reported by Yu et al. (2003) for alfalfa at bud stage. Similarly, concentration of WSC was comparable to previously reported values by Bolsen et al. (1992) for alfalfa at late bud stage. The chemical composition of the lignosulfonates tested in this study (Table 3-1) explained the increase in DM and ash and the decrease in CP observed at d-0 as application rates increased, especially at 1.5% (w/w, fresh basis). Similar results were reported by Killerby et al. (2020c). Furthermore, the addition of NaL linearly increased silage pH, in contrast, MgL did not. These effects are due to the initial pH of both lignosulfonates as shown in Table 3-1.

The initial LAB counts were high enough to ensure adequate spontaneous fermentation (~6.99 ± 0.09 log cfu/fresh g) and provide the minimum number of LAB required for clostridial suppression (Pahlow et al., 2003). The yeast count was comparable to what Moon et al. (1981) reported for fresh alfalfa (6.5 log cfu/fresh g) but higher than Lin et al. (1992; 5.35 log cfu/fresh g). The high yeast count may be related environmental factors and harvest conditions (Pahlow et al., 2003). In the case of molds, our results are comparable to what has been reported in other studies for alfalfa

at d 0 (4.82 log cfu/ fresh g, Silva et al., 2016; 4.1 log cfu/fresh g, Blajman et al., 2020). No effects of INO and both lignosulfonates were found on microbial counts with the exception of NaL on mold counts. The linear decrease of mold counts observed as the application rate of NaL increased can be explained by the antifungal properties of sodium lignosulfonate as reported by Jha and Kumar (2018), and explained by Reyes et al. (2020). These authors hypothesized that the antifungal mechanism of lignosulfonates are due to its surfactant properties, which interact with microbial structures and disrupt normal cellular functions (Núñez-Flores et al., 2012). McDonnell (2007) proposed that after adsorption and penetration of microbial cell wall, surfactants react with the cytoplasmic membrane and causes leakage of lower-molecular weight intracellular material, degradation of proteins and nucleic acids, and finally, cell lysis and death.

Silo Opening (229 d)

Nutritional composition, fermentation, and microbial counts. In our study, the application of INO increased the DM loss of alfalfa silage. Unfortunately, few studies have reported the effects of inoculation on DM losses of alfalfa silages (Oliveira et al., 2017; Blajman et al., 2020). Oliveira et al. (2017) reported an increase in DM recovery for grasses (+2.77%) but a decrease for sugarcane (-2.39%) with no effects on the category referred to as “others” in that silage meta-analysis (-1.39%). As also observed in this study, Arriola et al. (2015) reported that when an inoculant consisting of *L. plantarum* and *P. pentosaceus* was applied to bermudagrass (5 log cfu/ fresh g) it numerically decreased DM recovery compared with thecontrol (97.6 vs. 102%, respectively). Inoculation of legume silages can improve the fermentation processes by

accelerating the decrease of silage pH (Silva et al., 2016) which inhibits the growth of enterobacteria and clostridia (Pahlow et al., 2003). Fast acidification is crucial for legumes due to their relatively low WSC values and high buffering capacity, as mentioned previously. For instance, when Chen et al. (2019) applied a *L. plantarum* and *P. pentosaceus* mixture (6 log cfu/fresh g) to high moisture alfalfa silage, DM losses decreased when compared with the control (15.1 vs 17.6%) due to an increase in lactic acid (5.21 vs 3.53 % DM, respectively). In that study the LAB counts in the pre-ensiled alfalfa were 5.52 log cfu/fresh g and consequently the inoculation rate was higher than the epiphytic LAB population, which aided in the successful establishment of the inoculant. However, in our study, we did not observe the benefits from inoculation since the epiphytic LAB in untreated silage caused an extensive homolactic fermentation which decreased silage pH lower than the inoculated silage. According to Kung et al. (2003) an inoculant may not have effect on silage fermentation when is overwhelmed by epiphytic microflora. In that sense, Muck (1989) reported that inoculants improve silage fermentation when they are applied at 10% or more of the natural level of LAB but when they are applied at less than 1% of the epiphytic population there is no effect of inoculation, like in our study (0.99% of d-0 LAB count). Furthermore, Muck (1996) reported that 5 log cfu/g is the minimum required epiphytic LAB to minimize the losses during fermentation process. In our study, epiphytic LAB counts (6.99 log cfu/fresh g) were higher than this threshold (Muck, 1996).

The cut order and frequency across the growing season also have an effect on alfalfa quality (Guo et al., 2019) and microbial counts (Lin et al., 1992). For instance, relative to the first cut, the second cut of pre-ensiled alfalfa has a higher NDF, buffering

capacity, aerobic bacteria counts, and yeast and mold counts, but lower DM and WSC concentrations (Guo et al., 2019). In the same study, the ensiled second cut alfalfa had a higher pH, acetic acid, DMD, and NDFD relative to the first cut. Overall, the first cut alfalfa silage seems to have a higher fermentation quality at the same harvest stage than later cuts (McDonald et al., 1991). In our experiment we used a third cut alfalfa, which also has been reported to have higher LAB counts than earlier cuts (Lin et al., 1992). This fact may explain our relatively high counts of epiphytic LAB. More research needs to be conducted to optimize inoculant application rates across growing season cuts.

Application of INO did increase the LAB counts at silo opening and decreased WSC compared with the untreated silage, and consequently the NDF concentrations were increased. Similar results were reported by Paradhipta et al., (2019). Likewise, Hashemzadeh-Cigari et al. (2011) reported that the inoculation of *L. plantarum* decreased WSC in alfalfa silage compared with the control (5.8 vs 7.2 %DM, respectively). Tian et al. (2017) also reported that when certain strains of *L. plantarum* were applied to high moisture alfalfa silage (73.7%), the inoculum decreased WSC and increased ADF concentrations compared with the control (4.1 vs 4.6, and 22.9 vs 20.4% DM, respectively). A recent meta-analysis analyzed the effect of homofermentative LAB on alfalfa silage and reported a decrease in pH, NDF, ethanol, acetic acid, NH_3-N, WSC, and LAB, yeast and mold counts (-0.4, -1.57, -0.21, -0.25% DM, -4.53% of N, -0.6% DM and -0.4, -1.0 and -3.4 log cfu/g, respectively) and an increase in CP, lactic acid and IVDMD-48h (+0.53%, +0.49% DM, and +5.6%, respectively) compared with the control (Blajman et al., 2020).

Relative to other studies evaluating high moisture alfalfa silage, our NH_3-N levels (~11.0% of N) did not indicate that extensive proteolysis occurred. This was partially due by the absence of clostridia activity, since butyric acid was below detection limits in our study (< 0.014% of DM). For high moisture legume silages, Kung et al. (2018) reported that levels of up to 37.5% NH_3-N (% of N) and 2% (of DM) butyric acid can be observed with extensive clostridial fermentation. In contrast, our results are more in line with the typical concentrations of NH_3-N (10-15% of N) and butyric acid (<0.5% of DM) observed in high moisture legumes silages with negligible clostridial activity (Kung et al., 2018). Mills and Kung (2002) suggested that exposure to air for the first 24 h of ensiling may be necessary to create the ideal conditions for clostridia growth beyond just high moisture conditions and pH. The exposure to air allows for the full oxidation of sugars by aerobic microbes which then limits the capacity to produce the organic acids required to inhibit clostridia and other undesirable microbes (Mills and Kung, 2002). Conversely, we observed a thorough acidification in the untreated silage most likely carried by a very active wild-type homofermentative LAB, which reduced the pH even further than INO. This is the most likely explanation on why INO failed to decrease hay NH_3-N in this study.

Both lignosulfonates did not decrease hay NH_3-N concentrations further compared with the untreated silage despite their antiproteolytic properties reported for alfalfa hay (Reyes et al.,2018) and in the rumen environment (Mansfield and Stern, 1994;Petit et al., 1999), which is likely linked to their capacity to precipitate proteins (Cerbulis, 1978). Lignosulfonates have been commercially used to increase ruminal protein bypass of legume meals by up to 173% with no negative effects on performance

(Petit et al., 1999). Windschitl and Stern (1988) reported that when calcium lignosulfonate was applied to soybean meal, this lignosulfonate reduced ruminal protein degradation compared with the control (53.7 vs 70.6%, respectively). In the case of hay, Reyes et al. (2020) speculated that the antiproteolytic effect of lignosulfonates was due to a reduction or inhibition of the metabolic activity of microorganisms that cause aerobic spoilage. In our study, both lignosulfonates tested did not have an effect on proteolysis (measured as NH_3-N). The fact that pH was increased by NaL before ensiling and by both lignosulfonates at opening (Table 3-3) may have limited their antiproteolytic effects. We also observed that MgL decreased WSC concentrations but NaL did not affect it relative to untreated silage. This could partially explain the DM loss results at opening, as MgL increased it but NaL did not affect it. Furthermore, MgL decreased the L:A ratio suggesting that its fermentation was less homolactic compared with the untreated silage.

In our study, both lignosulfonates increased LAB counts during ensiling. Similar stimulatory effects on LAB have been reported for alternative uses. For instance, Flickinger et al. (1998) reported that when two types of lignosulfonates (derived from hardwood and softwood) were added to the diet of rats (3% inclusion rate, DM basis) *Bifidobacterium* and *Lactobacillus* counts were higher than the untreated diet in the colonic and cecal microflora (9.90 vs 9.60 and 8.22 vs 7.57 log cfu/ g of feces, respectively). Similarly, Baurhoo et al. (2007) reported that when alcell lignin (Alcell Technologies Inc., Montreal, Quebec, Canada) was added to the diet of broiler chickens, Lactobacilli and Bifidobacteria counts were higher than the untreated diet (8.75 vs 8.25, and 4.75 vs 3.25 log cfu/g, respectively). Therefore, NaL and MgL may

stimulate the growth of beneficial bacteria in silage, most likely heterofermentative LAB if we were to consider the L:A ratio and the increase in acetic acid for NaL.

In vitro Ruminal Digestibility. Our results did not show an improvement of DMD or NDFD by the treatments. In the case of lignosulfonates, only Reyes at el. (2020) has evaluated their effects on forages. That study reported that when sodium and magnesium lignosulfonates were added to high moisture alfalfa hay, DMD and NDFD were higher than the untreated silage. Furthermore, sodium lignosulfonate prevented the increase of NDF by decreasing hay spoilage, however, magnesium lignosulfonate did not have the same effect (Reyes et al., 2020). For that reason, Reyes et al. (2020) suggested that magnesium lignosulfonate could stimulate rumen fibrolytic bacteria activity by improving the adsorption of microbial enzymes onto feed particles while sodium lignosulfonate effects were mediated by the preservation of nutrients alone. Similarly, Standford et al. (1995) reported that when lignosulfonates were added to a barley-based and grass hay-based diets, there was an increase of NDFD during in situ ruminal fermentation. This improvement of NDFD may be due to longer availability of essential growth factors (NH_3-N, peptides, and branched-chain VFA) to cellulolytic bacteria after feeding (Veen, 1986). The reason why lignosulfonates did not improve digestibility in this study may be a consequence of the ensiling process, as this is the first study to assess their effects in silage. More studies are need to reach a conclusive explanation.

In agreement with our INO results, Kozelov et al. (2008) reported that when an inoculant from Pioneer Hi-Bred International (Johnson, IA, USA) consisting of *L. plantarum* (4 strains) and *Enterococcus faecium* (2 strains) was applied to high moisture

alfalfa silage (74%), the inoculum did not affect in vitro DMD and NDFD. Furthermore, a meta-analysis conducted by Oliviera et al. (2017) reported that inoculation with homofermentative and facultative heterofermentative LAB to forage did not affect in vitro or total-tract in vivo DMD across forage types assessed. The same response was reported in the meta-analysis conducted by Blajman et al. (2020) with alfalfa silage inoculated with homolactic inoculants.

In this study, treatments did not affect in vitro ruminal NH_3-N concentrations. However, several authors have reported that the application of lignosulfonates decreased ruminal NH_3-N under *in vivo* (Windschitl and Stern, 1988;Stanford et al., 1995;Wright et al., 2005) and *in vitro* conditions (McAllister et al., 1993;Reyes et al., 2020) compared with the control. Lignosulfonates effectively protected feed protein (canola and soybean meal) during ruminal degradation and increased rumen undegradable protein (Wright et al., 2005;Wang et al., 2009) probably due to their capacity to bind and precipitate proteins (Cerbulis, 1978). However, it is important to note that this is the first silage study evaluating lignosulfonates and the fermentation processes during storage may have affected the capacity of lignosulfonates to reduce ruminal NH_3-N. Furthermore, Reyes et al. (2020) did not observe a reduction of ruminal NH_3-N with MgL-treated hay, as observed for NaL-treated hay. More research should be conducted to understand better the effects across lignosulfonate types. In the case of INO, Sharp et al. (1994) reported that when an inoculant composed by *L. plantarum* and *S. faecatis* were applied to a mixture of high moisture silage (80.6%) consisting of predominantly *Lolium perenne* and *Trifolium repens,* the inoculum did not affect rumen pH, ruminal NH_3-N or molar proportions of acetate but increased propionate and

butyrate. Conversely, Chen et al. (2019) reported that when exogenous LAB strains were added to high moisture alfalfa silage, there was a decrease of *in vitro* ruminal NH_3-N concentrations and an increase of propionic acid but there was no effect on acetic or butyric acid concentrations. The decrease of ruminal NH_3-N concentrations manifested in a more efficient use of N since microbial crude protein was higher with the inoculum compared with the control (Chen et al., 2019).

In our study both lignosulfonates decreased ruminal total VFA, acetic, propionic, and butyric acid concentrations relative to untreated silage while Reyes et al. (2020) reported that when sodium lignosulfonate was added to high moisture alfalfa hay, there was an increase of ruminal total VFA, and acetic acid with doses as low as 0.5% (w/w) and higher doses were needed for an increase of propionic acid and butyric acid (1 and 3% w/w, respectively). Also, the same authors reported that when magnesium lignosulfonate was applied at 0.5% (w/w), total VFA and butyric acid decreased (78.0 vs 86.7, 8.79 vs 10.9 m*M*, respectively) but did not affect acetic acid compared with the the control (~47.8). Wright et al. (2005) reported that the application of a lignosulfonate (Lignotech US, Inc., Rothschild, WI) on canola meal decreased the total amounts of acetic and propionic acid compared with the control (57.4 vs 59.9 and 20.3 vs 22.2 m*M*, respectively) due to the numerical decrease of total VFA when lignosulfonates were applied (95.1 vs 100.6 m*M*, respectively). Also, Windschitl et al. (1988) reported that when Ca lignosulfonate (Reed Lignin, Inc., Rothschild, WI) was added to soybean meal, total ruminal VFA and propionic acid decreased but acetic acid increased compared with the control (105.5 vs 120.6, 18.6 vs 27.3, and 61.1 vs 58.8 m*M*, respectively). Since lignosulfonate-treated legume meals objective is to increase RUP, less rumen

fermentability and production of VFAs may have occurred in these studies. A similar effect may have occurred in this silage study. Differences between ensiling and haymaking may explain the differences between this study and Reyes et al. (2020).

Conclusion

Relative to untreated silage, both MgL and INO increased the DM losses of high moisture alfalfa silage during storage. Such results can be explained in part by a lower production of lactic acid in all treated silages, which resulted in a less acidification relative to untreated silage. This alongside no changes in acetic acid levels for both MgL and INO-treated silages resulted in a more homolactic fermentation process in untreated relative to all additives, as reflected in a higher L:A ratio for the former. The higher acidification in untreated silage may help explain why all additives tested failed to reduce the extent of proteolysis too, measured as NH_3N, relative to untreated silage. Despite reducing mold counts at d 0, increasing LAB counts at d 229, and not increasing DM losses relative to untreated silage, NaL failed to improve the nutrient preservation of high moisture alfalfa silage. Furthermore, lignosulfonates did not increase in vitro ruminal digestibility nor reduced ruminal NH_3N as reported in a previous hay study (Reyes et al., 2020). Our results may indicate that lignosulfonates exert contrasting effects on nutrient preservation across a gradient of moisture concentration, if we compare our findings with previous hay research (reference). Furthermore, silage fermentation may have affected lignosulfonates nutrient preservation efficacy.

In the case of INO, the silage fermentation profile seems to imply a relatively higher activity of epiphytic homofermentative LAB in untreated silage, if we were to consider that INO consisted of facultative heterofermentative LAB. Also, it is possible

that due to the high counts of epiphytic LAB, INO dosage was unable to shift the fermentation profile relative to untreated silage. However, the lower residual sugar concentration and higher LAB counts at silo opening in INO do not seem to support the latter explanation. More research needs to be conducted to understand the role of epiphytic populations on silage nutrient preservation, especially at high moisture concentrations, as well as, how moisture concentration affects the efficacy of lignosulfonates as feed preservatives.

Table 3-1. Chemical composition for NaL and MgL

Lignosulfonate[1]	Total soluble phenolics[2]	ORAC[3] (mmol of Trolox equivalents/g of DM)	DPPH Scavenging effect[4] (%)	% of DM			
				WSC[5]	Ash[6]	N[7]	pH
NaL	184.3	12.1	14.2	16.2	33.9	1.54	6.58
MgL	142.5	10.1	10.5	14.1	13.6	1.29	4.95

[1]NaL = sodium Lignosulfonate and MgL = magnesium lignosulfonate.
[2]Singleton and Rossi (1965)
[3]Hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC). NaL and MgL, were tested by hydrophilic ORAC (Dong et al., 2011).
[4]Wu et al. (2006) and method 2012.04 (AOAC International, 2012). DPPH= 2,2-diphenyl-1-picrylhydrazyl.
[5]WSC= water-soluble carbohydrates; DuBois et al. (1956).
[6]FAO (2008).
[7]N=nitrogen.

