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DEVELOPING ANIMAL FEED PRESERVATIVES FROM PAPER MILL BYPRODUCTS

By

Diana Carolina Reyes Gomez

B.S. National University of Colombia, 2016

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 2018

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DEDICATION

To my dear family

DEVELOPING ANIMAL FEED PRESERVATIVES FROM PAPER MILL BYPRODUCTS

By Diana Carolina Reyes Gomez

Thesis advisor: Dr. Juan Romero

An Abstract of the Thesis Presented
In Partial Fulfillment of the Requirements for the
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Our objectives were to evaluate the antifungal properties of technical lignins against 3 molds and 1 yeast causing hay spoilage, and for their ability to preserve alfalfa hay nutritive value. In experiment 1, 8 technical lignins and propionic acid (PRP; positive control) were tested at a dose of 40 mg/mL. The experiment had a randomized complete block design (RCBD, 4 runs) and a factorial arrangement of 3 molds × 10 additives (ADV). The effects of ADV on the yeast were also evaluated with a RCBD. Across fungi, sodium lignosulfonate (NaL) and PRP were the only treatments with a 100 ± 2.8% inhibition. In experiment 2, the minimum inhibitory (MIC) for selected technical lignins and PRP were determined. Among technical lignins, NaL had the lowest MIC across molds (< 33.3 mg/mL) and MgL for the yeast (26.7). However, PRP had values that were several fold lower across all fungi (< 3.33). In experiment 3, a RCBD (5 blocks) with a 3 (ADV; NaL, MgL, and PRP) × 4 (doses: 0, 0.5, 1, and 3% w/w fresh basis) factorial arrangement of treatments was used to evaluate the preservative effects of ADV in high moisture alfalfa hay inoculated with a mixture of the fungi previously tested and incubated under aerobic conditions. After 15 d, relative to untreated hay (14.9 ± 0.77%), DM losses were lessened by doses as low as 1% for NaL (3.39) and

0.5% for PRP (0.81). This was explained by a reduced mold count in both NaL at 3% (3.92 ± 0.55 log cfu/fresh g) and PRP as low as 0.5% (3.94) relative to untreated hay (7.76). Consequently, sugars were best preserved by NaL at 3% (10.1 ± 0.283% DM) and PRP as low as 0.5% (10.5) vs. untreated (7.99), while keeping NDF values lower in NaL (45.9 ± 0.66% DM) and PRP-treated (45.1) hays at the same doses, respectively, relative to untreated (49.7 ± 0.66% DM). Hay DMD was increased by doses as low as 3% for NaL (67.5± 0.77%), 1% MgL (67.0), and 0.5% PRP (68.5) vs. untreated hay (61.8). In the case of NDFD, 0.5% for MgL and PRP (30.5 and 30.1 ± 1.09% DM, respectively) and 1% for NaL (30.7) were the lowest doses increasing NDFD relative to untreated hay (23.3). Total volatile fatty acids were increased to the greatest extent by NaL at 3% (111.9 ± 1.3 mM) relative to spoiled hay (86.7). Across technical lignins, NaL was the best hay preservative. However, its effects were limited compared to PRP at equivalent doses. Despite not having an effect on preservation, MgL improved DMD by stimulating NDFD. Further research needs to be conducted to isolate the most antifungal fraction of NaL and to understand the stimulatory effects of MgL on fiber degradation.

Keywords: hay preservation, technical lignins, ruminal digestibility.

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADG	Average daily gain
ADIN	Acid detergent insoluble nitrogen
ADV	Additive
AI	Acetone insoluble Kraft lignin fraction
AKL	Alkali Kraft lignin
AMOL	Ammonium lignosulfonate
CFU	Colony forming units
CP	Crude protein
d	Day
DM	Dry matter
DMI	Dry matter intake
FCM	Fat corrected milk
IVDMD	*In vitro* dry matter digestibility
HEX	Hexane insoluble Kraft lignin fraction
LCC	Lignin-carbohydrate complex
LAB	Lactic acid bacteria
MIC	Minimum inhibitory concentration
MGL	Magnesium lignosulfonate
NAL	Sodium lignosulfonate
NDF	Neutral detergent fiber
NDFD	Neutral detergent fiber digestibility
NH_3-N	Ammonia nitrogen
MFC	Minimum fungicidal concentration

OM	Organic matter
PI	Hexane soluble Kraft lignin fraction
PRP	Propionic acid
RCBD	Randomized complete block design
TVFA	Total volatile fatty acids
UWKL	Unwashed Southern pine softwood Kraft lignin
VFA	Volatile fatty acid
WKL	Washed Southern pine softwood Kraft lignin
WSC	Water soluble carbohydrates

CHAPTER 1

INTRODUCTION

Forages are the largest dietary component for ruminant animals representing 61% and 83% of dairy and beef cattle diets, respectively (Barnes and Nelson, 2003). During periods of scarcity caused by limited pasture growth or inadequate pasture conditions, or when fed as a supplement, forages are preserved either as hay or silage (Romero et al., 2015). Despite the recent growth of silage production, haymaking remains the prevailing forage conservation method in the U.S. (NASS, 2017). The greatest issues in haymaking are the large losses of dry matter (DM) and nutritive value that occur during harvest due to increased leaf fragility below 20% moisture, and during storage if bales are stored with a moisture concentration above 20% (or 15% for large round bales), due to unpredictable weather. When hay is baled above the maximum recommended moisture levels (high moisture hay), microbial spoilage ensues by metabolizing the most nutritive fractions (e.g. sugars) and leaving behind the most recalcitrant ones (e.g. fiber), releasing heat in the process which will further reduce nutritional value due to the Maillard reaction (Rotz and Shinners, 2007). Furthermore, excessive mold growth in high moisture hays poses a health risk to farmers in the form of “farmer’s lung” (Emanuel et al., 1964) and mycotoxins that can get into the food chain potentially affecting consumers (Raymond et al., 2000).

Additives have been developed to prevent spoilage in high moisture hay. These include organic acids, anhydrous ammonia, and microbial inoculants (Rotz and Shinners, 2007). Anhydrous ammonia is effective at preventing spoilage but the high costs of application and caustic nature have prevented its widespread use (Muck and Shinners, 2001), and it cannot be used on high quality forages due to toxicity problems

(Rotz and Shinners, 2007). Organic acids such as propionic and acetic acid also prevent spoilage of hays with up to 35% moisture, but effects do not last more than 6 months and its acidic nature quickly corrodes farm equipment (McCartney, 2005). Buffered propionic acid such as ammonium propionate reduces damage to harvesting equipment (Rotz and Shinners, 2007). Results from propionic acid-based additives have been inconsistent and beneficial effects seem to be mostly limited to reducing spontaneous heating (i.e. microbial respiration; Coblentz et al., 2013). Microbial inoculants have had very limited effects on hay preservation which have limited their adoption in the field (Shinners, 2000). Because of the limitations listed above, there are no methods that have been widely implemented by hay producers due to high-cost, hazardousness, and minimal effectiveness. Further research needs to be conducted to evaluate alternative additives that can offer a low-cost (< $4,000/Mg) and effective hay preservative.

Technical lignins are byproducts of paper mills, approximately 50 million Mg/y are produced worldwide but only 2% are commercialized (Gosselink et al., 2004). Certain technical lignin types, such as kraft lignins and lignosulfonates, have been reported to have antibacterial (Dong et al., 2011), antifungal (Jha and Kumar, 2018), and antiviral activities (Gordts et al., 2015), and prebiotic effects (Flickinger et al., 1998). Furthermore, lignosulfonates have been long used to increase soybean protein bypass in the rumen (Borucki Castro et al., 2007) and as feed binders (Corey et al., 2014). These properties could be adapted to prevent hay spoilage and consequently increase animal performance.