Table 3-2. Effect of applying sodium (NaL) or magnesium (MgL) lignosulfonate at different rates or a homolactic inoculant (INO) on the nutritional composition and microbial counts of chopped alfalfa at d 0.

Item	pH	DM (%)	Ash (% of DM)	CP (% of DM)	NH_3-N[1] (% of DM)	WSC[2] (% of DM)	NDF (% of DM)	Hemicellulose (% of DM)	ADF (% of DM)	LAB[3] (log cfu/ fresh g)	Yeast (log cfu/ fresh g)	Molds (log cfu/ fresh g)
INO[4]	6.00	22.1	8.68	21.8	0.02	5.98	45.2	11.8	33.4	7.07	7.00	4.38
Untreated	5.97	21.9	8.78	22.4	0.02	6.30	43.4	11.0	32.3	6.99	6.73	4.48
	0.5% (w/w, fresh basis)											
NaL[5]	6.10	22.4	9.37	21.9	0.02	5.99	42.7	10.8	32.0	7.22	7.17	3.97
MgL[6]	6.03	22.3	8.78	21.9	0.02	6.03	44.3	11.0	33.2	7.07	7.20	4.29
	1% (w/w, fresh basis)											
NaL	6.19	22.5	10.1	22.0	0.02	5.95	42.6	10.5	32.1	7.05	7.09	3.98
MgL	6.04	22.7	9.04	21.2	0.01	6.54	42.7	10.7	32.0	6.94	6.53	4.32
	1.5% (w/w, fresh basis)											
NaL	6.24	22.7	10.6	21.6	0.03	6.26	41.9	10.5	31.4	7.10	7.00	3.62
MgL	5.99	23.2	9.10	20.8	0.01	6.18	42.7	10.5	32.2	6.85	6.80	4.44
SEM	0.039	0.12	0.08	0.39	0.001	0.21	0.84	0.27	0.68	0.09	0.19	0.14

Table 3-2. Continued.

Polynomial effects[7]												
NaL rate	L**	L**	L**	NS	CU**	NS	NS	NS	NS	NS	NS	L**
MgL rate	NS	L**	L**	L**	CU*	NS	NS	NS	NS	NS	CU*	NS
Contrasts												
INO vs. Untreated	0.657	0.232	0.343	0.296	0.402	0.295	0.144	0.074	0.272	0.547	0.325	0.626
INO vs. NaL	0.0001	0.002	< 0.001	0.949	0.001	0.719	0.009	0.001	0.053	0.602	0.691	0.003
INO vs. MgL	0.533	< 0.001	0.002	0.277	0.370	0.276	0.055	0.004	0.234	0.262	0.492	0.831

[1] NH_3-N = ammonia N.
[2] WSC = water-soluble carbohydrate.
[3] LAB = lactic acid bacteria.
[4] INO = *Lactobacillus plantarum* and *Pediococcus pentosaceus* at 10,000 and 90,000 cfu/g of fresh alfalfa, respectively.
[5] NaL = sodium Lignosulfonate.
[6] MgL = magnesium lignosulfonate.
[7] Linear (L) and quadratic (Q) effect. NS: no significant effect; *: $P < 0.05$; **: $P < 0.01$.

Table 3-3. Effect of applying sodium (NaL) or magnesium (MgL) lignosulfonate at different rates or a homolactic inoculant (INO) on the nutritional composition of alfalfa silage at d 229.

Item	DM (%)	Ash (% of DM)	CP (% of DM)	NH_3-N[1] (% of N)	WSC[2] (% of DM)	NDF (% of DM)	Hemicellulose (% of DM)	ADF (% of DM)
INO[3]	18.8	9.58	21.2	11.7	0.45	44.5	9.75	34.8
Untreated	18.9	9.70	21.0	11.4	0.81	42.7	8.55	34.1
0.5% (w/w, fresh basis)								
NaL[4]	18.8	9.89	20.5	10.1	0.65	42.8	9.09	33.7
MgL[5]	18.8	9.73	20.8	11.2	0.59	42.7	8.78	33.9
1% (w/w, fresh basis)								
NaL	19.6	10.5	20.4	11.2	0.65	42.4	8.53	33.9
MgL	19.3	10.1	20.2	10.3	0.62	43.2	10.09	33.1
1.5% (w/w, fresh basis)								
NaL	19.9	10.9	19.9	11.0	0.68	42.5	8.88	33.6
MgL	19.6	10.2	20.4	10.9	0.70	41.2	8.42	32.8
SEM	0.19	0.15	0.30	0.54	0.065	0.59	0.325	0.59

Table 3-3. Continued.

Polynomial effects[6]								
NaL rate	L**	L**	L**	NS	NS	NS	NS	NS
MgL rate	L**	L**	L*	NS	Q*	NS	CU**	L*
Contrasts								
INO vs. Untreated	0.503	0.552	0.669	0.642	0.0001	0.018	0.006	0.380
INO vs. NaL	0.002	<.0001	0.006	0.109	0.004	0.003	0.011	0.094
INO vs. MgL	0.021	0.012	0.028	0.117	0.009	0.001	0.058	0.020

[1] NH_3-N = ammonia N.
[2] WSC = water-soluble carbohydrate.
[3] INO = *Lactobacillus plantarum* and *Pedioccocus pentosaceus* at 10,000 and 90,000 cfu/g of fresh alfalfa, respectively.
[4] NaL = sodium Lignosulfonate.
[5] MgL = magnesium lignosulfonate.
[6] Linear (L) and quadratic (Q) effect. NS: no significant effect; *: $P < 0.05$; **: $P < 0.01$.

Table 3-4. Effect of applying sodium (NaL) or magnesium (MgL) lignosulfonate at different rates or a homolactic inoculant (INO) on fermentation measures and microbial counts of alfalfa silage at d 229.

Item	DM loss (%)	pH	Lactic Acid (% of DM)	Acetic Acid (% of DM)	L:A ratio[1]	Ethanol (% of DM)	LAB[2] (log cfu/ fresh g)	Yeast (log cfu/ fresh g)	Molds (log cfu/ fresh g)
INO[3]	13.7	4.46	7.83	4.15	1.89	0.53	6.98	<2	<2
Untreated	11.3	4.33	9.23	4.04	2.30	0.53	6.42	<2	<2
0.5% (w/w, fresh basis)									
NaL[4]	11.7	4.37	8.35	4.06	2.06	0.54	6.91	<2	<2
MgL[5]	13.4	4.36	7.99	4.17	1.93	0.52	6.94	<2	<2
1% (w/w, fresh basis)									
NaL	11.7	4.48	7.54	4.24	1.78	0.53	6.99	<2	<2
MgL	13.7	4.41	7.52	4.35	1.74	0.53	7.00	<2	<2
1.5% (w/w, fresh basis)									
NaL	12.7	4.56	7.58	4.47	1.71	0.53	6.90	<2	<2
MgL	14.1	4.45	7.15	4.30	1.67	0.55	7.06	<2	<2
SEM	0.67	0.018	0.235	0.133	0.065	0.019	0.136		

Table 3-4. Continued.

Polynomial effects[6]							
NaL rate	NS	L**	Q*	L*	L**	NS	Q*
MgL rate	L**	L**	Q*	NS	Q*	NS	L**
Contrasts							
INO vs. Untreated	0.008	<.0001	<.0001	0.528	<.0001	0.870	0.003
INO vs. NaL	0.023	0.832	0.968	0.449	0.520	0.995	0.725
INO vs. MgL	0.947	0.007	0.259	0.355	0.095	0.920	0.886

[1] L = lactic acid, A = acetic acid
[2] LAB = lactic acid bacteria
[3] INO = *Lactobacillus plantarum* and *Pedioccocus pentosaceus* at 10,000 and 90,000 cfu/g of fresh alfalfa, respectively.
[4] NaL = sodium Lignosulfonate.
[5] MgL = magnesium lignosulfonate.
[6] Linear (L) and quadratic (Q) effect. NS: no significant effect; *: $P < 0.05$; **: $P < 0.01$.

Table 3-5. Effect of applying sodium (NaL) or magnesium (MgL) lignosulfonate at different rates or a homolactic inoculant (INO) on the 24-h in vitro DM digestibility (DMD), NDF digestibility (NDFD), and rumen fermentation measurements of alfalfa silage at d 229.

Item	IVDMD[1] (%)	NDFD[2] (% of DM)	pH	NH_3-N (mg/dL)	Total VFA (m*M*)	Acetic Acid (m*M*)	Propionic Acid (m*M*)	A:P[3] ratio	Butyric (m*M*)	Isobutyric (m*M*)	Isovaleric (m*M*)	Valeric (m*M*)
INO[4]	69.5	31.5	7.06	59.8	97.4	53.8	21.7	2.48	12.0	2.31	4.30	3.25
Untreated	70.8	31.7	7.16	61.6	97.1	53.1	22.0	2.41	11.9	2.50	4.37	3.17
0.5% (w/w, fresh basis)												
NaL[5]	71.8	34.1	6.99	58.9	88.1	52.4	21.2	2.47	11.4	2.30	4.07	3.05
MgL[6]	70.5	30.9	7.08	60.4	94.6	52.2	21.0	2.48	11.7	2.40	4.20	3.10
1% (w/w, fresh basis)												
NaL	71.0	31.7	7.12	58.0	86.1	51.3	20.5	2.51	11.3	2.32	4.02	3.06
MgL	70.7	32.1	7.03	61.5	93.1	51.4	20.8	2.47	11.5	2.34	4.13	2.91
1.5% (w/w, fresh basis)												
NaL	71.6	33.0	7.09	60.4	86.8	52.0	20.7	2.52	11.0	2.42	3.97	3.13
MgL	72.3	32.7	7.13	61.2	91.5	52.3	20.7	2.53	11.6	2.52	4.02	2.95
SEM	0.82	1.85	0.057	1.32	0.893	0.39	0.27	0.025	0.13	0.116	0.093	0.091

Table 3-5. Continued.

Polynomial effects[7]												
NaL rate	NS	NS	NS	NS	Q**	L*	L**	L**	L**	NS	L**	NS
MgL rate	NS	NS	NS	NS	L**	Q*	L**	L**	L*	NS	L**	L*
Contrasts												
INO vs. Untreated	0.220	0.959	0.165	0.280	0.777	0.141	0.393	0.033	0.806	0.206	0.584	0.529
INO vs. NaL	0.030	0.464	0.904	0.599	<.0001	<.0001	0.002	0.532	<.0001	0.746	0.006	0.087
INO vs. MgL	0.064	0.837	0.693	0.380	<.0001	<.0001	0.004	0.659	0.010	0.364	0.056	0.009

[1] IVDMD = in vitro dry matter digestibility after 24 h of incubation
[2] NDFD = neutral detergent fiber digestibility after 24 h of incubation
[3] A = acetic acid, P = propionic acid
[4] INO = *Lactobacillus plantarum* and *Pediococcus pentosaceus* at 10,000 and 90,000 cfu/g of fresh alfalfa, respectively.
[5] NaL = sodium Lignosulfonate.
[6] MgL = magnesium lignosulfonate.
[7] Linear (L) and quadratic (Q) effect. NS: no significant effect; *: $P < 0.05$; **: $P < 0.01$.

CHAPTER 4

AN OPTIMIZED LIGNOSULFONATE-BASED PRODUCT MATCHED PROPIONIC ACID PRESERVATION EFFECTS ON HIGH-MOISTURE ALFALFA HAY

Introduction

Hay is the second most widely used method of forage conservation (NASS, 2020a) and the third most valuable crop in the U.S. (NASS, 2020b). In particular, alfalfa hay alone contributes $9 billion per year to the US economy (NASS, 2020b). However, significant interdependent nutrient losses can occur during hay harvest and storage (Coblentz and Hoffman, 2010). As hay moisture at baling is decreased below 15%, DM storage losses become negligible but losses during harvest increase as much as 15% (Collins, 1996). Conversely, as hay moisture is increased above 20% DM, harvest losses decrease below 5% but storage losses rise over 24% (Ball et al., 1998;Coblentz and Bertram, 2012). Storage losses are the direct consequence of nutrient oxidation by spoilage microbes, which results in a decrease in nutritive value (Coblentz and Hoffman, 2009), an increase in greenhouse gas emissions (Emery and Mosier, 2015), and the production of harmful mycotoxins (Roberts, 1995).

Preservatives typically have been used to prevent storage losses when hay is baled above 20 and 15% moisture for small and large bales, respectively (Collins et al., 1987;Coblentz and Bertram, 2012). Propionic acid alone or in mixtures has traditionally been used to prevent spoilage in high moisture hay. A recent meta-analysis conducted by our group (Killerby et al., 2020a) assessed the overall effect of propionic acid on hay preservation, and reported (as standardized mean differences) a decrease in DM losses (-1.65), visible moldiness (-58.8%), heat degree-days (-3.40) and ADIN (-0.42) but an

increase of sugars (+1.95) and dry matter digestibility (+3.41). However, propionic acid benefits are transient (6 months) due to volatilization losses and metabolization by aerobic microbes (McCartney, 2005;Coblentz et al., 2013b). Furthermore, propionic acid corrodes farm equipment and is hazardous to operators during handling (Perry and Cecava, 1995). Therefore, there is a need to develop a novel hay preservative for high moisture hay that is less expensive and safer to handle at the farm level.

Recently, Reyes et al. (2020) reported that the addition of sodium lignosulfonate applied at 4 doses (0, 0.5, 1, and 3% w/w, fresh basis) on high moisture alfalfa hay decreased DM losses with at least 1% (3.39 vs 14.9 ± 0.77%; respectively) and total mold counts at 3% (3.92 vs 7.76 ± 0.55 log cfu/fresh g, respectively), compared with the control. Lignosulfonates antifungal activity, especially at low pH, seem to partially explain the preservation effects. Furthermore, lignosulfonates antiproteolytic properties can help not only to preserve protein quality (Petit et al., 1999) but also potentially to increase RUP, which is beneficial for alfalfa usage (Mansfield and Stern, 1994).

Chitosan is a polycationic polymer that is non-toxic to humans (Olicón-Hernández et al., 2015). It has been used in the food and agricultural industry as a preservative due to its antimicrobial activity against fungi and bacteria (Kanatt et al., 2008;Olicón-Hernández et al., 2015). It causes permeabilization of the microbial membrane due to its polycationic structure that binds to the anionic components of microorganisms (cell surface proteins) (Kong et al., 2010). Furthermore, it inhibits fungal growth and respiratory activity, and causes swelling and destruction of the microbial membrane (Olicón-Hernández et al., 2015). However, chitosan remains to be tested as a potential hay preservative.

Our first objective was to compare the fungistatic and fungicidal activity of 5 lignosulfonates and 2 chitosans sources against fungi isolated from spoiled hay. The second objective was to evaluate the effects of an optimized lignosulfonate-based product (**LST,** UMaine), chitosan (**ChNv**, Sigma-Aldrich), and propionic acid (**PRP**, 99%; MP Biomedicals) on the preservation of high-moisture alfalfa hay using an in vitro aerobic incubation assay. We hypothesized that LST and ChNv can reduce DM losses and preserve the nutritive value of high moisture alfalfa hay during aerobic storage.

Materials and Methods

Experiment 1

Additives. Table 4-1 summarizes the set of lignosulfonates evaluated in this study. We also included in our test naïve chitosan (**ChNv**: Sigma-Aldrich Corp, St Louis, MO), chitosan microparticles (**ChMp**; provided by Dr. K.C. Jeong, University of Florida), and propionic acid (PRP, 99.8% w/v; MP Biomedicals, Solon, OH) and control (untreated). According to the manufacturer's information, ChNv presented the following technical specifications: molecular weight 50-190 kDa, viscosity 20-300 cP, and deacetylation level ranges from 75 to 85%. Chitosan microparticles were fabricated from ChNv, briefly, a cross-linker sodium sulfate was added to ChNv through sonication at 60 W for 20 min (Garrido-Maestu et al., 2018). As a result, ChMp had the following characteristics: particle size 241.8 nm, and poly-dispersity index 0.362 (Garrido-Maestu et al., 2018). The ash (FAO, 2008), crude protein (Baethgen and Alley, 1989), water soluble carbohydrates (Dubois et al., 1956), minerals (Beliciu et al., 2012), and total soluble phenolics concentrations (Dong et al., 2011) of the lignosulfonates tested are listed in Table 4-1.

Antifungal assay. The minimum inhibitory concentration (**MIC**) and minimum fungicidal concentration (**MFC**) of the listed ADV were determined against previously isolated strains of the molds *Aspergillus amoenus*, *Mucor circinelloides*, *Penicillium solitum*, and of the yeast *Debaromyces hansenii*, as outlined by Reyes et al. (2020). Macrodilution assays were carried out independently three times in duplicate and values are reported as mean concentrations (mg/mL ± standard deviation; SD). Stock solutions of lignosulfonates, ChNv, and ChMp were sonicated for 60, 120, and 180 min, respectively, in an 8510 Series Ultrasonic Cleaning Bath (Emerson, St. Louis, MO) at 40°C in order to inactivate microbes with minimal effect on chemical integrity (Piyasena et al., 2003). Naïve chitosan was initially dissolved in a solution containing 1% (v/v) HCl to increase its solubility in malt extract broth (Romanazzi et al., 2009).

Experiment 2

Substrate, Additives, and Design*.* The experimental site was located at the J. Franklin Witter Teaching and Research Center in Orono, Maine. Alfalfa (Medicago sativa, HybriForce-3420/Wet) was established and fertilized based on soil test results and recommendations for alfalfa production in Maine (Hoskins, 1997). During 2019, the alfalfa field was divided in five randomly located plots and mowed to 7.6 cm stubble height with a New Holland H6830 mower (CNH Industrial, Burr Ridge, IL, USA) at 1030 h on August 5. The alfalfa was then tedded with a Kuhn GF5001MH (Kuhn North America INC, Brodhead, WI, USA) at 1200h on August 6 and allowed to wilt in the field to a 60% DM concentration on August 7. On the same day, the wilted alfalfa collected from each plot was chopped with a chipper shredder (DR, Vergennes, VT), dried at 60°C in a convection oven for 48 h, and ground to pass through a 3-mm screen of a

Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) for later use in the in vitro aerobic incubation test.

The most antifungal lignosulfonate from experiment 1 (NaNew) was modified following a proprietary process developed at University of Maine to maximize its efficacy in forage substrates. The final product of this process is referred to as LST. Since negligible differences were found between ChNv and ChMp, the latter was not selected for further evaluation since it requires the added step of producing the microparticles. The effects of LST, the selected chitosan (ChNv), and a positive control (PRP) on ground high-moisture alfalfa hay (30% moisture concentration) were evaluated in vitro using a randomized complete block design (**RCBD**) with a 3 (ADV: LST, ChNv, and PRP) × 5 (0, 0.25, 0.5, 1, and 2% w/w fresh basis) factorial arrangement of treatments and 5 blocks (alfalfa stand plots).

Antifungal activity. The antifungal activity of ADV on high moisture alfalfa hay (30% moisture concentration) using the isolates from experiment 1 was evaluated according to the modified method outlined by Reyes et al. (2020) with an incubation period of 23 d. This incubation time was selected because most of the aerobic spoilage in hay occurs during the first 2 to 5 wk of storage (Collins and Coblentz, 2007).

Sampling Procedure. At d 0 and 23, samples were taken from each replicate to determine of nutritional value (25 g, fresh basis), and microbial counts (10 g, fresh basis). In the case of d 0, samples were obtained immediately after inoculation. At d 23, hay spoilage was visually evaluated for each replicate and ranked using a scale from 0 to 10 developed by Duchaine et al. (1995). The subjective score of 7-10 was given for

marked spoilage deterioration, 5-6 for the presence of mycelia or abundant spores, 3-4 for the presence of mold spores and dust, and 0-2 for no presence of mold.

Nutritional analysis. From samples taken at d 0 and 23, subsamples were processed for the determination of DM concentration by drying at 60°C until constant weight in a forced–air oven. Dried samples were ground to pass a 2 mm screen using a Foss Cyclotec mill (Foss, Hillerød, Denmark). Ground samples were analyzed for ash, CP, NDF, ADF, DMD, and NDFD as described by Reyes et al. (2020). Water extracts were prepared by mixing 10 g of fresh alfalfa from subsamples with 90 mL of 0.1% sterile peptone water in a 400C Stomacher blender for 3 min (Seward Ltd., Worthing, UK). The solution was processed and analyzed for pH, NH_3-N, and WSC as described by Reyes et al. (2020).

Microbiological analysis. An aliquot was taken immediately after filtering with sterilized cheesecloth and used for enumeration of fungal populations. Serial (10-fold) dilutions were done in 0.1% sterile peptone water and plated on Dichloran Rose Bengal Chloramphenicol (BD Difco, Franklin Lakes, NJ). Plates were incubated for 72 or 120 h at 25°C for yeast and molds, respectively.

In vitro ruminal digestibility and fermentation. All ADV were evaluated with a 24-h in vitro ruminal digestibility assay using alfalfa hay as the substrate, as described by Hall (2015), using 50 mL borosilicate glass tubes (Pyrex 8422; Corning NY) with phenolic screw caps fitted with a rubber liner. The ruminal fluid was representatively collected by aspiration 3 h after feeding (1200 h) from 3 lactating, ruminally cannulated Holstein cows consuming a ration consisting of grass haylage (13.2 kg), cornmeal (3.3 kg), and concentrate (12.3 kg, DM basis). The ruminal fluid collection protocol was

approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maine. Ruminal fluid was filtered through 2 layers of cheesecloth and flushed with CO_2, and 26 mL of medium containing rumen fluid inoculum and Goering and Van Soest (1970) medium were added to each tube and the suspension was incubated for 24 h at 39°C. The fermentations were terminated by placing tubes at 5°C. Tubes were processed for residue and filtrate analysis to determine DM and NDF digestibility, pH, NH_3-N, concentrations of VFA as described by Reyes et al. (2020). The digestible DM recovery was also calculated according to Reyes et al. (2020).

Statistical Analysis

In experiment 1, the determination of MIC and MFC were carried out independently 3 times in duplicate and values are reported as mean concentrations (mg/mL ± SD). For experiment 2, a RCBD with a 3 (ADV) × 5 (dose) factorial arrangement of treatments and 5 blocks (stand plots) was used to determine effects of ADV and dose on spoilage, nutritional composition, and rumen *in vitro* digestibility and fermentation measures of alfalfa hay.

The model used to analyze additive effects was:

$$Y_{ijkl} = \mu + ADV_i + DOSE_j + \beta_k + ADVDOSE_{ij} + E_{ijk}$$

where μ = the general mean, ADV_i = the effect of additive I, β_k = the effect of the block k, $DOSE_j$ = the effect of dose j, $ADVDOSE_{ij}$ = the effect of the ADV $_i$ × DOSE $_j$ interaction, and E_{ijk} = the experimental error.

The GLM procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC) was used to analyze the data. When an interaction was present the SLICE option was used. Polynomial contrasts were used to determine dose effects and PDIFF procedure of LSMEANS was

used to compare least squares means within dose and ADV. Data were tested for normality using the Shapiro-Wilk test. Significance was declared at $P \leq 0.05$.

Results

Experiment 1

The MIC and MFC of the additives against the fungi tested are shown on Table 4-2. Among the lignosulfonates at pH 4, NaSP had the lowest MIC across *A. amoenus*, *P. solitum*, *M. circinelloides*, and *D. hansenii*, followed by MgSP. It is important to point out that we could not determine the MIC of NaAL or NaBT solutions at pH 4 nor chitosan solutions at both pH levels due to the high turbidity of these solutions, with the exception of chitosan against *D. hansenii* due to the very low concentrations needed for inhibition. In the case of the lignosulfonates, none inhibited molds or yeast at pH 6. In contrast, PRP inhibited all fungal strains at both pH levels, but the inhibition was more potent at pH 4.

Across lignosulfonates, NaSP at pH 4 was the most fungicidal against *A. amoenus*, *P. solitum*, and *D. hansenii*. However, NaBT was the only lignosulfonate with fungicidal activity against *M. circinelloides*. The strongest fungicidal activity across fungi tested was observed for the chitosans at both pH 4 and 6. However, both chitosans failed to kill *M. circinelloides* in any condition tested. Overall, no differences were observed in fungicidal activity between ChNv and ChMp at both pH levels evaluated. In the case of PRP, a higher antifungal activity was observed relative to NaSP at both pH 4 and 6, but it was a less effective killer than chitosans except for *M. circinelloides*. In general, PRP had a higher antifungal activity at pH 4 than 6.

Experiment 2

DM Losses, Microbial Populations, and Visual Moldiness. Effects of treatments on DM loss, hay pH, microbial counts, and visual moldiness of alfalfa hay at d 23 are shown on Table 4-3. All these variables were affected by an ADV × dose effect ($P < 0.001$). Both LST and PRP halted DM losses to the same extent with a dose as low as 0.25% (w/w), relative to untreated hay (2.02 and 1.20 vs. 24.0 ± 0.451%, respectively; $P < 0.001$). This was in part a consequence of a decrease in total mold and yeast counts as the application rates of LST and PRP were increased. A dose as low as 0.5 and 0.25% decreased total mold counts for LST (3.89) and PRP (2.25) relative to untreated hay (6.76 log cfu/fresh g), respectively. Similarly, at least 0.5% LST and 0.25% PRP decreased yeast counts relative to untreated hay (2.25 and <2 vs. 6.10 log cfu/fresh g). However, ChNv did not affect total mold nor yeast counts (~6.59 and ~6.16 log cfu/fresh g; respectively), consequently DM losses were as high as untreated hay for all ChNv doses. The visual ranking results (0-10) were correlated with the mold counts, both LST and PRP prevented visual hay moldiness with a dose as low as 0.25% (w/w), relative to untreated hay (2.8 and 0.0 vs 9.4, respectively; $P < 0.001$). However, ChNv showed marked spoilage deterioration (~9.9).

Nutritional Composition. We found an interaction between ADV × dose on all nutritive value estimates at d 23 ($P < 0.02$) except for WSC and ADF ($P > 0.165$; Table 4-4). Spoilage processes resulted in untreated hay DM (62.5) being lower than values obtained for at least 0.25% LST and PRP (~75.5%). In the case of ChNv, all doses had similar DM as untreated hay, except for 1% (64.5%). Also due to spoilage, the ash concentration was higher in untreated hay relative to at least 0.25% LST and PRP (15.3

vs ~12.1 ± 0.124) and at least 1% ChNv (14.9% DM). The original concentration of CP (20.3) was preserved by LST and PRP with a dose of at least 0.25% (~20.7 ± 0.265), while decomposition increased it in both ChNv and untreated hay, which were no different to each other with the exception of ChNv at 0.25% (~24.2 vs 23.3% DM, respectively). As a consequence of antimicrobial properties, proteolysis (measured as NH_3-N) was halted by at least 0.25% for both LST and PRP (~1.11) relative to untreated hay (7.80 ± 0.190% of N). This increase in NH_3-N partially explained the increase in pH observed in untreated hay and ChNv (~8.88), relative to LST and PRP (~5.88), which kept the NH_3-N concentration closer to the value reported at d-0 (0.4 % of N; Table 4-5). A minor increase of WSC proportion for untreated hay was observed relative to the other doses across all ADV (6.76 vs ~6.36 ± 0.215 % DM, respectively). The concentration of NDF was not affected as doses increased for LST and PRP but in the case of ChNv it was increased with at least 1% (w/w, fresh basis).

In Vitro Ruminal Digestibility. We found an interaction effect of ADV × dose on all ruminal in vitro fermentation measures ($P < 0.001$; Table 4-6), except for ruminal pH, NDFD, NH_3-N, butyric, isobutyric, and isovaleric acid concentrations. An increased DMD was observed for at least 0.25% LST (71.1) and 1% PRP (71.4) compared with the untreated hay (69.3 ± 0.499%; $P < 0.001$). Similarly, LST and PRP at a dose as low as 0.25% increased digestible DM recovery (~69.7) to a greater extent compared with the untreated hay (52.7 ± 0.614%; $P < 0.001$). In the case of ChNv, at least 0.5% slightly increased digestible DM recovery (53.1%). Fiber digestibility increased across all ADV with at least 0.5% (w/w, fresh basis) relative to untreated hay (~31.6 vs. 28.5%, respectively). Both LST and PRP had higher NDFD than ChNv (32.5 and 31.3 vs 29.9 ±

1.649%, respectively). Hay treated with PRP and ChNv had a slightly higher ruminal fluid pH (~7.29) compared with LST (7.18 ± 0.069; $P < 0.05$). Across all ADV, ruminal NH_3-N decreased with at least 0.25% (w/w, fresh basis) relative to untreated hay (49.7 vs 52.8 mg/dL, respectively). Moreover, LST and PRP (~49.5) decreased ruminal NH_3-N concentration compared with ChNv across all doses (51.6 ± 0.817 mg/dL; $P < 0.001$).

At least 0.25% LST and PRP increased total VFA (**TVFA**), relative to untreated hay (~94.4 vs 83.8 ± 1.094 m*M*, respectively). At 1 and 2% PRP, TVFA levels were higher than LST (~ 96.5 vs ~92.4 m*M*, respectively). The addition of ChNv did not affect TVFA, acetic, and propionic acid ruminal concentrations (~ 82.8 m*M*, ~49.1± 0.607 and ~15.9 ± 0.240 m*M*, respectively). The acetic acid to propionic acid (A:P) ratio was decreased using at least 0.25% (w/w) for both LST and PRP relative to untreated hay (~2.95 vs 3.08 ± 0.018, respectively) but ChNv did not affect it (~3.10). At 1 and 2% doses, the A:P ratio for PRP was lower than LST (~2.83 vs ~2.95, respectively). No treatment combinations affected butyric acid concentrations (~10.2 ± 0.170 m*M*). In the case of isobutyric acid, across doses, PRP and ChNv had higher values than LST (~1.78 vs 1.71 ± 0.052 m*M*, respectively). For isovaleric acid, PRP and LST presented lower concentrations than ChNv (~2.92 vs 3.12 ± 0.068 m*M*, respectively). Furthermore, across all ADV isovaleric acid concentrations decreased with at least 0.25% (w/w, fresh basis) relative to untreated hay (~2.96 vs 3.18 m*M*, respectively). Moreover, ChNv decreased valeric acid concentrations with a dose as low as 1% (w/w) but LST and PRP did not affect it compared with untreated hay.

Discussion

Experiment 1

In our study, NaSP had the strongest inhibitory properties among all the lignosulfonates tested when evaluated against *A. amoenus*, *P. solitum*, *M. circinelloides* (molds), and *D. hansenii* (yeast) at pH 4. Reyes at al. (2020) reported MIC values at pH 4 for sodium lignosulfonate (NaL; Sappi North America, Skowhegan, ME) of 20.0, 33.3, 25.0, and 40.0 mg/mL for *A. amoenus*, *P. solitum*, *M. circinelloides*, and *D. hansenii*, respectively. Overall, in our study we had similar results as Reyes et al. (2020) with exception of lower MIC values for *M. circinelloides* and *D. hansenii.* Although we tested sodium lignosulfonate and magnesium lignosulfonate from the same manufacturer as did Reyes et al. (2020), the lignosulfonates were from different batches and that may explain slight differences observed.

We confirmed across 5 different lignosulfonate products from 4 different sources that acidic conditions are necessary to activate the antifungal properties of lignosulfonates. It is hypothesized that the antimicrobial activity of lignosulfonates is related to their strong surfactant properties (Núñez-Flores et al., 2012), being classified as anionic surfactants due to the presence of sulfonate ($R\text{-}SO_3^-$) substitutions (Zhang et al., 2019). Surfactants can interact with microbial constituents and disrupt their cellular functions (Hugo, 1992). These properties are related to the shape and distribution of charged as well as uncharged groups on the macromolecular surface (Vainio et al., 2012). For instance, de Freitas Ferreira et al. (2019) reported that rhamnolipids –a type of anionic surfactants- had a much higher antimicrobial activity at acidic conditions relative to neutral or alkaline. Under acidic conditions, rhamnolipids become non-ionic surfactants due to the protonation of polar groups which reduces the electrostatic

repulsion with anionic groups present on microbial cell surfaces. This in turn, increases the interaction of rhamnolipids with microbial membranes which results in a reduction of microbial cell surface hydrophobicity and an increase in cytoplasmic membrane damage (de Freitas Ferreira et al., 2019).

In the case of lignosulfonates, Yan et al. (2010) reported that as conditions become more acidic, the electrostatic repulsion of lignosulfonates decrease due to protonation of sulfonic, carboxyl, and phenolic hydroxyl groups (Figure 2-4). Consequently, we speculate that lignosulfonates may have a similar antimicrobial mode of action to rhamnolipids. However, it is unclear what factors explain the relative differences in antifungal activity observed across lignosulfonates tested. We speculate that differences in tree species used as raw material (softwood vs. hardwood; Flickinger et al., 1998) and the manufacturing process (neutral sulphite semi-chemical pulping vs sulphite process; Kuenen et al. 2009) may explain variation in antimicrobial activity across lignosulfonates. Recently, Peddinti et al. (2019) reported that a styrene-based midblock-sulfonated multiblock polymer antimicrobial activity can be boosted by increasing the degree of sulfonation, which results in a reduction of surface pH that stresses microbial membranes and causes microbial death (Peddinti et al., 2019). Another factor that may affect the antimicrobial properties of lignosulfonates is the composition of their phenolic units (Dumitriu and Popa, 2013). However, the concentration of total phenolics was not found to be correlated to the antimicrobial activity of lignins in several studies (Medina et al., 2016;Reyes et al., 2020). Further research is needed to expand our understanding of technical lignins antimicrobial properties.