The objectives of this study were to evaluate the antifungal properties of technical lignins against four fungi isolated from spoiled alfalfa (*Medicago sativa*) hay and their potential preservation properties on dry matter losses, nutritional composition, digestibility, and the fermentation profile of high moisture alfalfa hay.

CHAPTER 2

LITERATURE REVIEW

Hay Production

Forage can be preserved either as hay, haylage, or silage, depending on the crop type, available resources (e.g. storage capability), weather conditions, and the intended use of the conserved forage (Romero et al., 2015). In spite of the growth of the silage industry, haymaking remains the prevailing forage conservation method in the United States (NASS, 2017). This may be due to a better suitability of hay for long-term storage, easier transportation (reduced fresh weight per unit of dry matter; DM), and marketing (Collins and Owens, 2003). In fact, hay is the third most valuable crop in the U.S. and the second in the state of Maine worth $16 billion and $39 million per year, respectively (NASS, 2017) Although hay can be made from a variety of crops, alfalfa is the most important crop for production of high-quality hay. In the U.S. and in the state of Maine, alfalfa hay contributes $8 billion and $3 million per year to agricultural economies, respectively (NASS, 2016).

Definition

Hay is defined as the aerial portions of fine-stemmed forages, mostly legumes and grasses, which are dried, conserved, and stored under aerobic conditions (Horrocks and Valentine, 1999). With haymaking, the primary objective is to decrease the moisture content in no more than 3-5 d to a stable long-term storage level (<15-20%) while capturing most of the forage stand yield and nutrients (Rees, 1982). However, nutrient losses during hay harvest and storage are interdependent; as hay moisture decreases below 20%, leaf losses increase substantially during harvest (Rotz and Shinners, 2007) but above 20%, storage losses rise due to plant enzymatic and microbial activity (Muck

and Shinners, 2001; Coblentz and Hoffman, 2009). If hay could be baled above recommended moisture levels, harvested yield could be increased by up to 7% (Rotz et al., 1992). Considering leaves are the most nutritionally rich plant organ (Albert et al., 1989) and that most of the field losses are leaves (Rees, 1982), it is evident that a technology that could allow for the safe long-term storage of high moisture hay would have a great impact in the efficiency of hay production and consequently, in the profitability and environmental impact of this economic activity.

Hay production practices

Hay can be produced under a variety of systems combining several processes and equipment. The goal of these processes is to further increase the drying rate of the forage, produce a homogeneously dry crop, and reduce field exposure time (Rees, 1982). In the U.S., typical hay production involves mowing, curing, tedding, raking, baling, and storage, as described next.

Mowing

There are four major types of hay mowing equipment, sickle cutter bar, rotary disk, rotary drum, and flail mowers (McCartney, 2005). The sickle bar mower (reciprocating knives) is the traditional method used worldwide, due to its reliability and low cost (Fig. 2-1a; Collins and Owens, 2003). However, it has limited speed and swath width capacity, which led to the development of rotary disk mowers (Porter, 2017). Disk mowers cut forage with knives that are attached to rotating disks on a horizontal plane, which provides higher cutting capacity (Fig. 2-1b; Rotz and Shinners, 2007). However, these mowers require more power (4×) and fuel per hour of use relative to cutter bar mowers (Rotz, 2001).

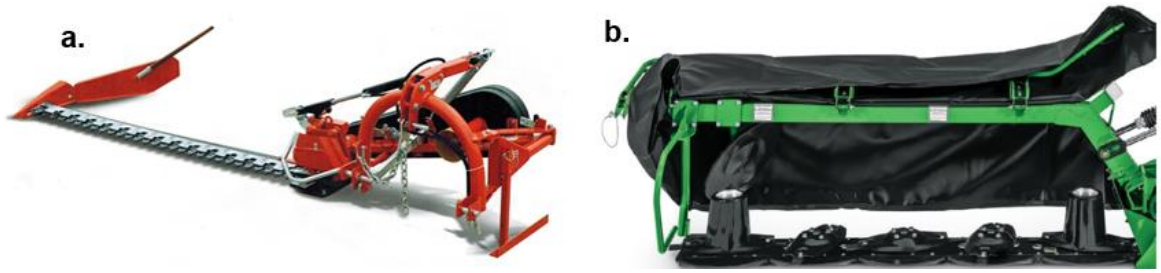

Figure 2-1a. Sickle bar and 2-1b. Rotary disc mower. Reproduced from Romero et al. (2015).

Curing

Fresh forage usually contains ~80% moisture, therefore, large amounts of water need to be removed through evaporation to produce hay with the desired moisture level (< 15-20%). The entire process usually takes 3 to 5 d under ideal weather conditions (Collins and Owen, 2003). However, the speed of the drying process will depend on the crop, weather, and management factors (McCartney, 2005). Relevant crop factors influencing the curing process include plant species, growth stage, and leaf:stem ratio (McCartney, 2005). Overall, grasses have a superior drying rate relative to legumes, due to the greater leaf:stem ratio in grasses because leaves dry faster than stems (reduced curing time; Rotz, 1995). Across legumes, alfalfa (*Medicago sativa*) dries faster than red clover (*Trifolium pratense*), due to the latter's thicker stems which increases radial distance from the stem core to the epidermis (Macdonald and Clark, 1987).

Weather is the most unpredictable variable influencing haymaking. Environmental factors are highly correlated with each other, and it is therefore difficult to separate the effects of each. Among these, ambient temperature, relative humidity (curing time increases when > 70%), wind speed (ideally < 19 km/h), solar radiation, and soil

moisture are the most important (Rotz and Shinners, 2007). Management factors will be discussed in detail next.

Losses during hay curing

Physical, biological, and chemical changes occur in forage as it dries in the field (Rees, 1982). After cutting the forage, the primary metabolic loss is derived from plant cell respiration, which ceases when forage moisture falls below 40% moisture. At this level the plant respiration is null (Wolf and Carson, 1973). Respiration losses of 4, 5 and 7% DM have been reported for alfalfa, ryegrass (*Lolium multiflorum*), and white clover (*Trifolium repens*) hay, respectively, dried under appropriate weather conditions (Rotz et al, 1987). However, during warm and humid conditions, alfalfa hay can lose > 10% DM due to plant respiration (Morris, 1972). On the other hand, DM and nutritional quality losses may occur during field curing due to rain damage. These losses have been reported to range from 2 to > 30% for grass (Dernedde and Wilmschen, 1969) and as much as 15.4% for alfalfa hay (Collins, 1996). The rapidly digestible water soluble carbohydrates (WSC) have been reported to be the most affected fraction of the crop, with 34 and 67% DM losses during curing reported for alfalfa and red clover, respectively (Collins, 1996). Therefore, a faster curing process is required in order to minimize DM respiration losses and the risk of damage due to unexpected precipitation events.

Conditioning

Conditioning is the mechanical or chemical process that helps accelerate field drying of the hay crop (Collins and Owens, 2003). After mowing, the rate of drying is fast at the beginning because the leaf stomata are open. However, after wilting, these pores close and moisture is lost only from the waxy epidermis of leaves and stems

(Rees, 1982). Therefore, mechanical conditioning is needed in most situations to allow additional water loss from openings in the waxy epidermis created during the crushing of the forage plant by the conditioner. This reduces curing time by 1 or 2 days (Rotz and Shinners, 2007). Currently, most mowing equipment include a mechanical conditioning device, with the most common being crimpers, crushing rollers, plastic brushes, or flails (McCartney, 2005). From these, impeller or flail conditioners are recommended for grass hay (Rotz, 2001), while roll conditioners are best suited for alfalfa and other legumes because these are less aggressive, and reduce leaf loss and crop damage (Greenlees et al., 2000). Mechanical treatment losses are noticeably larger for legume than for grass hay (4 vs. 1% DM; Rotz and Muck, 1994). This could be explained by the morphology of legumes (slender petiole) that makes it more susceptible to shattering losses than sheaths and blades of grasses (Savoie and Beauregard, 1991). On the other hand, chemical conditioners (potassium or sodium carbonate) are applied to the crop at the time of mowing to help speed the drying process by modifying the waxy materials of the cuticle layer allowing moisture to pass through (Rotz and Shinners, 2007). These compounds are more effective when used in legumes such as alfalfa and clover rather than orchardgrass and timothy (McCartney, 2005).

Tedding and Raking

The thickness and density of swaths influence the drying process; generally, more dense swaths generate a microclimate that limits moisture loss. Hence, exposing the wetter forage by mechanically turning the swath can speed the drying process (Porter, 2017). Hay tedders are wide machines with orbital wheels that spread and fluff the swath as they turn (Fig. 2-2a; Collins and Owens, 2003). These machines create a thinner forage layer that is more efficiently exposed to radiation, reducing drying times

up to 2 d (Rotz and Shinners, 2007). Tedding should be done when crop moisture concentration is > 40% in order to minimize leaf losses that can be > 10% DM in alfalfa tedded at 30% moisture (Rotz and Shinners, 2007).

Once the hay has been tedded and has almost reached the target moisture, the forage is raked into a windrow ready to be baled (Romero et al., 2015). Rake designs include parallel-bar, wheel, and rotary rakes (Fig. 2-2b). Typical raking losses range from 3-6% and are inversely correlated to the area density of the swath (Rotz and Abrams, 1988).

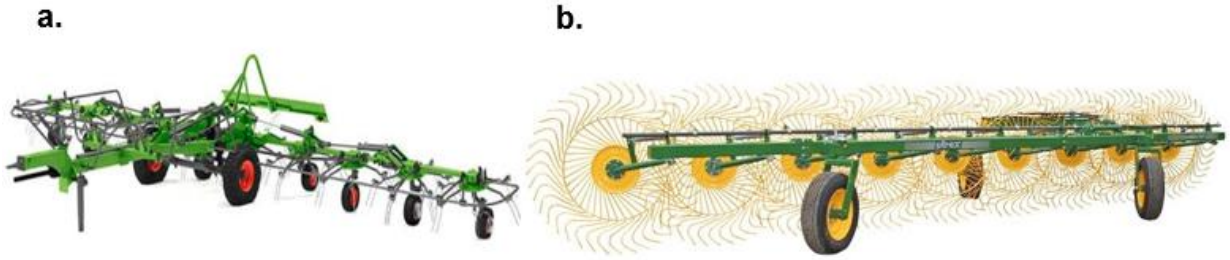

Figure 2-2a. Hay tedder, and 2-2b. Parallel bar raker. Reproduced from Fendt (2018) and John Deere (2018), respectively.

Baling

After the forage crop has reached an ideal moisture concentration (15-20%), forage can be packed into round, square, or rectangular bales to simplify handling and storage (Rotz and Shinners, 2007). Depending on their size and DM density, a safe baling moisture should be considered in order to maintain quality and avoid excessive DM losses during storage (Table 2-1; Rotz and Shinners, 2007). Losses during baling typically vary between 2-5% of hay yield for small rectangular bales (Rotz and Muck, 1994). However, for large round bales (the most common type across the U.S.) losses can surpass 10% (McCartney, 2005) due to heat accumulation in their compact core

(Porter, 2017). Yet, hay producers prefer round bales because they reduce costs of labor, infrastructure, and equipment (Huhnke, 2003).

Table 2-1. Typical dimensions, size, DM density, and baling moisture for hay bales. Adapted from Collins and Owens (2003).

Bale shape	Dimensions (m)[1]	Volume (m^3)	Weight (kg)	DM density (kg/m^3)	Safe baling moisture (%)
Rectangular	0.45 × 0.97, 0.36	0.15	27	128 – 176	20
	0.91 × 0.18, 2.0	1.6	408	224-256	12-16
	1.2 × 2.4, 1.2	3.2	816	224-256	12-16
Round	1.2, 1.2	1.4	227	160-208	18
	1.2, 1.5	1.8	386	160-208	18
	1.5, 1.2	2.2	454	160-208	18
	1.5, 1.5	2.8	590	160-208	18
	1.8, 1.5	3.9	862	160-208	18

[1]For rectangular bales, width × length, diameter. For round bales length, diameter.

Storage

Ideally, all types of hay bales should be stored under cover to prevent weather damage and reduce DM losses, particularly in high rainfall areas (McCartney, 2005). The economic value of storage losses is substantial (surpassing $2 billion/year; Ball et al., 1998) and can be described as a function of the extent of DM loss and the quality of the remaining forage (Rotz and Shinners, 2007). Even when bailing moisture is adequate and storage conditions are appropriate, typical DM losses during storage are 5% (Romero et al., 2015). On the other hand, if hay is baled above 20% (15% for large round bales), DM losses are expected to be greater relative to normal moisture hay (Coblentz et al., 2004). The internal temperature of the bale increases, due to the respiration of both plant and epiphytic microorganisms (Roberts, 1995). The initial heating spike lasts for approximately 5 d, and it is followed by an extended phase of heating led by storage microorganisms that can last for several weeks, which intensifies

in high moisture hays (Fig. 2-3; Coblentz and Bertram, 2012) resulting in a major decrease in nutritive value and increase in DM losses (Coblentz and Hoffman, 2009).

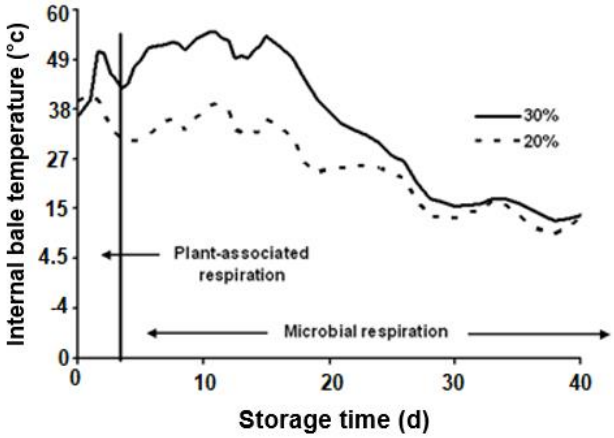

Figure 2-3. Internal bale temperature over storage time curved for conventional rectangular bales of alfalfa baled at 20 and 30% moisture. Reproduced from Coblentz et al. (1996).

Further losses can occur in unprotected hays depending on weather conditions (Rotz and Shinners, 2007). Storing hay outside in dry regions result in minimal DM losses compared to regions with high annual rainfall (> 76 cm) where round bales can lose up to one third of their initial DM (Collins, 1996). Typically, greater losses occur in the most exposed outer layer (10 - 20 cm) while the core of the bale has comparable losses to barn stored hay (Rotz and Shinners, 2007). Furthermore, rain damage reduces DM digestibility and palatability of hay (Ball et al., 1998). Alfalfa-grass hay exposed to rainfall had a marked reduction of DM digestibility compared to unweathered hay (34 vs. 57% DM digestibility, respectively (Lechtenberg et al., 1980). This decrease may be explained because most digestible fractions of the forage (WSC) are lost during storage rain damage (Collins and Owens, 2003). The following section focuses on how hay management practices affect microbial succession and its effects on hay nutritional value and voluntary intake across the different stages of making hay.

Hay Spoilage

A limited number of studies have described the microbial community of hay (Breton and Zwaenepoel, 1991; Taffarel et al., 2013). Moreover, most of the research efforts on hay microbiology have focused on depicting the microbial population changes that occur during the storage phase and that result in spoilage when moisture concentrations are above 20% at bailing (Undi et al., 1997). From a microbiological perspective, particular attention has been paid to molds over yeasts and bacteria, and spontaneous heating has been frequently used as a proxy of the aerobic respiration of plant nutrients (mainly nonstructural carbohydrates) by spoilage microorganisms (Coblentz et al., 2012). From a nutritional perspective, important changes including an increased concentration of fiber components, and reduced protein digestibility and energy density due to spoilage have been reported (Coblentz and Hoffman, 2009). Furthermore, excessive mold growth in high moisture hay poses a health risk for both farm workers and livestock in the form of pulmonary allergies (i.e. farmer's lung; Emanuel, 1964), and mycotoxins that can get into the food chain potentially affecting consumers (Raymond et al., 2000).