In this study, we reported that MIC values for chitosans ranged from 0.02 to 0.08 mg/ml. These values were within the values reported by Sahariah and Masson (2017) for different fungal species (MIC = 0.01-2.5 mg/mL) when chitosans of low or high molecular weight were applied. Although the antimicrobial activity of chitosan is not completely understood, it is widely accepted that the positive charges of chitosan interact with the negative charges present in microbial cell walls causing cell death (Sudarshan et al., 1992). Therefore, chitosan with higher degree of deacetylation – higher positive charge- is expected to show more antimicrobial activity (Jung et al., 2010). Also, Tayel et al. (2010) reported that the most antifungal chitosan against three *Candida albicans* strains had the lowest molecular weight (32 kDa) and the highest deacetylation degree (94%), and their MIC values ranged from 1.25 to 2 mg/ml. Also, Kong et al. (2010) reported that the antimicrobial activity of chitosans is affected by the target microorganism. For instance, Arancibia et al. (2015) reported that chitosan (molecular weight 3000 kDa and degree of acetylation 77%) applied at 1% against 26 microorganisms (including bacteria and fungi) had the highest inhibition against *D. hansenii* but that *Aspergilus niger* and *Penicilium expansum* were not inhibited (~12 vs ~5 and ~5 mm, respectively). In our study, both ChMp and ChNv showed similar antifungal activity, however, Yien Ing et al. (2012) reported that chitosan microparticles (crosslinked with tripolyphosphate) had a greater antifungal activity against *Candida albicans*, *Fusarium solani,* and *Aspergillus niger* than chitosan parent due to a higher affinity to fungal cell walls. This divergence could be due to differences in cross-linkers used (Garrido-Maestu et al., 2018) and fungal species evaluated (Arancibia et al., 2015). According to our results, chitosans showed fungicidal activity against all the

molds and yeast tested, with the exception of *M. circinelloides.* This could be explained by the presence of chitosanases in *M. circinelloides* (Struszczyk et al., 2009) which are enzymes that hydrolyze the β-1,4-linkages in partly *N*-acetylated chitosan (Ghinet et al., 2010). Furthermore, Allan and Hadwiger (1979) reported that chitosan inhibited the growth of many fungal species *in vitro* with the exception of Zygomycetes, a fungal class which includes *Mucor* sp., *Rhizopus nigricans*, *Circinella* sp. (s581bb), and *Conidiobolis* sp. (78-4a).

In agreement with our results, Reyes et al. (2020) reported values of MIC at pH 4 for propionic acid of 1.25 for both *A. amoenus* and *D. hansenii,* and 3.3 mg/ml for *M. circinelloides.* Furthermore, the same study reported MIC values at pH 6 of 5, 5, and 10 mg/ml for *A. amoenus, P. solitum,* and *M. circinelloides,* respectively. However, we reported a slightly higher MIC values for *P. solitum* at pH 4 and for *D. hansenii* at pH 6. In our study, PRP showed more antimicrobial activity at pH 4 than 6 as observed in Reyes et al. (2020). The antimicrobial activity of PRP is related to the reduction of pH, as well as its ability to enter the microbial cell because it is lipid soluble in its undissociated form (Haque et al., 2009). Since propionic acid has a pK_a of 4.88 (Haque et al., 2009), the proportion of its undissociated form increases when the pH is lower than 4.88. Once propionic acid passes the microbial membrane, it is dissociated which causes an accumulation of protons inside the cell (Brul and Coote, 1999). Therefore, microorganisms are induced to metabolize high amounts of ATP in order to mantain intracellular pH homeostasis (Bracey et al., 1998) which causes a reduction of energy for growth and metabolic functions (Brul and Coote, 1999). Particularly for fungi, Yu and Lee (2016) reported that PRP induces programmed cell death in these microorganisms.

Experiment 2

After 23 d of aerobic incubation, the alfalfa hay DM losses were mitigated to the same extent by at least 0.25% (w/w) LST and PRP, with no further benefit observed at higher doses compared with untreated hay. Reyes et al. (2020) reported that when sodium lignosulfonate (Sappi North America, Skowhegan, ME) was applied at 1% on high moisture alfalfa hay, there was a decrease of DM loss compared with the control (3.39 vs 14.9%, respectively). However, in this study we boosted the antifungal activity of the lignosulfonate treatment by using a proprietary low-cost procedure. Furthermore, the increased antifungal action of LST was reflected by a more extensive decrease of total mold counts compared with Reyes et al. (2020). Nonetheless, LST decreased yeast counts at the same dose as did the sodium lignosulfonate tested by Reyes et al. (2020). We speculate that the failure of ChNv to preserve hay nutrients, despite outstanding antimicrobial activity against several fungal isolates, is a consequence of the chitosanases present in *M. circinelloides*, which was the only isolate that could not be inhibited by ChNv in experiment 1. In fact, the initial hay pH (5.73; Table 4-5) was within the optimal pH range for this enzyme (5.5 to 6.0; (Struszczyk et al., 2009)

Both LST and PRP were equally effective at preventing major proteolysis during aerobic storage losses as was reported by Reyes et al. (2020), who observed that sodium lignosulfonate and propionic acid prevented proteolysis losses (expressed as NH_3-N) with a dose as low as 0.5% on high moisture alfalfa hay. This is most likely due to the antimicrobial properties of both treatments during aerobic storage, as shown in experiment 1. Lignosulfonates are also known to bind and precipitate proteins, which contributes to its antiproteolytic properties (Cerbulis, 1978). Conversely, ChNv failed to prevent the extensive proteolysis observed in untreated hay, most likely due to its

inactivation by *M. circinelloides*. Hlödversson and Kaspersson (1986) reported that when untreated high moisture alfalfa hay (37%) composed of 90% clover and 10% grass was stored during 21 d, there was an increase of N, ADF, and ash (4.2 vs 3.8, 35.7 vs 27.9, and 13.2 vs 10.1% DM; respectively), and a decrease of WSC (2.6 vs 11.2% DM) relative to initial composition, respectively. However, in our study, after 23 d of aerobic incubation, there was a negligible decrease in WSC across all treatments and for all doses relative to untreated hay(~6.36 vs. 6.76), with ChNV having slightly higher WSC relative to LST and PRP (6.70 vs. ~6.19% of DM). If we take into consideration the WSC concentration at d 0 (6.4 % of DM), the extensive DM loss, and the growth of fungi in the untreated hay and ChNV treatments, we speculate that the slightly increased sugar levels were due to pectin break down by fungal pectinases (Gundala and Chinthala, 2017).

The preservation of nutrients observed for LST manifested in maintaining, to some extent, the DMD and NDFD values observed at d 0 (71.4 and 34.7%, respectively). The LST consistently had a higher DMD relative to untreated hay across all doses, compared to PRP which only increased it at 1% (w/w). At 2%, ChNv even decreased DMD relative to untreated hay (66.6 vs. 69.3%, respectively). Fiber digestibility was higher in LST compared to PRP and ChNv, which were no different (32.5 vs. ~30.6%, respectively). Across all ADV, at least 0.5% (w/w) was needed to increase NDFD relative to untreated hay (~31.6 vs. 28.5%). It was surprising that ChNv increased NDFD despite not having an effect on nutrient preservation and this may be related to stimulatory effects on fiber digestibility (Henry et al., 2015). The decrease in NDFD between untreated and d 0 may be an indication of preferential degradation of

easily digestible fibers by molds (Coblentz et al., 1996). In agreement with our results, Reyes et al. (2020) reported that both sodium lignosulfonate and propionic acid increased DMD and NDFD compared with the control. Although it is difficult to separate preservation from stimulatory effects on digestion, lignosulfonates have been reported to stimulate ruminal NDFD. Standford et al. (1995) reported that when lignosulfonates were added to a barley-based and grass hay-based diets, there was an increase of NDFD during in situ ruminal fermentation. This improvement of NDFD may be related to the stimulation of surfactants on fibrolytic enzymes action (Reyes et al., 2020) and longer availability of essential growth factors (NH_3-N, peptides, and branched-chain VFA) to cellulolytic bacteria after feeding (Veen, 1986).

Both LST and PRP increased total VFA, acetic, and propionic acid ruminal concentrations with doses as low as 0.25% (w/w) but did not affect butyric acid concentration compared with the untreated hay. Reyes et al. (2020) reported that sodium lignosulfonate increased total VFA, acetic acid, and propionic acid ruminal concentrations but decreased butyrate with doses as low as 0.5% (w/w) compared with the control. Windschitl and Stern (1988) reported that when calcium lignosulfonate was applied to soybean meal, acetic acid was increased (64.2 vs 58.4 mol/100 mol) and propionic acid was decreased compared with the control (64.2 vs 58.4, and 19.6 vs 27.1 mol/100 mol, respectively). Similarly, Wright et al. (2005) reported that the application of lignosulfonate (Lignotech US, Inc., Rothschild, WI) on canola meal increased acetate and decreased propionate but did not affect butyrate concentration. Furthermore, these authors speculated that the changes in ruminal VFA reflect the increase of fiber digestibility by addition of the lignosulfonates. Differences in the effect of lignosulfonates

on ruminal fermentation profiles may be due to differences in substrates across studies. Overall, the increase of acetate and propionic acid are beneficial since the increase of ruminal acetate linearly increases milk fat concentration (Urrutia and Harvatine, 2017), and propionic acid increases the lactose and milk yield (Seymour et al., 2005).

LST and PRP decreased ruminal NH_3-N with doses as low as 0.25% (w/w) but ChNv did not affect it. Reyes et al. (2020) reported that sodium lignosulfonate at 3% (w/w) decreased ruminal NH_3-N, which could be reflected in an increase of rumen undegradable protein in vivo. Several authors have reported that the application of lignosulfonates can decrease ruminal NH_3-N under in vivo (Windschitl and Stern, 1988;Stanford et al., 1995;Wright et al., 2005) or in vitro conditions (McAllister et al., 1993;Reyes et al., 2020) relative to the control. Lignosulfonates effectively protected feed protein (canola and soybean meal) during ruminal degradation and increased rumen undegradable protein (Wright et al., 2005;Wang et al., 2009) probably due to their capacity to bind and precipitate proteins (Cerbulis, 1978).

Conclusions

In experiment 1, NaSP was the most antifungal lignosulfonate among all tested in this study at pH 4. However, no lignosulfonates had antifungal activity at pH 6, which indicated that protonation of lignosulfonates is a pre-requisite for effectiveness as has been observed in previous studies. On the other hand, both chitosans had the strongest fungicidal activity against *A. amoenus*, *P. solitum*, and *D. hansenii*, but presented no antifungal activity against *M. circinelloides* at both pH evaluated which was most likely due to the presence of chitosanases in the latter fungal species. Negligible differences were observed between ChNv and ChMp across all fungi and pH did not affect their

effectiveness in this study. In the case of PRP, we found a higher antifungal activity than NaSP for all fungi at both pH evaluated, but its effectiveness was reduced as pH was increased across all fungi. PRP was the most effective antifungal against *M. circinelloides* but trailed both chitosans for all other fungi.

In experiment 2, we observed that by optimizing the most antifungal lignosulfonate from experiment 1 (NaSP), we obtained comparable results to PRP for DM losses, hay proteolysis inhibition, in vitro ruminal digestibility, and in vitro ruminal NH_3N levels of high-moisture alfalfa hay incubated under aerobic conditions. However, PRP was superior to LST in reducing mold counts, visual moldiness, an in vitro ruminal TVFA concentration. Both LST and PRP preserved hay nutrients and in vitro ruminal digestibility and fermentation profile relative to untreated hay, which was deteriorated by fungal spoilage. Conversely, ChNv was ineffective at preserving the nutritional value of high-moisture alfalfa hay most likely due to inactivation by *M. circinelloides* chitosanases. However, it seemed to have stimulated NDFD, as reported in other studies, despite microbial spoilage damage. Field testing across a variety of conditions and forage crops is necessary to confirm the effectiveness of optimized lignosulfonates as high-moisture hay preservatives.

Table 4-1. Chemical composition of lignosulfonates

Lignosulfonates[1]	% of DM			Magnesium[5]	Sodium	Sulfur	pH
	Ash[2]	N[3]	WSC[4]				
NaSP	32.83	0.18	28.1	n.d.[6]	n.d.	n.d.	5.91
MgSP	13.29	0.12	14.0	n.d.	n.d.	n.d.	5.04
NaAL	45.57	1.00	12.5	0.10	12.85	7.07	8.68
NaUM	23.29	0.08	24.3	0.96	6.81	6.37	6.66
NaBT	53.41	1.06	7.8	4.57	0.05	6.34	10.17

[1]NaSP = sodium lignosulfonate - new (Sappi North America, Boston, MA); MgSP = magnesium lignosulfonate - new(Sappi North America, Boston, MA); NaAL = lignosulfonic acid sodium salt (Sigma-Aldrich Corp, St Louis, MO); NaUM = sodium lignosulfonate (Spectrum Chemical MFG Corp, New Brunswick, NJ); and NaBT = lignosulfonic acid sodium salt (BeanTown Chemical, Hudson, NH).
[2]FAO (2008).
[3]N=nitrogen.
[4]WSC= water-soluble carbohydrates; DuBois et al. (1956).
[5]Beliciu et al. (2012).
[6]n.d.=not determined.

Table 4-2. Minimal inhibitory concentration (MIC, mg/ml) and minimal fungicidal concentration (MFC, mg/ml) of additives against fungi isolated from spoiled hay as a function of media pH

		A. amoenus		*P. solitum*		*M. circinelloides*		*D. hansenii*	
ADV	pH	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
NaSP	4	16.0±4.2[2]	29.0±2.2	15.0±0.0	31.0±5.5	15.0±0	>60	13.8±2.5	13.8±2.5
	6	>60	n.c.[4]	>60	n.c.	>60	n.c.	>60	n.c.
MgSP	4	35.0±0	>60	48.8±2.5	>60	45.0±0	>60	30.0±0	33.3±2.9
	6	>60	n.c.	>60	n.c.	>60	n.c.	>60	n.c.
NaAL	4	n.d.[3]	>60	n.d.	>60	n.d.	>60	n.d.	20.0±0
	6	>60	n.c.	>60	n.c.	>60	n.c.	>60	n.c.
NaUM	4	>60	n.c.	>60	n.c.	>60	n.c.	58.3±2.9	>60
	6	>60	n.c.	>60	n.c.	>60	n.c.	>60	n.c.
NaBT	4	n.d.	38.6±2.4	n.d.	40.0±0	n.d.	45.0±0	n.d.	28.8±2.5
	6	>60	n.c.	>60	n.c.	>60	n.c.	>60	n.c.
ChMp	4	n.d.	5.1±1.1	n.d.	1.6±0.9	>8	n.c.	0.07±0.02	0.16±0.0
	6	n.d.	2.9±0.7	n.d.	0.23±0.02	>8	n.c.	0.08±0.0	0.67±0.28
ChNv	4	n.d.	1.9±0.7	n.d.	0.06±0	>10	n.c.	0.02±0.0	0.04±0.0
	6	n.d.	1.1±0.7	n.d.	2.2±0.6	>10	n.c.	0.02±0.0	0.07±0.02
PRP	4	1.25±0.0	7.5±2.0	2.5±0.0	5.0±0.0	3.3±0.0	18.8±2.5	1.25±0.0	2.5±0.0
	6	12.0±2.7	>60	16.3±4.8	>60	12.5±2.9	>60	14.0±2.2	15.0±0.0

[1]*Aspergillius amoenus*, *Penicillium solitum*, *Mucor circinelloides* (molds), and *Debaryomyces* hansenii (yeast). NaSP= Sodium lignosulfonate (Sappi North America), MgSP= Magnesium lignosulfonate (Sappi North America), NaAL= Sodium *lignosulfonate* (Sigma-Aldrich Corp), NaUM= Sodium lignosulfonate (Spectrum Chemical MFG Corp), ChMp= Chitosan nanoparticles (provided by the University of Florida), ChNv= Chitosan (Sigma-Aldrich Corp), PRP= Propionic acid (MP Biomedicals).

[2]Mean ± standard deviation.

[3]n.d. = Cannot be determined visually.

[4]n.c. = Not calculated.

Table 4-3. Dry matter losses (%), pH, and microbial counts of alfalfa hay as a function of additive (ADV) and dose at d 23

Item	Dose (%, w/w)					Mean	SEM	*P*-value			
	0	0.25	0.5	1	2			ADV	Dose	ADV × Dose	Contrast[1]
DM loss, %											
LST[2]	24.0^{a}	2.02^{B,b}	1.24^{B,b}	0.92^{B,b}	1.08^{B,b}	5.85^{B}	0.451	<.0001	<.0001	<.0001	CU**
ChNv[3]	24.0	23.4^{A}	23.0^{A}	22.3^{A}	23.3^{A}	23.2^{A}					NS
PRP[4]	24.0^{a}	1.20^{B,b}	1.20^{B,b}	0.73^{B,b}	-0.06^{B,b}	5.41^{B}					CU**
Mean	24.0^{a}	8.86^{b}	8.50^{bc}	7.99^{c}	8.11^{c}						
Hay pH											
LST	8.89^{ab}	5.32^{B,b}	5.08^{C,c}	5.02^{B,c}	4.98^{B,c}	5.86^{C}	0.035	<.0001	<.0001	<.0001	CU**
ChNv	8.89	8.95^{A}	8.87^{A}	8.87^{A}	8.83^{A}	8.88^{A}					NS
PRP	8.89^{a}	5.41^{B,b}	5.28^{B,c}	5.08^{B,d}	4.86^{C,e}	5.90^{B}					CU**
Mean	8.89^{a}	6.56^{b}	6.41^{c}	6.32^{d}	6.23^{e}						
D. hansenii, log cfu/fresh g											
LST	6.10^{a}	5.77^{A,a}	2.24^{B,b}	0.0^{B,c}	0.0^{B,c}	2.82^{B}	0.368	<.0001	<.0001	<.0001	QU**
ChNv	6.10	6.14^{A}	6.16^{A}	6.09^{A}	6.31^{A}	6.16^{A}					NS
PRP	6.10^{a}	0.0^{B,b}	0.0^{C,b}	0.0^{B,b}	0.0^{B,b}	1.22^{B}					CU**
Mean	6.10^{a}	3.97^{b}	2.8^{c}	2.03^{d}	2.10^{d}						
Molds, log cfu/fresh g											
LST	6.76^{a}	6.39^{A,a}	3.89^{B,b}	0.0^{B,d}	0.56^{B,d}	3.84^{B}	0.317	<.0001	<.0001	<.0001	CU**
ChNv	6.76	6.43^{A}	6.47^{A}	6.59^{A}	6.72^{A}	6.59^{A}					NS
PRP	6.76^{a}	2.25^{B,b}	0.0^{C,c}	0.0^{B,c}	0.0^{B,c}	1.74^{C}					CU**
Mean	6.76^{a}	5.02^{b}	3.45^{c}	2.42^{d}	2.20^{d}						
Visual moldiness											
LST	9.4^{a}	2.8^{B,b}	1.0^{B,c}	0.0^{B,d}	0.0^{B,d}	2.6^{B}	0.320	<.0001	<.0001	<.0001	CU**
ChNv	9.4	10^{A}	10^{A}	10^{A}	10^{A}	9.9^{A}					NS
PRP	9.4^{a}	0.0^{C,b}	0.0^{C,b}	0.0^{B,b}	0.0^{B,b}	1.9^{C}					CU**
Mean	9.4^{a}	4.3^{b}	3.7^{c}	3.3^{c}	3.3^{c}						

^{A,B,C} Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$)
^{a,b,c,d} Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$)

Table 4-3. Continued.