Microbiology of Hay

The microbial community structure of conserved forages is dynamic and changes during harvest, storage, and feeding (Muck, 2013). The dynamics of those changes are affected by factors such as the phyllosphere at mowing (Mogodiniyai Kasmaei et al., 2017), forage type (Duniere et al., 2017), plant variety (Romero et al., 2018), plant physiological stage (Gdanetz and Trail, 2017), moisture concentration (Undi et al., 1997), environmental temperature (Rotz and Muck, 1994), storage conditions (Taffarel et al., 2013), and feeding management (Belyea et al., 1985). Next, we will review the

microbial communities that characterize each stage of hay production and how they relate to changes in forage nutritional value.

Epiphytic community

In conserved forages, the composition of the epiphytic community influences the stability and feeding value, particularly of silage, being less important for the successful preservation of hay (Lin et al., 1992). Epiphytic populations across forage crops have been described (Table 2-2; Pahlow et al., 2003). For alfalfa, *Enterobacteriaceae*, *Lactobacillaceae*, and fungal populations of 6.06, 3.76, and 5.07 log cfu/fresh g have been reported, respectively (Lin et al., 1992). Typically *Enterobacteriaceae* is the predominant family, while *Lactobacillaceae* populations are less abundant (McDonald et al., 1991), and much less when the community is described using amplicon-based NGS (next generation sequencing; Romero et al., 2018). In fact, many microbes part of the phyllosphere are viable but unculturable, thus, the complexity and diversity of epiphytic communities is often underestimated (Rastogi et al., 2012). Only few recent studies have used amplicon-based NGS to describe the epiphytic community structure associated with forages. Across studies, the main family in the phyllosphere seems to be *Enterobacteriaceae* (~50% relative abundance; McGarvey et al., 2013; Romero et al., 2018; Dunière et al., 2017). Among the most abundant genera, *Cronobacter*, *Erwinia*, and *Sphingobacterium* have been reported for corn (Ni et al., 2017; Romero et al., 2018), *Erwinia*, *Escherichia*, *Pseudomonas*, and *Enterobacter* for alfalfa, *Sphingomonas* for timothy (*Phleum pratense*)-meadow fescue (*Festuca pratensis*), and *Pseudomonas* predominated in red clover (Mogodiniyai et al., 2016). Numerous bacterial species belonging to these genera are described as important pathogens in plants and animals (Toth et al., 2016).

Fewer studies have described the fungal phyllosphere. Using curated fungal databases (e.g. UNITE), most of the sequences remain unidentified (~45.0% relative abundance; Romero et al., 2018). The small grain (oats, barley and triticale) fungal microbiome was dominated by unidentified members of the order *Capnodiales*, *Pucciniales* (rust fungi), and *Tremellales* with relative abundance of 31, 21, and 18%, respectively (Duniere et al., 2017). For corn, the most abundant taxa were Incertae sedis *Tremellales* (12.5%), followed by unidentified *Ascomycota,* and *Debaryomycetaceae* (both at ~11.3%) and unidentified *Pleosporales* (9.1%; Romero et al., 2018). Among the most abundant genera in corn, yeasts such as *Hannaella, Bullera, Bulleromyces, Meyerozyma,* and molds such as *Fusarium* and *Gibberella* were reported (Romero et al., 2018). Members of these genera are reported as plant, human, and animal pathogens (Kurtzman et al., 2011).

Table 2-2. Typical populations of epiphytic organisms in forage crops. Adapted from Pahlow et al. (2003).

Microorganism	Population (CFU[1]/g fresh forage)
Total aerobic bacteria	1×10^7
Enterobacteria	1×10^3 to 1×10^6
Molds	1×10^3 to 10^4
Yeasts	1×10^3 to 1×10^5

[1]Colony forming units

Wilting community

The microbial community of hay during wilting and baling primarily comprises mesophilic organisms that propagate on high moisture plant tissues (Magan and Lacey, 1986). Typically, a greater fungal diversity during wilting is observed relative to the community found during storage, where few species dominate (Wittenberg, 1997). A small number of studies have described wilting community dynamics (Breton and Zwaenepoel, 1991; Taffarel et al., 2013). The fungal community of wilted tall fescue

grass hay consisted primarily of molds such as *Cladosporium herbarum*, *Fusarium poae*, *Alternaria alternata* (6×10^4, 6×10^3, and 6×10^3 CFU/g fresh hay, respectively), and fewer yeasts (1×10^2 CFU/g fresh hay) belonging to the genera *Sporobolomyces* and *Trichosporon* (Breton and Zwaenepoel, 1991). The presence of these molds may have adverse effects on humans and livestock health, including respiratory allergies caused by *C. herbarum* and *A. alternata* (Breitenbach and Simon-Nobbe, 2002), and production of mycotoxins (type A trichothecenes) by *F. poae* (Dinolfo and Stenglein, 2014).

Storage community

Field fungi rapidly disappear upon exposure to high temperatures (> 50°C) after the second or third day of storage due to their thermo sensitivity (Breton and Zwaenepoel, 1991). Simultaneously, species belonging to the genera *Rhizopus*, *Absidia*, *Aspergillus*, and *Penicillium* thrive during the storage phase (Undi et al., 1997). These fungi are described to proliferate on dryer plant tissues and at higher temperatures (Roberts, 1995). The microbial community of Tifton 85 bermudagrass (*Cynodon dactylon*) hay dried under field or shed conditions consisted of the molds *Fusarium*, *Penicillium*, and *Aspergillus* across cutting, baling, and storage (Taffarel et al., 2013). *Fusarium* was the most abundant genus across all stages and storage conditions with populations reaching up to 9×10^3 CFU/g fresh forage after the storage period (Fig. 2-4), followed by *Penicillium* and *Aspergillus* with populations reaching 10^3 and 10^2 CFU/g fresh forage, respectively during baling (Taffarel et al., 2013).

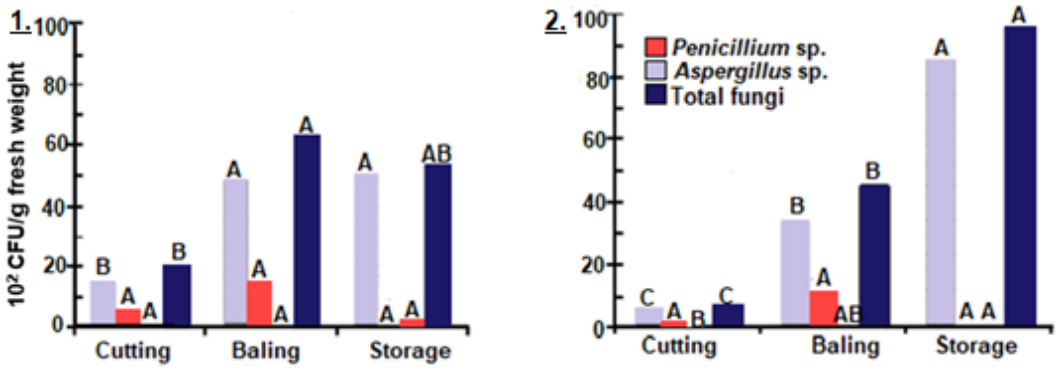

Figure 2-4. Total fungi during haymaking phases (cutting baling and storage) dried under field conditions (1) or under shed (2). Modified from Taffarel et al. (2013).

The moisture content of forage at the time of baling is an important factor influencing microbial counts and species diversity during storage (Undi et al., 1997). Kaspersson et al. (1984) described those dynamics for an unspecified grass species hay stored under high moisture conditions (30%). Relative to the initial nutritional composition, increased concentrations of acid detergent fiber (ADF) and acid detergent insoluble nitrogen (ADIN; 20.0 and 16.2% DM, respectively), lower levels of metabolizable energy (-8%), and a simultaneous increase in bale temperature were observed due to spoilage ensued by microbial succession (Kaspersson et al., 1984). During the first 3 days of storage, the population of mesophilic bacteria, primarily members of the genus *Micrococcus* decreased from an initial population of more than 10^7 to approximately10^2 cfu/g forage. On the other hand, populations of thermophilic bacteria increased two logs until they reached a maximum of 10^9 cfu/g forage after six days and remained high throughout the storage phase (Fig. 2-5). Also, the fungal community during baling was dominated by mold species from the genera *Cladosporium* and *Fusarium* with populations of 10^6 cfu/g forage. After five days of

storage, there was a shift to species predominantly from the genus *Aspergillus* (*A. flavus* and *A. glaucus*).

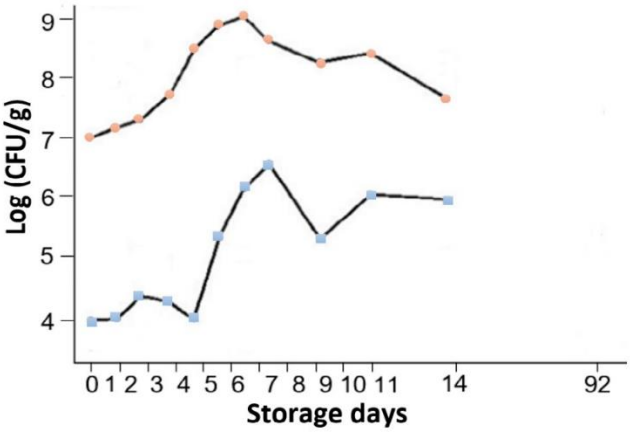

Figure 2-5. Changes in the number of bacteria during storage of hay CFU/g forage of thermophilic bacteria; CFU/g forage of mesophilic bacteria. Adapted from Kaspersson et al. (1984).

Similarly, Gregory et al. (1963) investigated these dynamics for mixed grass hay baled at high (30%) and low (15%) moisture concentration. Compared to high moisture hay, low moisture bales were characterized as having a diverse fungal community, including members of *Cladosporium*, *Penicillium*, and *Aspergillus*, with total fungal populations of log 10^4 spores/g DM (Fig. 2-6). On the other hand, high moisture hay was dominated by thermophilic fungi, particularly members from *Absidia* and *Mucor* as well as by diverse actinomycetes (mainly *Micromonospora vulgaris*), with populations of 10^7 and 10^8 spores/g DM for total fungi and total actinomycetes, respectively (Gregory et al., 1963). Conversely, higher populations of bacteria were found in low moisture bales (10^8 vs. 10^5 CFU/g DM). In addition, low moisture hay remained stable and no substantial changes were observed in pH (6), sugar content (4% DM), as well as volatile (2% total N) and soluble N (35% total N) over the storage phase for low moisture hay (Fig. 2-6; Gregory et al., 1963). In contrast, for high moisture hays, moisture content

decreased overtime and the temperature reached a maximum of 55°C (Gregory et al., 1963). As for nutritional components, sugar content decreased considerably (from 4% to 0.09% DM), while volatile N increased from the first day of baling until reaching 6% of total N after 77 d of storage (Gregory and Lacey, 1963).

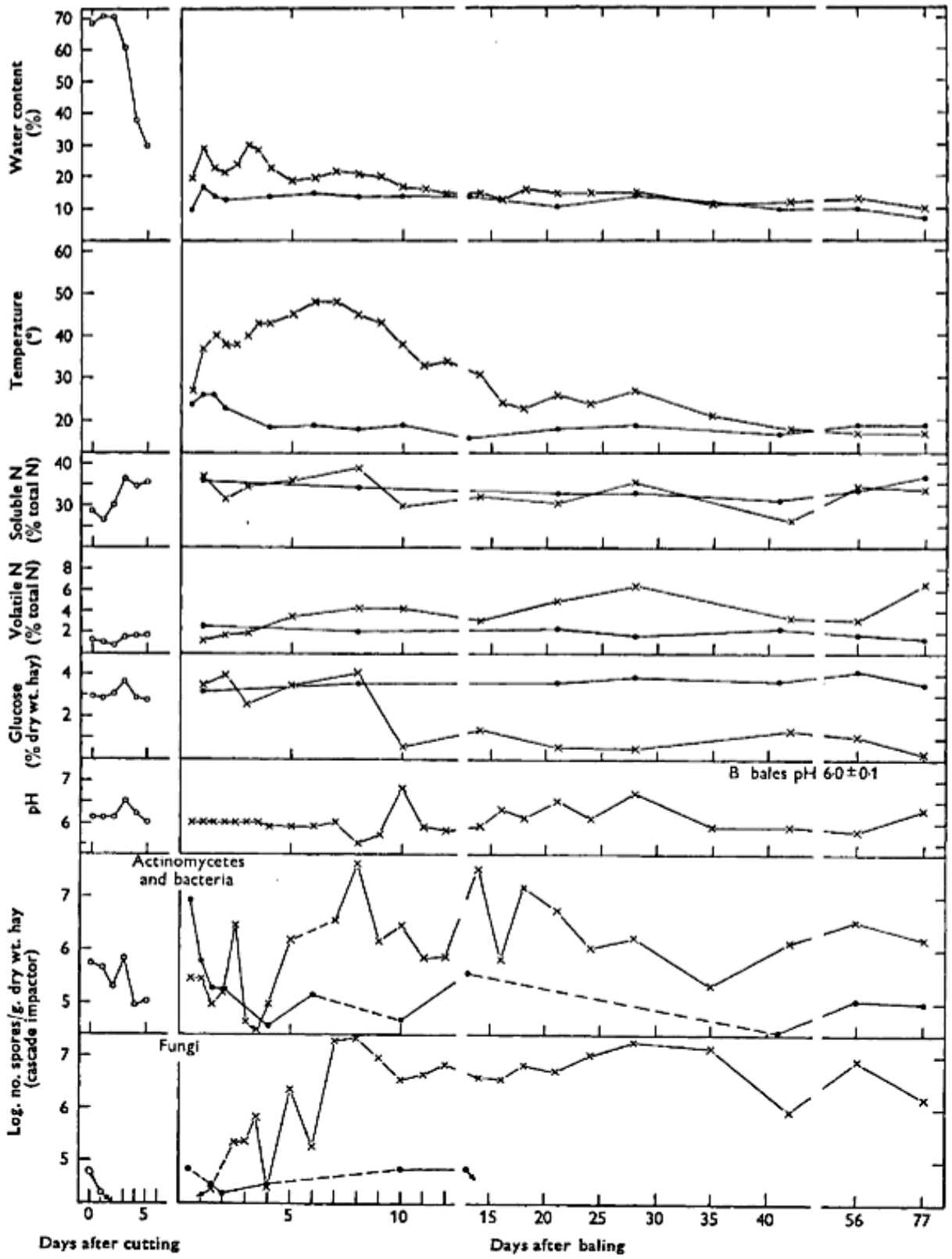

Figure 2-6. Changes in wet (A, x), dry (B, ●) baled hay, and grass dried in the field (○). Modified from Gregory et al. (1963).