[1]Linear (L), quadratic (QU) and cubic (CU) effect ($P \leq 0.05$). *$P < 0.05$; **$P < 0.01$.
[2]Optimized lignosulfonate-based product
[3]Chitosan
[4]Propionic acid

Table 4-4. Nutritional composition of alfalfa hay as a function of additive (ADV) and dose at d 23

Item	Dose (%, w/w)					Mean	SEM	*P*-value			
	0	0.25	0.5	1	2			ADV	Dose	ADV × Dose	Contrast[1]
DM , %											
LST[2]	62.5[a]	75.6[A,b]	74.9[A,b]	76.0[A,b]	75.8[A,b]	73.0[A]	0.468	<.0001	<.0001	<.0001	CU**
ChNv[3]	62.5	63.5[B]	63.7[B]	64.5[B]	63.6[B]	63.5[B]					NS
PRP[4]	62.5[a]	75.3[A,b]	75.3[A,b]	74.7[A,b]	74.8[A,b]	72.5[A]					CU**
Mean	62.5[a]	71.5[b]	71.3[b]	71.7[b]	71.4[b]						
Ash, % of DM											
LST	15.3[a]	12.1[B,bc]	12.2[B,bc]	12.3[B,bc]	12.6[A,c]	12.9[B]	0.124	<.0001	<.0001	<.0001	CU**
ChNv	15.3[a]	15.4[A,a]	15.1[A,ab]	14.9[A,b]	14.9[B,b]	15.1[A]					L*
PRP	15.3[a]	12.0[B,b]	12.0[B,b]	12.0[B,b]	11.9[C,b]	12.6[C]					CU**
Mean	15.3[a]	13.2[b]	13.1[b]	13.1[b]	13.1[b]						
CP, % of DM											
LST	24.1[a]	20.7[B,b]	20.7[B,b]	20.1[B,b]	20.1[B,b]	21.1[B]	0.265	<.0001	<.0001	<.0001	CU**
ChNv	24.1[a]	23.3[A,b]	23.8[A,a]	24.2[A,a]	24.7[A,a]	24.0[A]					CU*
PRP	24.1[a]	20.6[B,b]	20.4[B,b]	20.1[B,b]	20.7[B,ba]	21.2[B]					CU**
Mean	24.1[a]	21.5[b]	21.6[b]	21.5[b]	21.8[b]						
NH_3-N, % of N											
LST	7.80[a]	1.26[B,b]	1.03[B,b]	1.07[B,b]	0.97[B,b]	2.43[B]	0.190	<.0001	<.0001	<.0001	CU*
ChNv	7.80[a]	6.94[A,b]	7.11[A,b]	6.96[A,b]	7.24[A,b]	7.21[A]					QU*
PRP	7.80[a]	0.96[B,b]	0.98[B,b]	0.99[B,b]	0.92[B,b]	2.33[B]					CU**
Mean	7.80[a]	3.05[b]	3.04[b]	3.01[b]	3.04[b]						
WSC, % of DM											
LST	6.76	5.77	6.12	6.09	6.37	6.22[B]	0.215	0.0002	0.009	0.239	CU*
ChNv	6.76	6.69	6.65	6.65	6.75	6.70[A]					NS
PRP	6.76	5.88	6.29	6.09	5.73	6.15[B]					L*
Mean	6.76[a]	6.11[b]	6.35[b]	6.28[b]	6.28[b]						
NDF, % of DM											
LST	42.9	43.7	43.0[B]	43.0[B]	42.8[B]	43.1[B]	0.621	<.0001	<.0001	<.0001	NS

Table 4-4. Continued.

ChNv	42.9[a]	44.6[ab]	45.2[A,b]	47.5[A,c]	47.2[A,c]	45.5[A]					QU*
PRP	42.9	43.0	42.9[B]	42.9[B]	43.6[B]	43.1[B]					NS
Mean	42.9	43.8	43.7	44.5	44.5						
ADF, % of DM											
LST	28.7	31.0	30.0	30.1	29.6	29.9	0.554	0.720	0.0007	0.165	NS
ChNv	28.7	30.0	30.5	31.1	29.6	30.0					QU*
PRP	28.7	29.5	31.3	30.2	31.1	30.2					L*
Mean	28.7[a]	30.2[ab]	30.6[ab]	30.5[bc]	30.1[bc]						
Hemicellulose, % of DM											
LST	14.2	12.7	13.0[A]	12.9[B]	13.2[B]	13.2[B]	0.716	<.0001	0.182	0.020	NS
ChNv	14.2[a]	14.6[ab]	14.7[A,ab]	16.5[A,]	17.6[A]	15.5[A]					L**
PRP	14.2[a]	13.5[ab]	11.6[B,b]	12.7[B,ab]	12.5[B,ab]	12.9[B]					NS
Mean	14.2	13.6	13.1	14.03	14.4						

A,B,C Means with different uppercase superscripts within a column are significantly different (P ≤ 0.05)
a,b,c Means with different lowercase superscripts within a row are significantly different (P ≤ 0.05)
[1]Linear (L), quadratic (QU) and cubic (CU) effect (P ≤ 0.05). *P < 0.05; **P < 0.01.
[2]Optimized lignosulfonate-based product.
[3]Chitosan.
[4]Propionic acid.

Table 4-5. Microbial counts, nutritional composition, and 24 h in vitro digestibility and rumen fermentation parameters of alfalfa hay at d 0

Item	Value (mean ± standard deviation)
Microbial counts, log cfu/fresh g	
Total mold counts	5.8 ± 0.04
Debaromyces hansenii counts	5.3 ± 0.24
Aspergillus amoenus counts	5.3 ± 0.41
Mucor circinelloides counts	5.3 ± 0.24
Penicillium solitum counts	5.2 ± 0.13
Nutritional value	
DM, %	71.3 ± 0.23
Hay pH	5.73 ± 0.037
Ash, % DM	11.29 ± 0.18
NDF, % DM	43.9 ± 2.75
ADF, % DM	32.1 ± 1.47
CP, % DM	20.3 ± 0.94
Hay ammonia nitrogen (NH_3-N), % DM	0.013 ± 0.0013
NH_3-N, %N	0.400
WSC (water soluble carbohydrates), % DM	6.4 ± 0.52
In vitro digestibility and rumen fermentation parameters	
24 h IVDMD, %	71.4 ± 0.51
24 h NDFD, % DM	34.7 ± 3.62
Total VFA, m*M*	91.9
Acetic acid, m*M*	54.9
Propionic acid, m*M*	20.1
Butyric acid, m*M*	9.7
Isobutyric acid, m*M*	1.6
Isovaleric acid, m*M*	2.7
Valeric acid, m*M*	2.8
Acetic-to-propionic acid ratio	2.7
Ruminal pH	7.26 ± 0.101
Ruminal NH_3-N, mg/dL	47.4 ± 2.21

Table 4-6. The 24-h in vitro DM digestibility (DMD), digestible DM recovery, and rumen fermentation measurements of ground alfalfa hay as a function of additive (ADV) and dose after a 23-d in vitro aerobic incubation[1]

	Dose (%, w/w)							*P*-value			
Item[2]	0	0.25	0.5	1	2	Mean	SEM	ADV	Dose	ADV × Dose	Contrast[3]
DMD (%)											
LST	69.3^{a}	71.1^{A,b}	71.7^{A,b}	71.9^{A,b}	71.6^{A,b}	71.1^{A}	0.499	<.0001	0.022	0.001	QU**
ChNv	69.3^{a}	67.9^{B,ab}	68.9^{B,a}	68.3^{B,a}	66.6^{B,b}	68.2^{C}					L**
PRP	69.3	70.4^{A}	70.4^{A}	71.4^{A}	70.5^{A}	70.4^{B}					NS
Mean	69.3^{a}	69.8^{ab}	70.3^{b}	70.5^{b}	69.6^{a}						
Digestibility DM recovery (%)											
LST	52.7^{a}	69.7^{A,b}	70.8^{A,b}	71.2^{A,b}	70.8^{A,b}	67.1^{A}	0.614	<.0001	<.0001	<.0001	CU**
ChNv	52.7^{ab}	52.1^{B,ab}	53.1^{B,b}	53.0^{B,b}	51.1^{B,a}	52.4^{B}					NS
PRP	52.7^{a}	69.6^{A,b}	69.6^{A,b}	70.9^{A,b}	70.6^{A,b}	66.7^{A}					CU**
Mean	52.7^{a}	63.8^{b}	64.5^{bc}	65.0^{c}	64.1^{bc}						
NDFD (%)											
LST	28.5	33.8	32.4	34.5	33.5	32.5^{A}	1.649	0.051	0.009	0.747	QU*
ChNv	28.5	27.8	31.2	33.0	29.1	29.9^{B}					NS
PRP	28.5	31.2	31.1	33.3	32.4	31.3^{AB}					L*
Mean	28.5^{a}	30.9^{ab}	31.6^{b}	33.6^{b}	31.7^{b}						
pH											
LST	7.26	7.27	7.09	7.17	7.12	7.18^{B}	0.069	0.029	0.461	0.142	NS
ChNv	7.26	7.35	7.32	7.17	7.30	7.28^{A}					NS
PRP	7.26	7.27	7.16	7.39	7.36	7.29^{A}					NS
Mean	7.26	7.30	7.19	7.24	7.26						
NH_3-N (mg/dL)											
LST	52.8	49.1	49.4	47.7	48.1	49.4^{B}	0.817	<.0001	<.0001	0.422	QU**
ChNv	52.8	51.2	51.5	51.7	50.7	51.6^{A}					NS
PRP	52.8	48.9	48.5	48.6	48.8	49.5^{B}					CU*
Mean	52.8^{a}	49.7^{b}	49.8^{b}	49.3^{b}	49.2^{b}						

Table 4-6. Continued.

Total VFA (m*M*)											
LST	83.8^{a}	93.6^{A,b}	93.4^{A,b}	93.2^{B,b}	91.6^{B,b}	91.1^{B}	1.094	<.0001	<.0001	<.0001	CU**
ChNv	83.8	83.4^{B}	82.8^{B}	83.2^{C}	81.0^{C}	82.8^{C}					NS
PRP	83.8^{a}	95.1^{A,b}	93.4^{A,b}	97.0^{A,c}	95.9^{A,bc}	93.0^{A}					CU*
Mean	83.8^{a}	90.7^{b}	89.9^{b}	91.1^{b}	89.5^{b}						
Acetic acid (m*M*)											
LST	49.4^{a}	56.6^{A,b}	56.6^{A,b}	56.4^{B,b}	55.6^{B,b}	54.9^{B}	0.607	<.0001	<.0001	<.0001	CU**
ChNv	49.4	49.4^{B}	48.9^{B}	49.4^{C}	48.2^{C}	49.1^{C}					NS
PRP	49.4^{a}	57.7^{A,b}	56.3^{A,b}	58.0^{A,c}	57.3^{A,b}	55.7^{A}					CU**
Mean	49.4^{a}	54.5^{bc}	53.9^{bc}	54.6^{b}	53.7^{c}						
Propionic acid (m*M*)											
LST	16.1^{a}	19.1^{A,b}	19.2^{A,b}	19.1^{B,b}	18.8^{B,b}	18.9^{B}	0.240	<.0001	<.0001	<.0001	CU**
ChNv	16.1	15.9^{B}	15.8^{B}	16.0^{C}	15.5^{C}	15.9^{C}					NS
PRP	16.1^{a}	19.5^{A,b}	19.3^{A,b}	20.3^{A,c}	20.4^{A,c}	19.1^{A}					CU**
Mean	16.1^{a}	18.2^{bc}	18.1^{b}	18.5^{c}	18.2^{bc}						
A:P ratio											
LST	3.08^{a}	2.96^{B,b}	2.95^{B,b}	2.94^{B,b}	2.96^{B,b}	2.98^{B}	0.018	<.0001	<.0001	<.0001	CU*
ChNv	3.08	3.11^{A}	3.10^{A}	3.10^{A}	3.11^{A}	3.10^{A}					NS
PRP	3.08^{a}	2.94^{B,b}	2.91^{B,b}	2.86^{C,c}	2.80^{C,c}	2.92^{C}					CU**
Mean	3.08^{a}	3.00^{b}	2.99^{bc}	2.97^{bc}	2.96^{c}						
Butyric acid (m*M*)											
LST	10.2	10.5	10.2	10.1	9.85	10.2	0.170	0.317	0.077	0.474	NS
ChNv	10.2	10.2	10.1	10.2	10.0	10.1					NS
PRP	10.2	10.4	10.0	10.5	10.3	10.3					NS
Mean	10.2	10.4	10.1	10.3	10.0						
Isobutyric acid (m*M*)											
LST	1.81	1.76	1.69	1.67	1.62	1.71^{B}	0.052	0.017	0.094	0.541	NS
ChNv	1.81	1.81	1.82	1.77	1.76	1.79^{A}					NS
PRP	1.81	1.77	1.73	1.83	1.72	1.77^{A}					NS
Mean	1.81	1.78	1.75	1.76	1.70						
Isovaleric acid(m*M*)											
LST	3.18	2.89	2.85	2.84	2.76	2.90^{B}	0.068	<.0001	<.0001	0.147	CU*

Table 4-6. Continued.

ChNv	3.18	3.10	3.14	3.09	3.08	3.12^{A}					NS
PRP	3.18	2.90	2.80	2.94	2.81	2.93^{B}					CU*
Mean	3.18^{a}	2.96^{b}	2.93^{b}	2.96^{b}	2.88^{b}						
Valeric acid (m*M*)											
LST	3.11	2.82	2.80^{B}	2.98^{B}	2.91^{B}	2.92^{B}	0.132	<.0001	0.624	0.010	NS
ChNv	3.11^{a}	3.03^{ab}	3.03^{AB,ab}	2.71^{B,bc}	2.60^{B,c}	2.90^{B}					L**
PRP	3.11	3.13	3.17^{A}	3.41^{A}	3.41^{A}	3.25^{A}					NS
Mean	3.11	2.99	3.00	3.03	2.97						

$^{A-C, a-c}$ Means with different uppercase letters within a column and within a row are significantly different ($P \leq 0.05$)

[1]LST = Optimized lignosulfonate-based product; ChNv = Chitosan; and PRP = propionic acid

[2]A = acetic acid; P = propionic acid,

[3]Linear (L), quadratic (QU) and cubic (CU) effect ($P \leq 0.05$).

*$P < 0.05$; **$P < 0.01$.

REFERENCES

Abdoun, K.,F. Stumpff and H. Martens. 2006. Ammonia and urea transport across the rumen epithelium: A review. Animal health research reviews / Conference of Research Workers in Animal Diseases 7:43-59.

Albrecht, K. A. and R. E. Muck. 1991. Proteolysis in Ensiled Forage Legumes That Vary in Tannin Concentration. Crop Science 31(2):464-469.

Albretch, K. A. and K. A. Bearchemin. 2003. Alfalfa and Other Perennial Legume Silage. Pages 633-664 in Silage Science and Technology D. R. Buxton, Muck, R.E. and Harrison, J.H., ed. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA.

Ali, J.,D. C. Sharma,A. Bano,A. Gupta,S. Sharma,P. Bajpai and N. Pathak. 2019. Chapter 29 - Exploiting Microbial Enzymes for Augmenting Crop Production. Pages 503-519 in Enzymes in Food Biotechnology. M. Kuddus, ed. Academic Press.

Allan, C. R. and L. A. Hadwiger. 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. Exp Mycol 3(3):285-287.

Arancibia, M. Y.,M. E. López-Caballero,M. C. Gómez-Guillén,M. Fernández-García,F. Fernández-Martín and P. Montero. 2015. Antimicrobial and rheological properties of chitosan as affected by extracting conditions and humidity exposure. LWT - Food Science and Technology 60(2, Part 1):802-810.

Arriola, K. G.,O. C. M. Queiroz,J. J. Romero,D. Casper,E. Muniz,J. Hamie and A. T. Adesogan. 2015. Effect of microbial inoculants on the quality and aerobic stability of bermudagrass round-bale haylage. J. Dairy Sci. 98(1):478-485.