Aerobic spoilage in other harvested high fiber crops

Spoilage community in silage crops

Due to the limited information on hay microbiota, the fungal and bacterial communities of silages during aerobic exposure will be described to draw a parallel with high moisture hay. As silage is exposed to oxygen during the feeding phase, undesirable aerobic microorganisms thrive, causing spoilage (Driehuis et al., 2001). The fungal community of corn silage after 14 d of aerobic exposure was characterized using denaturing gradient gel electrophoresis (Dolci et al., 2011). Among the identified species, the mold *Aspergillus fumigatus*, and the yeasts *Kazachstania exigua* and *Pichia kudrivzevii* were described (Dolci et al., 2011). These findings agree with Li and Nishino (2011b), who identified the yeasts *P. kudriavzevii* and *Saccharomyces exiguous* after 14 and 56 d of aerobic exposure in corn silage. In the case of ryegrass silage, marked changes in the fungal communities after 56 d of aerobic spoilage were observed using denaturing gradient gel electrophoresis (Li and Nishino, 2011). This was characterized by the disappearance of *Fusarium equiseti* and *Paecilomyces* sp. after aerobic exposure, while *Penicillium roquefortii*, *Pichia anomala*, *Pichia burtonii*, and *S. cerevisiae* appeared (Li and Nishino, 2011).

Spoilage community in vegetables

Likewise, some useful parallels can be drawn between the characterized microbial communities in spoiled vegetables and in high moisture hay. In the study of Harding et al. (2017), 386 and 344 bacterial and fungal isolates, respectively, were isolated from a wide variety of fresh produce. Then, representative isolates were sequenced and characterized by their spoilage potential after being re-inoculated in fruits and vegetables. Species from the genera *Fusarium*, *Penicillium*, and *Aspergillus* were the

most frequently identified for having spoilage potential for fresh produce (Harding et al., 2017). Likewise, the fungal communities of spoiled seeds across a wide range of soybean varieties were determined using next generation sequencing for the nuclear ribosomal internal transcribed spacer-1 (ITS1; Liu et al., 2017). Most fungi belonged to the *Mycosphaerellaceae* family (59.7% relative abundance) in spoiled black soybeans, while for yellow soybeans the most abundant fungi belonged to the genera *Giberella* (*Fusarium graminareum*) and *Alternaria* (39.5 and 32.01% relative abundance, respectively; Liu et al., 2017). In addition, *Aspergillus flavus*, *A. niger*, *Fusarium monoliforme*, and *Penicillium chrysogenum* were isolated and identified for their potential role in spoilage (Liu et al., 2017).

Methods to measure fungal contamination in hay

Researchers have relied on both qualitative and quantitative methods to describe the degree of microbial spoilage in hay, with most procedures aiming to estimate mold presence (Roberts, 1995). These procedures can be classified as visual estimation, viable counting (dilution plating), and chemical methods (Wittenberg, 1997).

Visual estimation

Traditionally, fungal contamination of hay has been evaluated through visual assessment of mycelia and spores presence related to a defined scale of contamination which may range from 1 to 5, where 1 means no visible spores or mycelia and 5 substantial evidence of mycelia throughout the bale (Wittenberg, 1995). Hay appearance is also a factor to be considered, due to the changes in hay color that occur along with microbial growth and heating. Depending upon the magnitude of spoilage (i.e. heating), hay may change from green color at baling to different shades of brown at the end of the storage period (Collins, 1996). However, some drawbacks can occur with

this method. First, visual estimation is a subjective measurement that poses inconveniences for comparison across quantitative studies. In addition, in some cases it is difficult to differentiate fungal spores from dust (e.g. red clover hay).

Enumeration techniques

Among quantitative methods, plate and spore counts have been frequently used to detect and enumerate fungi from a hay sample (Roberts, 1995). However, these techniques may underestimate the true microbial diversity since many of the microorganisms associated with forages are viable but not culturable (McAllister et al., 2018). Traditionally, all-purpose media such as malt extract (MEA), yeast glucose, Sabouraud, and peptone dextrose agar have been described as standard media to isolate and enumerate fungi from spoiled hay (Lacey and Dutkiewicz, 1976). Malt extract agar has been the most consistently used and no other medium was reported to grow more total fungal colonies (Lacey and Dutkiewicz, 1976). More recent reports of media used for isolation, enumeration and identification of food spoilage fungi have described dichloran rose bengal chloramphenicol (DRBC) agar as an ideal medium to count more accurately, since it contains rose bengal and dichloran, which limit rapidly spreading molds (e.g. *Rhizopus* and *Mucor*) without disturbing spore germination (Pitt and Hocking, 2009).

Chemical methods

Chemical methods consist of quantifying fungal constituents such as ergosterol or chitin, which are used as markers for total mycelial dry matter (Roberts, 1995). Chitin is a simple polysaccharide present in the fungal cell wall (Latge, 2007). It is widely used as a marker for mold contamination in different crops including hay, it is stable for years, and it has been reported to accurately measure mycelial contamination in several

forages (Wittenberg et al., 1989). However, relationships between chitin concentration and fungal growth can be biased by the different chitin concentrations that exist across fungal species (Roberts et al., 1987) and the stage of mycelial development (Plassard et al., 1982). Additionally, chitin coming from the exoskeleton of arthropods can potentially interfere with the accuracy of the method (Roberts, 1995).

Ergosterol on the other hand, is the predominant sterol present in fungal membranes and it is specific to fungi (Seitz, 1979). However, it fluctuates among species and mycelia growth phase (Tothill et al., 1992). Ergosterol markers are particularly used in moldy grain analysis. However, it has been suggested that concentrations could decrease over the long storage period of hay bales, as ergosterol is easily oxidized, particularly in southern climates (Roberts, 1995).

Forage quality losses due to spoilage

During storage, microbial and plant respiration and the resultant heating affect the nutritional value and voluntary intake of high moisture hay (Rotz and Muck, 1994). These changes have been reported to occur within 12 d of baling (highest microbial activity), with most of the DM loss arising from the oxidation of water soluble carbohydrates (WSC, Turner, 2002). The concentration of WSC declines linearly throughout the storage phase and its extent is driven by hay moisture content (Coblentz, 2004). For low moisture alfalfa hay, WSC decreased from 7.29 at d 0 to 4.21% DM at d 60, while for high moisture hay (30%), WSC went from 5.96 to 2.07% DM (Coblentz et al., 1996). Consequently, an increase in NDF and ADF fractions is expected due to a reduced concentration of non-fiber components (e.g. WSC; Coblentz et al., 2000). Changes in concentration of crude protein (CP) are influenced by storage time. During the first 60 d of storage, protein loss is minor and its concentration can

even increase because of oxidation of WSC (Coblentz et al., 2013). On the other hand, long term storage (6 months) of alfalfa hay bales will result in losses of 0.25% DM of CP per month due to volatilization of ammonia (Rotz and Muck, 1994). Other nitrogen (N) losses are derived from the formation of insoluble N components through Maillard reactions, which are measured as acid detergent insoluble nitrogen (ADIN) that is essentially indigestible in ruminants (Guerrero and Shenvood, 1997). Concentrations of ADIN of 0.6 and 1.4% of total N were reported for large round alfalfa hay baled at normal (15%) and high (27%) moisture concentrations, respectively (Jin et al., 2018). Consequently, the digestibility of hay DM and nutrient constituents declines, mainly due to the oxidation of highly digestible components (e.g. WSC), and to the reduction of CP digestibility through the formation of ADIN (Rotz and Muck, 1994). A comprehensive review of typical losses and forage quality changes during haymaking processes for legume and grass hay is summarized in Table 2-3.

Table 2-3. Typical dry matter (DM) losses and changes in nutrient composition during haymaking practices. Adapted from Rotz and Muck, 1994

Practice	DM loss (average %)	Changes in nutrient concentration (% DM)		
		Crude protein	Neutral detergent fiber	DM digestibility
Legume hay				
Mowing	1	-0.4	0.6	-0.7
Conditioning	2	-0.7	1.2	-1.4
Tedding	3	-0.5	0.9	-1.2
Raking	5	-0.5	1	-1.2
Baling				
Small rectangular	4	-0.9	1.5	-2.0
Large rectangular	3	-0.7	1.0	-1.5
Large round	6	-1.7	3.0	-4.0
Storage				
Inside	5	-0.7	2.1	-2.1
Outside	15	0.0	5.0	-7.0
Grass hay				
Mowing	1	0	0	0

Conditioning	1	0	0	0
Tedding	1	-0.2	0.4	-0.4
Raking	5	-0.3	0.5	-0.6
Baling				
Small rectangular	4	-0.5	0.9	-1.0
Large rectangular	3	-0.4	0.7	-0.8
Large round	6	-1.0	1.8	-2.0
Storage				
Inside	5	-1.3	3.2	-1.8
Outside	12	0.0	8.0	-4.8

As described earlier, field losses are greatly reduced when hay is baled at high moisture concentrations. However, this type of hay can deteriorate rapidly during storage due to fungal spoilage and resultant heating. Consequently, safe and inexpensive preservatives are needed to allow producers to bale hay above safe moisture levels, reducing the risk of weathering and decreasing field losses (Rotz and Much, 1994). However, current hay preservatives are mostly hazardous compounds, costly, and inconsistent in their effects (Rotz and Shinners, 2007).

Hay preservation

Definition and importance

Preservatives are defined as compounds added to products such as food or feed to prevent spoilage (Wittenberg, 1997). Hay preservatives inhibit the growth of undesirable microorganisms to minimize storage losses and maintain pre-storage nutritive quality (Rotz and Shinners, 2007). Ideally, hay preservatives should neither impact livestock health and productivity nor leave residue in milk or meat, and should be safe to handle and have a return on investment of at least 3:1. In Canada and the United States, about 134 additives are registered as hay preservatives (Manitoba-Agriculture, 2015) including organic acids (primarily propionic acid), ammonia-based compounds (anhydrous ammonia and urea), and microbial inoculants, which are

predominantly homofermentative lactic acid bacteria (LAB) inoculants (McCartney, 2009). For the purpose of assessing the effects of the three major hay preservative categories on forage quality, preservation, and animal performance, a literature search was conducted using the Web of Science database on August 2018. A total of 94 peer reviewed publications were retrieved using the terms “hay” and “preservative”. The inclusive criteria for selecting studies were as follows. Studies had to (1) be published in English language peer-reviewed journals; (2) report the preservative application rate; and (3) measure a proxy variable for spoilage (spontaneous heating measurements or DM loss). For each preservative category, the relative differences between means for treated (TRT_m) and untreated (CON_m) hay were estimated using the following formula, Relative Difference (%) = $((TRT_m - CON_m)/CON_m) \times 100$. Factors such as moisture concentration, type of bale, and application dose were not controlled statistically. However, these data will be useful to understand the changes and draw comparisons between preservatives relative to untreated hay values.

Organic acids

Organic acids and their salts constitute the major additive class used to improve preservation of high moisture hay (Coblentz et al., 2013). In most commercially available products, propionic acid (PRP) is the active (i.e. antimicrobial) and most abundant ingredient (typically present at ~67% v/v), although some mixtures may contain acetic or formic acid (McCartney, 2005). Currently, the use of buffered PRP products (ammonium or sodium propionate) containing 10 to 20% undissociated acid is recommended over PRP because these reduce corrosion damage to baler machines (EFSA, 2011). The antimicrobial mechanism of action of PRP consists of the disruption of the electrochemical proton gradient when undissociated acid molecules penetrate the

cell wall and dissociate internally. This process depletes cellular energy and therefore, limits cellular growth and metabolic functions (Davidson, 2001). In fungi, recent research suggested that PRP induces the generation of reactive oxygen species and mitochondrial dysfunction leading to oxidative stress and apoptosis (Yun and Lee, 2016). In addition, because the proportion of undissociated acid declines as pH increases, preservatives are more effective at lower pH (Lück and Jager, 1997), in fact PRP fungistatic properties are enhanced at low pH (< 4.5; (Woolford, 1984), Therefore, it has been suggested that PRP effectiveness is determined by the interaction between forage buffering capacity, epiphytic microbial communities (as some can metabolize the free acid form), and the application rate (Mahanna and Soderlund, 1990). Typically, PRP-based preservatives are applied at 1 to 2% (w/w) to hay of up to 35% moisture (Rotz, 2003).

Across 25 studies (Table A-1), PRP-based preservatives decreased DM losses, visible moldiness, maximum internal bale temperature, and NDF concentration (-32.2, -18.9, -19.0, and -5.8%, respectively), relative to untreated hay values. In addition, this additive class increased WSC concentration and DMD (+8.9 and +4.0%, respectively). Differences observed for ADF, CP, and DMI (-2.8, +2.4, and -2.0%, respectively) were considered not biologically meaningful.

Anhydrous ammonia

Ammonia-based compounds, including anhydrous ammonia (NH_3) and urea have been used effectively as hay preservatives (McCartney, 2005). However, ammoniation is primarily used as a method to improve forage quality by increasing the rate and extent of digestibility (Brown, 1988). Additionally, NH_3 increases the concentration of CP by adding non-protein nitrogen (Lines and Weiss, 1996). Ammonia is applied to a stack

of bales through a pipe connected to a tank (Rotz and Shinners, 2007). The period of treatment varies from 48 h (Knapp et al., 1976) to 14 weeks (Mir et al., 1991), and treated hay must be covered with plastic throughout the storage time to maximize the reaction between NH_3 and hay, increasing labor and materials cost (Rotz and Shinners, 2007). The precise mechanism of antimicrobial action of anhydrous ammonia has not been fully elucidated; it has been suggested that NH_3 can cross the cell membrane by simple diffusion, cause a rapid alkalization of the cytoplasm, and form NH_4^+, which results in low concentration of protons and depletion of ATP (Park and Diez-Gonzalez, 2003).

Due to its caustic nature, improper handling of anhydrous ammonia can cause chemical burns, blindness, and death upon direct exposure, which has limited its adoption by hay producers in the US (Muck and Shinners, 2001; Rotz and Shinners, 2007). In addition, when applied to forage with high soluble sugar concentrations, NH_3 can potentially cause toxicity in livestock due to formation of 4-methyl imidazole (Mir et al., 1991).

Across 11 peer-reviewed articles, hay treated with anhydrous ammonia had reduced DM losses, visual moldiness, as well as NDF concentration (-25.8, -54.2, and -4.08%, respectively), relative to untreated hay values (Table A-2). Furthermore, increased CP concentration, DMD, and DMI (+35.1, +10.4, and +4.49%, respectively) were observed. However, marginal differences were observed for maximum internal bale temperature (+0.5%), ADF (-0.21%), and ADG (-0.91%).

Microbial inoculants

Currently marketed microbial products were originally developed as silage inoculants (Mahanna and Soderlund, 1990). These consist of strains including

Lactobacillus plantarum, *Pediococcus pentosaceous*, *Bacillus subtilis*, and *Bacillus pumulus*. To be effective, inoculants must be applied at a much higher rate relative to epiphytic LAB (Wittenberg, 1995). The typical recommendation is log 5 cfu/g forage DM for both hay and silage (Manitoba-Agriculture, 2015). The antifungal activity of LAB is explained by mechanisms including nutrient competition and synthesis of antagonistic products (Magnusson et al., 2003). In the case of homofermentative LAB, undissociated lactic acid molecules penetrate and dissociate inside the fungal cell membrane, releasing H^+ ions that reduce pH, and consequently inhibit growth (Stratford and Eklund, 2003). Furthermore, this reduction of intracellular pH has an effect on the extent of inhibition by acetic and propionic acid produced by heterofermentative LAB (Schnürer and Magnusson, 2005). Acetic acid's mode of action is similar to the mechanism described for PRP. In hay, inoculants have shown inconsistent results (Shinners, 2000). Table A-3 summarizes the effect of homofermentative LAB inoculants (mostly combo inoculants of *L. plantarum* and *P. pentosaceous*) on the preservation of high moisture hay relative to untreated hay values across 6 studies. Inoculants only increased visible moldiness (+32.3%) and negligible differences were observed for variables such as DM loss, maximum internal bale temperature, NDF, ADF, CP, DMD, and DMI (+0.28, +2.99, +2.85, -0.44, +1.27, +2.4, and -2.5%, respectively).