Ávila, C. L. S. and B. F. Carvalho. 2020. Silage fermentation—updates focusing on the performance of micro-organisms. J Appl Microbiol Biochem 128(4):966-984.

Baethgen, W. and M. Alley. 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. Communications in Soil Science and Plant Analysis 20(9-10):961-969.

Ball, D.,D. Bade,G. Lacefield,N. Martin and B. Pinkerton. 1998. Minimizing losses in hay storage and feeding. National Forage Information Circular 98:16.

Barry, T. N. and W. C. McNabb. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. British Journal of Nutrition 81(4):263-272.

Beliciu, C. M.,A. Sauer and C. I. Moraru. 2012. The effect of commercial sterilization regimens on micellar casein concentrates. J. Dairy Sci. 95(10):5510-5526.

Blajman, J. E.,G. Vinderola,R. B. Páez and M. L. Signorini. 2020. The role of homofermentative and heterofermentative lactic acid bacteria for alfalfa silage: a meta-analysis. J Agric Sci 158(1-2):107-118.

Bleakley, B. H. and J. M. Tiedje. 1982. Nitrous Oxide Production by Organisms Other than Nitrifiers or Denitrifiers. Appl Environ Microbiol 44(6):1342-1348.

Bolsen, K. K.,G. Ashbell and Z. G. Weinberg. 1996. Silage fermentation and silage additives - Review. Asian-Australas J Anim Sci 9(5):483-494.

Bolsen, K. K.,C. Lin,B. E. Brent,A. M. Feyerherm,J. E. Urban and W. R. Aimutis. 1992. Effect of Silage Additives on the Microbial Succession and Fermentation Process of Alfalfa and Corn Silages. J. Dairy Sci. 75(11):3066-3083.

Borreani, G. and E. Tabacco. 2008. Low Permeability to Oxygen of a New Barrier Film Prevents Butyric Acid Bacteria Spore Formation in Farm Corn Silage. J. Dairy Sci. 91(11):4272-4281.

Borreani, G.,E. Tabacco,R. J. Schmidt,B. J. Holmes and R. E. Muck. 2018. Silage review: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101(5):3952-3979.

Bracey, D.,C. Holyoak and P. Coote. 1998. Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? J Appl Microbiol Biochem 85(6):1056-1066.

Brady, G. 1965. Nitrogen redistribution during ensilage at low moisture level. J Sci Food Agric 16(9):508-513.

Broderick, G. A.,A. F. Brito and J. J. O. Colmenero. 2007. Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on the Production of Lactating Dairy Cows. J. Dairy Sci. 90(3):1378-1391.

Broderick, G. A.,J. H. Yang and R. G. Koegel. 1993. Effect of Steam Heating Alfalfa Hay on Utilization by Lactating Dairy Cows1. J. Dairy Sci. 76(1):165-174.

Brul, S. and P. Coote. 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. Int J Food Microbiol 50(1-2):1-17.

Cavallarin, L.,S. Antoniazzi,G. Borreani and E. Tabacco. 2005. Effects of wilting and mechanical conditioning on proteolysis in sainfoin (*Onobrychis viciifolia* Scop) wilted herbage and silage. J Sci Food Agric 85(5):831-838.

Cerbulis, J. 1978. Precipitation of proteins from whey with bentonite and lignosulfonate. J Agric Food Chem 26(4):806-809.

Chen, L.,J. Li,Z. Dong andT. Shao. 2019. Effects of lactic acid bacteria inoculants and fibrolytic enzymes on the fermentation quality, in vitro degradability, ruminal variables and microbial communities of high-moisture alfalfa silage. Grassl Sci 65(4):216-225.

Cherney, D. 2000. 14 Characterization of Forages by Chemical Analysis. in Forage evaluation in ruminant nutrition. D. I. Givens, Owen, E., Omed, H.M. and Axford, R.F.E., ed.

Chrenková, M.,Z. Ceresnakova,M. R. Weisbjerg,Z. Formelova,M. Polacikova andM. Vondrakova. 2014. Characterization of proteins in feeds according to the CNCPS and comparison to in situ parameters. Czech J Anim Sci 59(6):288-295.

Church, D. C. 1993. The Ruminant animal: Digestive physiology and nutrition. Waveland Press, Prospect Heights, IL.

Coblentz, W. K. 2015. Managing fermentation with baled silage. in World Dairy Expo 2015. United Stated Department of Agriculture, Marshfield, WI.

Coblentz, W. K. and M. G. Bertram. 2012. Effects of a propionic acid-based preservative on storage characteristics, nutritive value, and energy content for alfalfa hays packaged in large round bales. J. Dairy Sci. 95(1):340-352.

Coblentz, W. K.,K. P. Coffey,A. N. Young and M. G. Bertram. 2013a. Storage characteristics, nutritive value, energy content, and in vivo digestibility of moist, large rectangular bales of alfalfa-orchardgrass hay treated with a propionic acid-based preservative. J. Dairy Sci. 96(4):2521-2535.

Coblentz, W. K.,K. P. Coffey,A. N. Young and M. G. Bertram. 2013b. Storage characteristics, nutritive value, energy content, and in vivo digestibility of moist, large rectangular bales of alfalfa-orchardgrass hay treated with a propionic acid-based preservative. J. Dairy Sci. 96(4):2521-2535.

Coblentz, W. K.,J. O. Fritz and K. K. Bolsen. 1994. Performance comparisons of conventional and laboratory-scale alfalfa hay bales in small haystacks. Agronomy Journal 86(1):46-54.

Coblentz, W. K.,J. O. Fritz,K. K. Bolsen and R. C. Cochran. 1996. Quality Changes in Alfalfa Hay During Storage in Bales1. J. Dairy Sci. 79(5):873-885.

Coblentz, W. K.,J. O. Fritz,R. C. Cochran,W. L. Rooney and K. K. Bolsen. 1997. Protein Degradation in Response to Spontaneous Heating in Alfalfa Hay by In Situ and Ficin Methods1. J. Dairy Sci. 80(4):700-713.

Coblentz, W. K. and P. C. Hoffman. 2009. Effects of bale moisture and bale diameter on spontaneous heating, dry matter recovery, in vitro true digestibility, and in situ disappearance kinetics of alfalfa-orchardgrass hays. J. Dairy Sci. 92(6):2853-2874.

Coblentz, W. K. and P. C. Hoffman. 2010. Effects of spontaneous heating on estimates of total digestible nutrients for alfalfa-orchardgrass hays packaged in large round bales1. J. Dairy Sci. 93(7):3377-3389.

Coblentz, W. K.,J. E. Turner,D. A. Scarbrough,K. E. Lesmeister,Z. B. Johnson,D. W. Kellogg,K. P. Coffey,L. J. McBeth and J. S. Weyers. 2000. Storage Characteristics and Nutritive Value Changes in Bermudagrass Hay as Affected by Moisture Content and Density of Rectangular Bales. Crop Science 40(5):1375-1383.

Collins, M. 1996. Advances in Harvesting and Storing High Quality Alfalfa.

Collins, M.,C. J. Nelson,K. J. Moore and R. F. Barnes. 2017. An Introduction to Grassland Agriculture (Vol. 1.) John Wiley & Sons.

Collins, M.,W. Paulson,M. Finner,N. Jorgensen and C. Keuler. 1987. Moisture and storage effects on dry matter and quality losses of alfalfa in round bales. Transactions of the ASAE 30(4):913-0917.

Colombari, G.,G. Borreani and G. M. Crovetto. 2001. Effect of Ensiling Alfalfa at Low and High Dry Matter on Production of Milk Used to Make Grana Cheese. J. Dairy Sci. 84(11):2494-2502.

Colombini, S.,G. Colombari,G. M. Crovetto,G. Galassi and L. Rapetti. 2009. Tannin treated lucerne silage in dairy cow feeding. Ital J Anim Sci 8(sup2):289-291.

Contreras-Govea, F. E.,R. E. Muck,D. R. Mertens and P. J. Weimer. 2011. Microbial inoculant effects on silage and in vitro ruminal fermentation, and microbial biomass estimation for alfalfa, bmr corn, and corn silages. Anim Feed Sci Technol 163(1):2-10.

Corey, A. M.,K. G. S. Wamsley,T. S. Winowiski and J. S. Moritz. 2014. Effects of calcium lignosulfonate, mixer-added fat, and feed form on feed manufacture and broiler performance. J Appl Poult Res 23(3):418-428.

Cortés, J. E.,B. Moreno,M. L. Pabón,P. Avila,M. Kreuzer,H. D. Hess and J. E. Carulla. 2009. Effects of purified condensed tannins extracted from Calliandra, Flemingia and Leucaena on ruminal and postruminal degradation of soybean meal as estimated in vitro. Anim Feed Sci Technol 151(3):194-204.

Crowley, J. 1985. Effects of Nitrate on livestock. American Society of Agricultural Engineer:Paper Number 80-20026.

Davies, D. R.,R. J. Merry,A. P. Williams,E. L. Bakewell,D. K. Leemans and J. K. S. Tweed. 1998. Proteolysis During Ensilage of Forages Varying in Soluble Sugar Content. J. Dairy Sci. 81(2):444-453.

de Freitas Ferreira, J.,E. A. Vieira and M. Nitschke. 2019. The antibacterial activity of rhamnolipid biosurfactant is pH dependent. Food Res Int 116:737-744.

Dewhurst, R. J.,L. Delaby,A. Moloney,T. Boland and E. Lewis. 2009. Nutritive value of forage legumes used for grazing and silage. Irish Journal of Agricultural and Food Research 48(2):167-187.

Dewhurst, R. J.,W. J. Fisher,J. K. S. Tweed and R. J. Wilkins. 2003. Comparison of Grass and Legume Silages for Milk Production. 1. Production Responses with Different Levels of Concentrate. J. Dairy Sci. 86(8):2598-2611.

Doelle, H. W.,J. S. Rokem and M. Berovic. 2009. BIOTECHNOLOGY-Volume VIII: Fundamentals in Biotechnology. Vol. 8. EOLSS Publications.

Dong, X.,M. Dong,Y. Lu,A. Turley,T. Jin and C. Wu. 2011. Antimicrobial and antioxidant activities of lignin from residue of corn stover to ethanol production. Ind. Crops Prod. 34(3):1629-1634.

Driehuis, F. and S. Oude Elferink. 2000. The impact of the quality of silage on animal health and food safety: A review. The Veterinary quarterly 22:212-216.

Driehuis, F.,J. M. Wilkinson,Y. Jiang,I. Ogunade and A. T. Adesogan. 2018. Silage review:Animal and human health risks from silage. J. Dairy Sci. 101(5):4093-4110.

Drouin, P.,L. J. Mari and R. J. Schmidt. 2019. Lactic acid bacteria as microbial silage additives: current status and future outlook. in New Advances on Fermentation Processes. IntechOpen.

Dubois, M.,K. A. Gilles,J. K. Hamilton,P. t. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry 28(3):350-356.

Duchaine, C.,M. C. Lavoie and Y. Cormier. 1995. Effects of a Bacterial Hay Preservative (Pediococcus pentosaceus) on Hay under Experimental Storage Conditions. Appl Environ Microbiol 61(12):4240-4243.

Dulphy, J. and C. Demarquilly. 1981. Problèmes particuliers aux ensilages. Previsión de la valeur nutritive des aliments des ruminants. INRA. Francia 81.

Dumitriu, S. and V. Popa. 2013. Polymeric Biomaterials. Vol. 2. CRC Press.

Dunière, L.,J. Sindou,F. Chaucheyras-Durand,I. Chevallier and D. Thévenot-Sergentet. 2013. Silage processing and strategies to prevent persistence of undesirable microorganisms. Anim Feed Sci Technol 182(1):1-15.

EFSA. 2015. Panel on Additives and Products or Substances used in Animal Feed. Scientific Opinion on the safety and efficacy of lignosulphonate as a feed additive for all animal species. EFSA Journal 13(7):17.

Emery, I. and N. Mosier. 2015. Direct emission of methane and nitrous oxide from switchgrass and corn stover: implications for large-scale biomass storage. GCB Bioenergy 7(4):865-876.

Fahey, G. C.,M. Collins,D. R. Mertens and L. E. Moser. 1994. PLant factors affecting forage quality. *Forage quality, evaluation, and utilization*, 115-154.

FAO. 2008. Joint FAO/WHO Expert Committee on Food Additives. in 69th Meeting. . Food and Agriculture Organization, Rome, Italy., Compendium of food additive specifications.

Feller, U. K.,T.-S. T. Soong and R. H. Hageman. 1977. Leaf Proteolytic Activities and Senescence during Grain Development of Field-grown Corn (Zea mays L.). Plant Physiology 59(2):290-294.

Fuller, M. F. and P. J. Reeds. 1998. Nitrogen cycling in the gut. Annual review of nutrition 18(1):385-411.

Garcia, A. D.,W. G. Olson,D. E. Otterby,J. G. Linn and W. P. Hansen. 1989. Effects of Temperature, Moisture, and Aeration on Fermentation of Alfalfa Silage. J. Dairy Sci. 72(1):93-103.

Garrido-Maestu, A.,Z. Ma,S.-Y.-R. Paik,N. Chen,S. Ko,Z. Tong and K. C. Jeong. 2018. Engineering of chitosan-derived nanoparticles to enhance antimicrobial activity against foodborne pathogen *Escherichia coli* O157:H7. Carbohydr Polym 197:623-630.

Ghinet, M. G.,S. Roy,D. Poulin-Laprade,M.-E. Lacombe-Harvey,R. Morosoli and R. Brzezinski. 2010. Chitosanase from Streptomyces coelicolor A3(2): biochemical properties and role in protection against antibacterial effect of chitosan. Biochemistry and Cell Biology 88(6):907-916.

Gibson, T.,A. C. Stirling,R. Keddie and R. Rosenberger. 1958. Bacteriological changes in silage made at controlled temperatures. Microbiology 19(1):112-129.

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analyses: apparatus, reagents, procedures, and some applications. No. 379. Agricultural Research Service, US Department of Agriculture.

Gopinatha Kurup, G.,B. Adhikari and B. Zisu. 2019. Recovery of proteins and lipids from dairy wastewater using food grade sodium lignosulphonate. Water Resour Ind 22:100114.

Gosselink, R. J. A.,E. de Jong,B. Guran and A. Abächerli. 2004. Co-ordination network for lignin—standardisation, production and applications adapted to market requirements (EUROLIGNIN). Ind. Crops Prod. 20(2):121-129.

Grabber, J. H. 2009. Protein fractions in forage legumes containing protein-binding polyphenols: Freeze-drying vs. conservation as hay or silage. Anim Feed Sci Technol 151(3):324-329.

Greenhill, W. 1959. The respiration drift of harvested pasture plants during drying. J Sci Food Agric 10(9):495-501.

Gundala, P. B. and P. Chinthala. 2017. Extremophilic pectinases. Pages 155-180 in Extremophilic Enzymatic Processing of Lignocellulosic Feedstocks to Bioenergy. Springer.

Guo, G.,C. Shen,Q. Liu,S. L. Zhang,C. Wang,L. Chen,Q. F. Xu,Y. X. Wang and W. J. Huo. 2019. Fermentation quality and in vitro digestibility of first and second cut alfalfa (*Medicago sativa* L.) silages harvested at three stages of maturity. Anim Feed Sci Technol 257:114274.

Guo, L.,D. Yao,D. Li,Y. Lin,S. Bureenok,K. Ni and F. Yang. 2020. Effects of Lactic Acid Bacteria Isolated From Rumen Fluid and Feces of Dairy Cows on Fermentation Quality, Microbial Community, and in vitro Digestibility of Alfalfa Silage. Front Microbiol 10(2998).

Guo, X. S.,W. Cheng,Y. Zhang,F. Y. Yang and H. Zhou. 2011. Contribution of endopeptidases to the formation of nonprotein nitrogen during ensiling of alfalfa. Anim Feed Sci Technol 168(1):42-50.

Guo, X. S.,W. R. Ding,J. G. Han and H. Zhou. 2008. Characterization of protein fractions and amino acids in ensiled alfalfa treated with different chemical additives. Anim Feed Sci Technol 142(1):89-98.

Gustafsson, A. H. and J. Carlsson. 1993. Effects of silage quality, protein evaluation systems and milk urea content on milk yield and reproduction in dairy cows. Livest Prod Sci 37(1):91-105.

Hall, M. 2003. Challenges with nonfiber carbohydrate methods. J. Anim. Sci. 81(12):3226-3232.

Hall, M. B. 2015. Comparisons of in vitro fermentation and high moisture forage processing methods for determination of neutral detergent fiber digestibility. Anim Feed Sci Technol 199:127-136.

Han, K. J.,W. D. Pitman and A. Chapple. 2014. Moisture concentration variation of silages produced on commercial farms in the South-central USA. Asian-Australas J Anim Sci 27(10):1436-1442.

Hao, W.,P. Tian,M. Zheng,H. Wang and C. Xu. 2019. Characteristics of proteolytic microorganisms and their effects on proteolysis in total mixed ration silages of soybean curd residue. Asian-Australas J Anim Sci 33(1):100-110.

Haque, M.,R. Chowdhury,K. Islam and M. Akbar. 2009. Propionic acid is an alternative to antibiotics in poultry diet. Bangladesh J. Anim. Sci. 38(1-2):115-122.

Henderson, N. 1993. Silage additives. Anim Feed Sci Technol 45(1):35-56.

Henry, D. D.,M. Ruiz-Moreno,F. M. Ciriaco,M. Kohmann,V. R. G. Mercadante,G. C. Lamb and N. DiLorenzo. 2015. Effects of chitosan on nutrient digestibility, methane emissions, and in vitro fermentation in beef cattle 1,2. J. Anim. Sci. 93(7):3539-3550.

Heron, S. J. E.,R. A. Edwards and P. Phillips. 1989. Effect of pH on the activity of ryegrass Lolium multiflorum proteases. J Sci Food Agric 46(3):267-277.

Higgs, R. J.,L. E. Chase,D. A. Ross and M. E. Van Amburgh. 2015. Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. J. Dairy Sci. 98(9):6340-6360.

Hlödversson, R. and A. Kaspersson. 1986. Nutrient losses during deterioration of hay in relation to changes in biochemical composition and microbial growth. Anim Feed Sci Technol 15(2):149-165.

Holzer, M.,E. Weidenholzer,H. Danner and R. Braun. 2003. The role of Lactobacillus buchneri in forage preservation. Trends in biotechnology 21:282-287.

Hoskins, B. R. 1997. Soil testing handbook for professionals in agriculture, horticulture, nutrient and residuals management. Maine Agriculture and Forest Experimental Station: Orono, ME, USA.

Hristov, A. N. and S. G. Sandev. 1998. Proteolysis and rumen degradability of protein in alfalfa preserved as silage, wilted silage or hay. Anim Feed Sci Technol 72(1):175-181.

Hugo, W. 1992. Disinfection mechanisms. Principles and practice of disinfection, preservation and sterilization:187-210.

Jarrige, R.,C. Danarqainy and J. P. Dulphy. 1981. Forage Conservation. Page 363 in Proc. Symposium Nutr. Limits Anim. Rod. Pastures. J.B. Backer, ed. Bridgne, Queensland, Australia.

Jha, A. and A. Kumar. 2018. Deciphering the role of Sodium Lignosulfonate against Candida spp. as persuasive anticandidal agent. Int J Biol Macromol 107:1212-1219.

Jones, B. A.,R. D. Hatfield and R. E. Muck. 1995. Characterization of Proteolysis in Alfalfa and Red Clover. Crop Science 35(2):537-541

Jones, B. A.,L. D. Satter and R. E. Muck. 1992. Influence of bacterial inoculant and substrate addition to lucerne ensiled at different dry matter contents. Grass Forage Sci 47(1):19-27.

Jorritsma, R.,T. Wensing,T. A. M. Kruip,P. L. A. M. Vos and J. P. T. M. Noordhuizen. 2003. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Vet. Res. 34(1):11-26.

Jovaisiene, J.,B. Bakutis,V. Baliukoniene and G. Gerulis. 2016. Fusarium and Aspergillus mycotoxins effects on dairy cow health, performance and the efficacy of Anti-Mycotoxin Additive. Pol J Vet Sci19(1).

Jung, E. J.,D. K. Youn,S. H. Lee,H. K. No,J. G. Ha and W. Prinyawiwatkul. 2010. Antibacterial activity of chitosans with different degrees of deacetylation and viscosities. Int J Food Sci Technol 45(4):676-682.

Kanatt, S. R.,R. Chander and A. Sharma. 2008. Chitosan and mint mixture: A new preservative for meat and meat products. Food Chemistry 107(2):845-852.

Ke, W.,F. Yang,D. Undersander and X. Guo. 2015. Fermentation characteristics, aerobic stability, proteolysis and lipid composition of alfalfa silage ensiled with apple or grape pomace. Anim Feed Sci Technol 202:12-19.

Killerby, M.,S. T. R. Almeida,R. Hollandsworth,B. C. Guimaraes,A. Y. Leon-Tinoco,Z. X. Ma,D. Coffin,B. Perkins,S. Annis,C. Knight,C. Wu,J. Bolton and J. J. Romero. 2020c. Effect of chemical and biological preservatives on the dry matter loss, nutritional composition, microbial counts, and aerobic stability of ensiled wet brewer's grain. in Proc. ADSA Virtual Annual Meeting.

Killerby, M.,R. White,D. C. Reyes,A. Y. Leon-Tinoco,S. A. Rivera,H. Paz,J. A. Jendza and J. J. Romero. 2020a. Meta-analysis of the effects of preservatives on hay spoilage. I: Chemical treatments. in Proc. ADSA Virtual Annual Meeting.

Killerby, M.,R. White,D. C. Reyes,A. Y. Leon-Tinoco,S. A. Rivera,H. Paz,J. A. Jendza and J. J. Romero. 2020b. Meta-analysis of the effects of preservatives on hay spoilage II: Microbial inoculants. in Proc. ADSA Virtual Annual Meeting.

King, C.,J. McEniry and P. O'Kiely. 2012. A note on the fermentation characteristics of red clover silage in response to advancing stage of maturity in the primary growth. Irish Journal of Agricultural and Food Research:79-84.

Knicky, M. and R. Spörndly. 2011. The ensiling capability of a mixture of sodium benzoate, potassium sorbate, and sodium nitrite. J. Dairy Sci. 94(2):824-831.

Kong, M.,X. G. Chen,K. Xing and H. J. Park. 2010. Antimicrobial properties of chitosan and mode of action: A state of the art review. Int J Food Microbiol 144(1):51-63.

König, W.,M. Lamminen,K. Weiss,T. T. Tuomivirta,S. Sanz Muñoz,H. Fritze,K. Elo,L. Puhakka,A. Vanhatalo and S. Jaakkola. 2017. The effect of additives on the quality of white lupin–wheat silage assessed by fermentation pattern and qPCR quantification of clostridia. Grass Forage Sci 72(4):757-771.

Kozelov, L. K.,F. Iliev,A. N. Hristov,S. Zaman and T. A. McAllister. 2008. Effect of fibrolytic enzymes and an inoculant on in vitro degradability and gas production of low-dry matter alfalfa silage. J Sci Food Agric 88(14):2568-2575.

Krízek, M. 1993. Biogenic amines in silage. 1. The occurrence of biogenic amines in silage. Archiv fur Tierernahrung 43(2):169.

Kuenen, J.,M. Deslauriers,C. Trozzi and M. Woodfield. 2009. Pulp and paper. EMEP/EEA emmision inventory guidebook

Kung, L., Jr. 2010. Understanding the biology of silage preservation to maximize quality and protect the environment. In Proceedings, California Alfalfa & Forage Symposium and Corn/Cereal Silage Conference, p. 1-2.

Kung, L., Jr.,R. D. Shaver,R. J. Grant and R. J. Schmidt. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101(5):4020-4033.

Kung, L. K.,M. R. Stokes and C. J. Lin. 2003b. Silage Additives. Pages 305-360 in Silage Science and Technology D. R. Buxton, Muck, R.E. and Harrison, J.H. , ed. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA.

Lee, M. R. F. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Front Plant Sci. 5(694).

Lee, M. R. F.,J. K. S. Tweed,F. R. MinchinandA. L. Winters. 2009. Red clover polyphenol oxidase: Activation, activity and efficacy under grazing. Anim Feed Sci Technol 149(3):250-264.

Leibensperger, R. and R. Pitt. 1987. A model of clostridial dominance in ensilage. Grass Forage Sci 42(3):297-317.

Licitra, G.,T. M. Hernandez and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim Feed Sci Technol 57(4):347-358.

Lin, C.,K. K. Bolsen,B. E. Brent,R. A. Hart,J. T. Dickerson,A. M. Feyerherm and W. R. Aimutis. 1992. Epiphytic Microflora on Alfalfa and Whole-Plant Corn1. J. Dairy Sci. 75(9):2484-2493.

Lingaas, F. and B. Tveit. 1992. Etiology of Acetonemia in Norwegian Catle. 2. Effect of Butyric Acid, Valeric Acid, and Putrescine. J. Anim. Sci. 75:7.

Liu, C.,Y.-j. Lai,X.-n. Lu,P.-t. GuoandH.-l. Luo. 2016. Effect of lactic acid bacteria inoculants on alfalfa (*Medicago sativa* L.) silage quality: assessment of degradation (*in situ*) and gas production (*in vitro*). J. Integr. Agric. 15(12):2834-2841.

Machado de Castro, A.,A. Fragoso dos Santos,V. Kachrimanidou,A. A. Koutinas and D. M. G. Freire. 2018. Chapter 10 - Solid-State Fermentation for the Production of

Proteases and Amylases and Their Application in Nutrient Medium Production. Pages 185-210 in Current Developments in Biotechnology and Bioengineering. A. Pandey, C. Larroche, and C. R. Soccol, ed. Elsevier.

Maeda, Y. 1993. Changes in ADIN (AcidDetergent InsolubleNitrogen) during Hay Making. Japanese Society of Grassl Sci 38(4):506-509.

Mahanna, B. and L. E. Chase. 2003. Practical Applications and Solutions to Silage Problems. Pages 855-895 in Silage Science and Technology. D. R. Buxton, Muck, R.E. and Harrison, J.H., ed.

Mallarino, A. P. and W. F. Wedin. 1990. Nitrogen fertilization effects on dinitrogen fixation as influenced by legume species and proportion in legume-grass mixtures in Uruguay. Plant and Soil 124(1):127-135.

Mansfield, H. R. and M. D. Stern. 1994. Effects of Soybean Hulls and Lignosulfonate-Treated Soybean Meal, on Ruminal Fermentation in Lactating Dairy Cows1. J. Dairy Sci. 77(4):1070-1083.

Matheis, G. and J. R. Whitaker. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. J. Food Biochem. 8(3):137-162.

Mayer, A. M. 2006. Polyphenol oxidases in plants and fungi: Going places? A review. Phytochemistry (Oxford) 67(21):2318-2331.

McAllister, T. and A. Hristov. 2000. The fundamentals of making good quality silage. Adv. Dairy Technol 12:318-399.

McAllister, T. A.,K. J. Cheng,K. A. Beauchemin,D. R. C. Bailey,M. D. Pickard and R. P. Gilbert. 1993. Use of lignosulfonate to decrease the rumen degradability of canola meal protein. Canadian J. Anim. Sci. 73(1):211-215.

McCartney, D. 2005. Prospects for haying systems. Pages 133-157 in Grasslands: Developments opportunities perspectives around the world. S. G. Reynolds and J. Frame, ed. Science Publishers, INC., Enfield, New Hampshire 0378, U.S.A.

McDonald, P.,A. R. Henderson and S. J. E. Heron. 1991. The biochemistry of silage. Chalcombe Publications. Marlow Bucks, UK 340.

McDonnell, G. E. 2007. Antiseptics, disinfection, and sterilization. Types, action, and resistance. Inter Microb 10:295-298.

McGarvey, J. A.,R. B. Franco,J. D. Palumbo,R. Hnasko,L. Stanker and F. M. Mitloehner. 2013. Bacterial population dynamics during the ensiling of Medicago sativa (alfalfa) and subsequent exposure to air. J Appl Microbiol Biochem 114(6):1661-1670.

McKersie, B. D. 1981. Proteinases and peptidases of alfalfa herbage. Can J Plant Sci 61:7.

Medina, J. D. C.,A. L. Woiciechowski,A. Zandona Filho,L. Bissoqui,M. D. Noseda,L. P. de Souza Vandenberghe,S. F. Zawadzki and C. R. Soccol. 2016. Biological activities and thermal behavior of lignin from oil palm empty fruit bunches as potential source of chemicals of added value. Ind. Crops Prod. 94:630-637.

Michalet-Doreau, B. and M. Y. Ould-Bah. 1992. Influence of Hay Making on In Situ Nitrogen Degradability of Forages in Cows. J. Dairy Sci. 75(3):782-788.

Miller, W. J. 2012. Dairy cattle feeding and nutrition. Academic Press, INC., New York, San Francisco,.

Mills, J. A. and L. Kung. 2002. The Effect of Delayed Ensiling and Application of a Propionic Acid-Based Additive on the Fermentation of Barley Silage1, 2. J. Dairy Sci. 85(8):1969-1975.

Mlejnkova, V.,P. Horky,M. Kominkova,J. Skladanka,L. Hodulikova,V. Adam,J. Mlcek,T. Jurikova and J. Sochor. 2016. Biogenic amines and hygienic quality of lucerne silage. Open Life Science 11:7.

Moon, N. J.,L. O. Ely and E. Max Sudweeks. 1981. Fermentation of Wheat, Corn, and Alfalfa Silages Inoculated with *Lactobacillus acidophilus* and *Candida* sp. at Ensiling. J. Dairy Sci. 64(5):807-813.

Muck, R. 1988a. Factors influencing silage quality and their implications for management. J. Dairy Sci. 71(11):2992-3002.

Muck, R. 1996. Inoculation of silage and its effects on silage quality. Pages 43-51 in Proc. Informational conference with dairy and forage industries. US Dairy Forage Res Center.

Muck, R. and L. Kung. 2007. Silage Production. Vol. 2. Forages: The Science of Grassland Agriculture. Blackwell Publishing, Ames, IA.

Muck, R. E. 1988b. Storage Temperature Effects on Proteolysis in Alfalfa Silage. Transactions of the ASAE 31(4):1005-1009.

Muck, R. E. 1989. Effect of Inoculation Level on Alfalfa Silage Quality. Transactions of the ASAE 32(4):1153-1158.

Muck, R. E. 2010. Silage microbiology and its control through additives. Revista Brasileira de Zootecnia 39:183-191.

Muck, R. E.,L. E. Moser and R. E. Pitt. 2003. Postharvest Factors Affecting Ensiling. Pages 251-304 in Silage Science and Technology. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA. ed. D. R. Buxton, Muck, R.E. and Harrison, J.H., ed.

Muck, R. E. and P. O'kiely. 1992. Aerobic deterioration of lucerne (*Medicago sativa*) and maize (*Zea mais*) silages—effects of fermentation products. J Sci Food Agric 59(2):145-149.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. J Sci Food Agric 86(13):2010-2037.

Musa, M. and M. Mustafa. 2020. Livestock Feeds and Feeding Practices in Pakistan. Page 280 in Livestock Feeds and Feeding Practices in South Asia. S. Ashis Kumar, B. Shaikh Mohammad, and A. Mohammad Younus, ed, SAARC Agriculture Centre, Dhaka, Bangladesh,.

Nagel, S. A. and G. A. Broderick. 1992. Effect of Formic Acid or Formaldehyde Treatment of Alfalfa Silage on Nutrient Utilization by Dairy Cows1. J. Dairy Sci. 75(1):140-154.

NASS. 2004. Field Crops Final Estimates 1997-2002. USDA, Washington, DC.

NASS. 2020a. Crop Production 2019 Summary. USDA, Washington, DC.

NASS. 2020b. Crop Values 2019 Summary. USDA, Washington D. C.

Nolan, J. V. and R. C. Dobos. 2005. Nitrogen Transactions in Ruminants. in Quantitative Aspects of Ruminant Digestion and Metabolism. J. Dijkstra, Forbes, J., and France, J., ed. Available from: ProQuest Ebook Central. CABI, Wallingford.

Núñez-Flores, R.,B. Giménez,F. Fernández-Martín,M. E. López-Caballero,M. P. Montero and M. C. Gómez-Guillén. 2012. Role of lignosulphonate in properties of fish gelatin films. Food Hydrocolloids 27(1):60-71.

Ogunade, I. M.,Y. Jiang,D. H. Kim,A. A. P. Cervantes,K. G. Arriola,D. Vyas,Z. G. Weinberg,K. C. Jeong and A. T. Adesogan. 2017. Fate of *Escherichia coli* O157:H7 and bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives. J. Dairy Sci. 100(3):1780-1794.

Ogunade, I. M.,D. H. Kim,Y. Jiang,Z. G. Weinberg,K. C. Jeong and A. T. Adesogan. 2016. Control of *Escherichia coli* O157:H7 in contaminated alfalfa silage: Effects of silage additives. J. Dairy Sci. 99(6):4427-4436.

Olicón-Hernández, D. R.,A. N. Hernández-Lauzardo,J. P. Pardo,A. Peña,M. G. Velázquez-del Valleand,G. Guerra-Sánchez. 2015. Influence of chitosan and its derivatives on cell development and physiology of Ustilago maydis. Int J Biol Macromol 79:654-660.

Oliveira, A. S.,Z. G. Weinberg,I. M. Ogunade,A. A. P. Cervantes,K. G. Arriola,Y. Jiang,D. Kim,X. Li,M. C. M. Gonçalves,D. Vyas and A. T. Adesogan. 2017. Meta-analysis of effects of inoculation with homofermentative and facultative

heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. J. Dairy Sci. 100(6):4587-4603.

Ortolani, E. L.,C. S. Mori and J. A. Rodrigues Filho. 2000. Ammonia toxicity from urea in a Brazilian dairy goat flock. Veterinary and human toxicology 42(2):87.

Pahlow, G.,R. E. Muck,F. Driehuis,S. J. W. H. Oude Elferink and S. F. Spoelstra. 2003. Microbiology of Ensiling. in Silage Science and Technology D. R. Buxton, Muck, R.E. and Harrison, J.H., ed. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA.

Pahlow, G.,D. Slotther,C. Rammer and M. Tuori. 2002. Ensiling of legumes.5.

Palmonari, A.,M. Fustini,G. Canestrari,E. Grilli and A. Formigoni. 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97(12):7729-7734.

Papadopoulos, Y. and B. McKersie. 1983. A comparison of protein degradation during wilting and ensiling of six forage species. Canadian Journal of Plant Science 63(4):903-912.

Peddinti, B. S. T.,F. Scholle,M. G. Vargas,S. D. Smith,R. A. Ghiladi and R. J. Spontak. 2019. Inherently self-sterilizing charged multiblock polymers that kill drug-resistant microbes in minutes. Materials horizons 6(10):2056-2062.

Pepys, J.,P. A. Jenkins,G. N. Festenstein,P. H. Gregory,M. E. Lacey and F. A. Skinner. 1963. Farmer's lung. Thermophilic actinomycetes as a source of " farmer's lung hay " antigen. Lancet 2(7308):607-611.

Perry, T. W. and M. J. Cecava. 1995. Beef Cattle Feeding and Nutrition. Vol. 2nd ed. Animal Feeding and Nutrition. Academic Press, San Diego.

Petit, H. V.,G. F. Tremblay,M. Marcotte and R. Audy. 1999. Degradability and digestibility of full-fat soybeans treated with different sugar and heat combinations. Canadian J. Anim. Sci. 79(2):8.

Phuntsok, T.,M. A. Froetschel,H. E. Amos,M. Zheng and Y. W. Huang. 1998. Biogenic Amines in Silage, Apparent Postruminal Passage, and the Relationship Between Biogenic Amines and Digestive Function and Intake by Steers. J. Anim. Sci. 81:11.

Pichard, G. R.,B. R. Tesser,C. Vives,C. Solari,A. Hott and R. E. Larraín. 2006. Proteolysis and Characterization of Peptidases in Forage Plants. Agronomy Journal 98(6):1392-1399.

Piyasena, P.,E. Mohareb and R. C. McKellar. 2003. Inactivation of microbes using ultrasound: a review. Int J Food Microbiol 87(3):207-216.

Purich, D. L. 2010. Enzyme kinetics: catalysis and control: a reference of theory and best-practice methods. Elsevier.

Queiroz, O. C. M.,I. M. Ogunade,Z. Weinberg and A. T. Adesogan. 2018. Silage review: Foodborne pathogens in silage and their mitigation by silage additives. J. Dairy Sci. 101(5):4132-4142.

Raymond, S. L.,M. Heiskanen,T. K. Smith,M. Reiman,S. Laitinen and A. F. Clarke. 2000. An investigation of the concentrations of selected Fusarium mycotoxins and the degree of mold contamination of field-dried hay. Journal of Equine Veterinary Science 20(10):616-621.

Reed, J. D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73(5):1516-1528.

Rees, D. V. H. 1982. A discussion of sources of dry matter loss during the process of haymaking. Journal of Agricultural Engineering Research 27(6):469-479.

Reyes, D. C.,S. L. Annis,S. A. Rivera,A. Y. Leon-Tinoco,C. Wu,L. B. Perkins,J. J. Perry,M. S. Castillo and J. J. Romero. 2020. Increased high-moisture alfalfa hay preservation, in vitro ruminal digestibility, fermentability, and decreased proteolysis with the novel use of technical lignins. J. Dairy Sci. 103 (7):6114-6134.

Reynal, S. M., Broderick, G.A. Effect of Dietary Level of Rumen-Degraded Protein on Production and Nitrogen Metabolism in Lactating Dairy Cows. J. Dairy Sci 88(11):4045-4064.

Roberts, C. A. 1995. Microbiology of Stored Forages. Page 21 in Post-Harvest Physiology and Preservation of Forages. K. J. Moore and M. A. Peterson, ed, CSSA Spec. Publ. 22. ASA and CSSA, Madison WI.

Romanazzi, G.,F. M. Gabler,D. Margosan,B. E. Mackey and J. L. Smilanick. 2009. Effect of Chitosan Dissolved in Different Acids on Its Ability to Control Postharvest Gray Mold of Table Grape. Phytopathology® 99(9):1028-1036.

Romero, J. J.,Y. Joo,J. Park,F. Tiezzi,E. Gutierrez-Rodriguez and M. S. Castillo. 2018. Bacterial and fungal communities, fermentation, and aerobic stability of conventional hybrids and brown midrib hybrids ensiled at low moisture with or without a homo- and heterofermentative inoculant. J. Dairy Sci. 101(4):3057-3076.

Rooke, J. A. and R. D. Hatfield. 2003. Biochemistry of Ensiling. Pages 95-139 in Silage Science and Technology. D. R. Buxton, Muck, R.E. and Harrison, J.H., ed. Madison, WI: Agron. Monogr. ASA, CSSA and SSSA.

Rotz, C. A. 1995. Field curing of forages. Post-Harvest Physiology and Preservation of Forages 22:39-66.

Rotz, C. A. and R. E. Muck. 1994. Changes in Forage Quality During Harvest and Storage. Pages 828-868 in Forage Quality, Evaluation, and Utilization.

Rotz, C. A.,R. E. Pitt,R. E. Muck,M. Allen and D. R. Buckmaster. 1993. Direct-cut harvest and storage of alfalfa on the dairy farm. Transactions of the ASAE 36(3):621-628.

Rotz, C. A. and K. J. Shinners. 2007. Hay Harvest and Storage. Pages 601-616 in The Science of Grassland Agriculutre. Sixth ed. R. F. Barnes, K. J. Moore, C. J. Nelson, and M. Collins, ed. Ames, Iowa: Blackwell Publishing.

Sahariah, P. and M. Masson. 2017. Antimicrobial chitosan and chitosan derivatives: a review of the structure–activity relationship. Biomacromolecules 18(11):3846-3868.

Seymour, W. M.,D. R. Campbell and Z. B. Johnson. 2005. Relationships between rumen volatile fatty acid concentrations and milk production in dairy cows: a literature study. Anim Feed Sci Technol 119(1):155-169.

Sharp, R.,P. G. Hooper and D. G. Armstrong. 1994. The digestion of grass silages produced using inoculants of lactic acid bacteria. Grass & Forage Science 49(1):42-53.

Siegfried, V.,H. Ruckemann and G. Stumpf. 1984. Eine HPLC-methode zur bestimmung organischer säuren in silagen. Landwirtsch forsch 37:298-304.

Singleton, V., and J. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:416-417.

Silva, V. P.,O. G. Pereira,E. S. Leandro,T. C. Da Silva,K. G. Ribeiro,H. C. Mantovani and S. A. Santos. 2016. Effects of lactic acid bacteria with bacteriocinogenic potential on the fermentation profile and chemical composition of alfalfa silage in tropical conditions. J. Dairy Sci. 99(3):1895-1902.

Sinclair, K. D.,M. Kuran,F. E. Gebbie,R. Webb and T. G. McEvoy. 2000. Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen1. J. Anim. Sci. 78(10):2670-2680.

Sniffen, C. J.,J. O'connor,P. Van Soest,D. Fox and J. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70(11):3562-3577.

Spoelstra, S. F. 1987. Degradation of nitrate by enterobacteria during silage fermentation of grass. Netherlands Journal of Agriculture Science 35(1):12.

Stanford, K.,T. McAllister,Z. Xu,K.-J. Cheng and M. Pickard. 1995. Comparison of lignosulfonate-treated canola meal and soybean meal as rumen undegradable protein supplements for lambs. Canadian J. Anim. Sci. 75(3):371-377.

Stokes, M. R. and J. Chen. 1994. Effects of an Enzyme-Inoculant Mixture on the Course of Fermentation of Corn Silage1. J. Dairy Sci. 77(11):3401-3409.

Struszczyk, K.,M. Szczęsna-Antczak,M. Walczak,E. Pomianowska and T. Antczak. 2009. Isolation and purification of *Mucor circinelloides* intracellular chitosanolytic enzymes. Carbohydr Polym 78(1):16-24.

Sudarshan, N. R.,D. G. HooverandD. Knorr. 1992. Antibacterial action of chitosan. Food Biotechnology 6(3):257-272.

Sullivan, M. L. and R. D. Hatfield. 2006. Polyphenol Oxidase and o-Diphenols Inhibit Postharvest Proteolysis in Red Clover and Alfalfa. Crop Science 46(2):662-670.

Tabacco, E.,G. Borreani,G. M. Crovetto,G. Galassi,D. Colombo and L. Cavallarin. 2006. Effect of Chestnut Tannin on Fermentation Quality, Proteolysis, and Protein Rumen Degradability of Alfalfa Silage. J. Dairy Sci. 89(12):4736-4746.

Tabacco, E.,S. Piano,L. Cavallarin,T. F. Bernardes and G. Borreani. 2009. Clostridia spore formation during aerobic deterioration of maize and sorghum silages as influenced by *Lactobacillus buchneri* and *Lactobacillus plantarum* inoculants. J Appl Microbiol Biochem 107(5):1632-1641.

Tao, L.,X. S. Guo,H. Zhou,D. J. Undersander and A. Nandety. 2012. Short communication: Characteristics of proteolytic activities of endo- and exopeptidases in alfalfa herbage and their implications for proteolysis in silage. J. Dairy Sci. 95(8):4591-4595.

Tao, L.,H. Zhou,N. Zhang,B. Si,Y. Tu,T. Ma and Q. Diao. 2017. Effects of different source additives and wilt conditions on the pH value, aerobic stability, and carbohydrate and protein fractions of alfalfa silage. Animal Science Journal 88(1):99-106.

Tayel, A. A.,S. Moussa,W. F. El-Tras,D. Knittel,K. Opwis and E. Schollmeyer. 2010. Anticandidal action of fungal chitosan against Candida albicans. Int J Biol Macromol 47(4):454-457.

Tian, J.,Z. Li,Z. Yu,Q. Zhang and X. Li. 2017. Interactive effect of inoculant and dried jujube powder on the fermentation quality and nitrogen fraction of alfalfa silage. Animal Science Journal 88(4):633-642.

Tremblay, G. F.,G. Bélanger,K. B. McRae and R. Michaud. 2001. Proteolysis in alfalfa silages made from different cultivars. Canadian Journal of Plant Science 81(4):685-692.

Turner, J. E.,W. K. Coblentz,D. A. Scarbrough,K. P. Coffey,D. W. Kellogg,L. J. McBeth and R. T. Rhein. 2002. Changes in Nutritive Value of Bermudagrass Hay during Storage. Agronomy Journal 94(1):109-117.

Undersander, D.,D. Combs,R. Shaver and D. Thomas. 1999. Nitrate poisoning in cattle, sheep and goats. University of Wisconsin-Madison Extension Cooperative Service, University of Wisconsin-Madison, Madison, WI.

Urrutia, N. L. and K. J. Harvatine. 2017. Acetate Dose-Dependently Stimulates Milk Fat Synthesis in Lactating Dairy Cows. The Journal of Nutrition 147(5):763-769.

Vainio, U.,R. A. Lauten,S. Haas,K. Svedström,L. S. Veiga,A. Hoell and R. Serimaa. 2012. Distribution of counterions around lignosulfonate macromolecules in different polar solvent mixtures. Langmuir 28(5):2465-2475.

Van Amburgh, M. E.,E. A. Collao-Saenz,R. J. Higgs,D. A. Ross,E. B. Recktenwald,E. Raffrenato,L. E. Chase,T. R. Overton,J. K. Mills and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98(9):6361-6380.

Van Amburgh, M. E.,K. L. Russomanno,R. A. Higgs and L. E. Chase. 2019. Invited Review: Modifications to the Cornell Net Carbohydrate and Protein System related to environmental issues—Capability to evaluate nitrogen and phosphorus excretion and enteric carbon dioxide and methane emissions at the animal level. Applied Animal Science 35(1):101-113.

Van Soest, P. J. 1982. Nutritional ecology of the ruminant. O & B Books. Inc., Corvallis, OR 374.

Varshavsky, A. 2001. Proteolysis. Pages 1573-1575 in Encyclopedia of Genetics. S. Brenner and J. H. Miller, ed. Academic Press, New York.

Veen, W. 1986. The influence of slowly and rapidly degradable concentrate protein on a number of rumen parameters in dairy cattle. NJAS wageningen journal of life sciences 34(2):199-216.

Walker, R. S. and J. Vendramini. 2018. Characteristics of and Animal Responses to Warm- and Cool-Season Forage Baleage. J. Anim. Sci. 96:64-64.

Wambacq, E.,J. P. Latré and G. Haesaert. 2013. The effect of *Lactobacillus buchneri* inoculation on the aerobic stability and fermentation characteristics of alfalfa-ryegrass, red clover and maize silage. Agricultural and Food Science 22(1):127-136.

Wang, Y.,T. Marx,J. Lora,L. E. Phillip and T. A. McAllister. 2009. Effects of purified lignin on in vitro ruminal fermentation and growth performance, carcass traits and fecal shedding of *Escherichia coli* by feedlot lambs. Anim Feed Sci Technol 151(1):21-31.

Weatherburn, M. 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical chemistry 39(8):971-974.

Weinberg, Z. G.,Y. Chen,R. Pinto and S. Sela. 2007. Fate of inoculated *Escherichia coli* in hay. J Appl Microbiol Biochem 102(6):1537-1543.

Weinberg, Z. G.,G. Szakacs,G. Ashbell and Y. Hen. 2001. The effect of temperature on the ensiling process of corn and wheat. J Appl Microbiol Biochem 90(4):561-566.

Wen, A.,X. Yuan,J. Wang,S. T. Desta and T. Shao. 2017. Effects of four short-chain fatty acids or salts on dynamics of fermentation and microbial characteristics of alfalfa silage. Anim Feed Sci Technol 223:141-148.

Whiter, A. G. and L. Kung. 2001. The Effect of a Dry or Liquid Application of Lactobacillus plantarum MTD1 on the Fermentation of Alfalfa Silage. J. Dairy Sci. 84(10):2195-2202.

Wilkinson, J. M. and D. R. Davies. 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci 68(1):1-19.

Windschitl, P. M. and M. D. Stern. 1988. Evaluation of calcium lignosulfonate-treated soybean meal as a source of rumen protected protein for dairy cattle. J. Dairy Sci. 71(12):3310.

Wu, C., F. Chen, X. Wang, H. Kim, G. He, V. Haley-Zitlin, and G. Huang. 2006. Antioxidant costituents in fevefew (*Tabacetum parthenium*) extract and their chromatographic quatification. Food Chem. 96:220-227.

Wright, C. F.,M. A. G. von Keyserlingk,M. L. Swift,L. J. Fisher,J. A. Shelford and N. E. Dinn. 2005. Heat- and Lignosulfonate-Treated Canola Meal as a Source of Ruminal Undegradable Protein for Lactating Dairy Cows. J. Dairy Sci. 88(1):238-243.

Xie, Z.,J. Huang,X. Xu and Z. Jin. 2008. Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry 111(2):370-376.

Yan, M.,D. Yang,Y. Deng,P. Chen,H. Zhou and X. Qiu. 2010. Influence of pH on the behavior of lignosulfonate macromolecules in aqueous solution. Colloids and Surfaces A: Physicochemical and Engineering Aspects 371(1):50-58.

Yari, M.,R. Valizadeh,A. A. Naserian,G. R. Ghorbani,P. Rezvani Moghaddam,A. Jonker and P. Yu. 2012. Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the afternoon and morning. Anim Feed Sci Technol 172(3):162-170.

Yien, L.,N. M. Zin,A. Sarwar and H. Katas. 2012. Antifungal activity of chitosan nanoparticles and correlation with their physical properties. Int J Biomater 2012.

Yitbarek, M. B. and B. Tamir. 2014. Silage Additives: Review. Journal of Applied Sciences 4:258-274.

Yu, P.,D. Christensen,J. McKinnon and J. Markert. 2003. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, in vitro rumen degradability and energy values of timothy and alfalfa. Canadian J. Anim. Sci. 83(2):279-290.

Zhang, X.,A. L. Licon,T. I. Harris,P. F. Oliveira,B. J. McFarland,B. E. Taurone,B. J. Walsh,B. E. Bell,C. T. Walker and R. V. Lewis. 2019. Silkworms with spider silklike

fibers using synthetic silkworm chow containing calcium lignosulfonate, carbon nanotubes, and graphene. ACS omega 4(3):4832-4838.

BIOGRAPHY OF AUTHOR

Angela Leon was born in Lima, Peru in 1987. She graduated from Union Latinoamericana High School in 1999 with her favorite courses being biology, math, and art. She grew up with great affection for animals which encouraged her to enroll in the animal science program at Universidad Nacional Agraria La Molina (UNALM). At the end of 2010 she finished her studies and got her B.S. in Animal Science at the top of her class. In the next year, she conducted a study “Effect of cysteamine as a growth promoter in the diet on productive performance of broiler chickens” under the supervision of Dr. Carlos Vilchez in UNALM. This study was published in the VII International Seminar of Association of Veterinarians Specialists in Birds in Peru. After that, she worked as formulator of cattle projects, and as manager of guinea pig and pig farms. In 2014, she was awarded a full scholarship for the Master’s degree program on Animal Production from the National Council for Science, Technology and Technological Innovation. In this program, she conducted an original study “Stock and Quality of Carbon in High Andean Wetlands” which was published in the “X International Rangeland Congress Proceedings” Canada, 2016. Two years after, she was admitted to the University of Maine to pursue a second M.S. in Animal Science under the supervision of Dr. Juan Romero. The fact that she was going to work on the preservation of forage was particularly exciting to her. During her time at U. Maine, she presented her research findings on her country at UNALM and at American Dairy Science Virtual Annual Meeting 2020. Angela is a candidate for the Master of Science degree in Animal Science from the University of Maine in December 2020.