In conclusion, PRP has been shown to be the most effective additive at preventing spoilage and thus the most popular type of preservative used on high moisture hay. However, PRP effects do not last for more than 6 months and its acidic nature quickly corrodes farm equipment (Rotz and Shinners, 2007). Buffered propionic acid such as ammonium propionate reduces damage to harvesting equipment. Overall, results from

propionic acid-based additives have been inconsistent and beneficial effects seem to be mostly limited to reducing spontaneous heating (i.e. microbial respiration; Coblentz et al., 2013). Furthermore, propionic acid and ammonium propionate cannot be used in organic hay production (NOP, 2016). Anhydrous ammonia is an effective hay preservative, but the high costs of application and caustic nature have prevented its widespread use (Rotz and Shinners, 2007), and it cannot be used on high quality forages due to toxicity problems. Extra costs are incurred during the application of ammonia and urea due to the need to cover the forage with a plastic sheet. Because of the limitations listed above, there are no methods that have been widely implemented by hay producers due to high cost, hazardousness, and lack of effectiveness. Further research needs to be conducted to evaluate alternative additives that can offer a low cost and effective hay preservation.

Lignin

Chemical properties

Lignin is defined as a complex phenolic polymer formed by the oxidative coupling of 4-hydroxyphenylpropanoids (Ralph et al., 2004). The primary lignin precursors are coniferyl, sinapyl, and *p*-coumaryl alcohols (Fig. 2-7a), which undergo enzyme-initiated dehydrogenative polymerization during biosynthesis, generating some interunit ether and carbon–carbon linkages within the lignin macromolecule (Kai et al., 2016). The most predominant being β-*O*-4 (Fig. 2-7b), which comprises approximately 50% of the total linkages (Hatfield and Vermerris, 2001). Lignin polymer is considerably more reactive than cellulose or other natural polymers, since it does not have a repetitive order of units and because of the presence of different functional groups in lignin including methoxyl, carboxyl, carbonyl, hydroxyl, and some minor terminal aldehyde

groups (Adler, 1977). The presence and quantities of these functional groups change depending on the lignin's origin and extraction process (Gosselink et al., 2004).

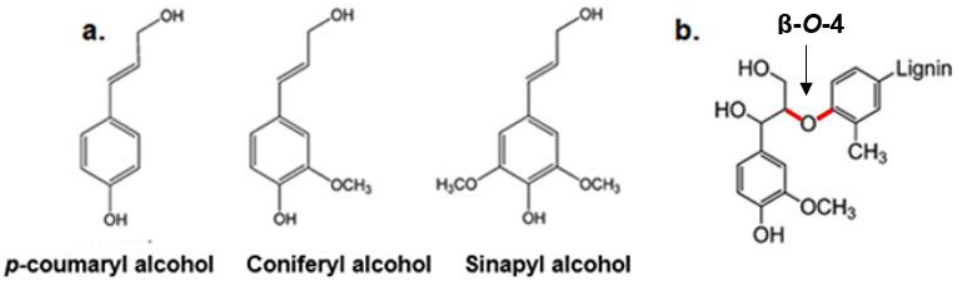

Figure 2-7a. Lignin monomeric building blocks. b. β-*O*-4 ether lignin bonds. Adapted from Xu et al. (2014).

Lignin is found in higher plant tissues as a cell wall component that provides rigidity and strength, controls water conduction, and protects the plant from microbial degradation (Boerjan et al., 2003). After cellulose and hemicellulose, lignin is one of the most abundant natural terrestrial polymers (Saito et al., 2012) with approximately 300 billion Mg found at any given time on earth (Buranov and Mazza, 2008), with an annual biosynthetic production increase of around 20 billion Mg (Argyropoulos and Menachem, 1998). In plant tissue, lignin does not exist as an independent polymer but it is linked with hemicellulose through covalent bonds, forming carbohydrate polymer matrixes termed lignin-carbohydrate complexes (Ralph et al., 1995).

The plant cell wall is a metabolically active, dynamic compartment with different layers, and each layer has different composition and attributes (Evert, 2006). The composition of a typical softwood cell wall (Fig. 2-8) includes the primary wall (P), the secondary wall which is divided into three sub layers (outer layer S1, middle S2, and inner S3), and the middle lamella (ML). In plant tissues that undergo lignification such as the sclerenchyma, epidermis (not lignified in legumes), xylem, and

nonclorenchymatous parenchyma, lignin synthesis starts during secondary cell wall formation at the cell corners in the ML and the P when S1 formation initiates (Cazacu et al., 2013; Terashima, 1993). As observed in Fig. 2, ML and P as well as S1 are mostly composed of lignin (80 and 52.7% of the total weight), while S2 is mostly cellulose (54.3%). However, because the secondary wall occupies a larger portion of the wall, it is recognized for having the highest lignin content (Baucher et al., 1998).

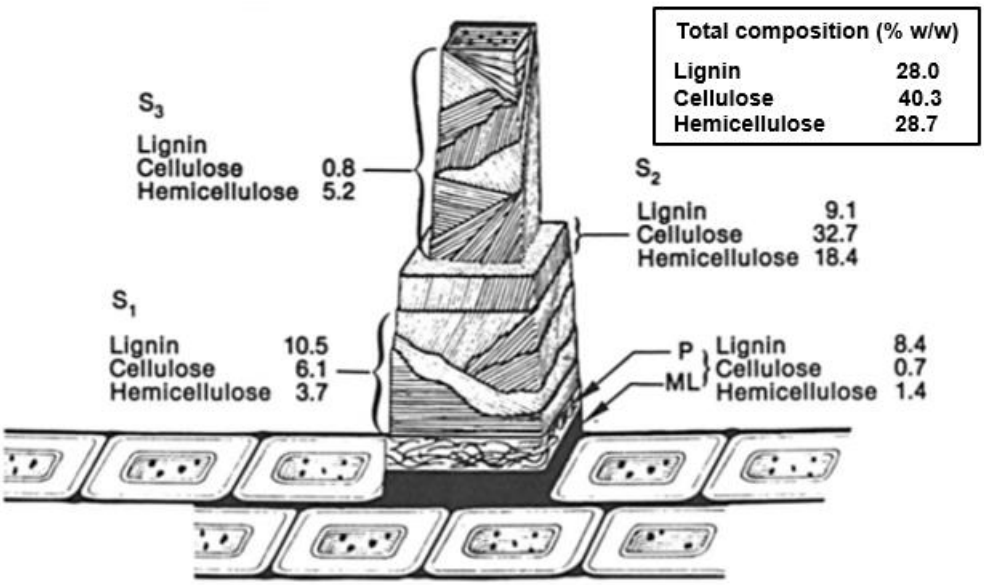

Figure 2-8. Representation of a Scotch pine cell wall with the chemical composition as a percent of total weight. Adapted from Winandy and Rowell (1984).

During lignin deposition, monolignols (coniferyl, sinapyl, and *p*-coumaryl alcohols) are synthetized in the cytoplasm from phenylalanine, via general phenyl-propanoid and monolignol-specific pathways (Fig. 2-9) and transported to the cell wall where they undergo oxidation and polymerization reactions to form lignin (i.e. lignification). The formation of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units occurs when the respective monolignols are being incorporated into the lignin polymer (Cazacu et al., 2013).

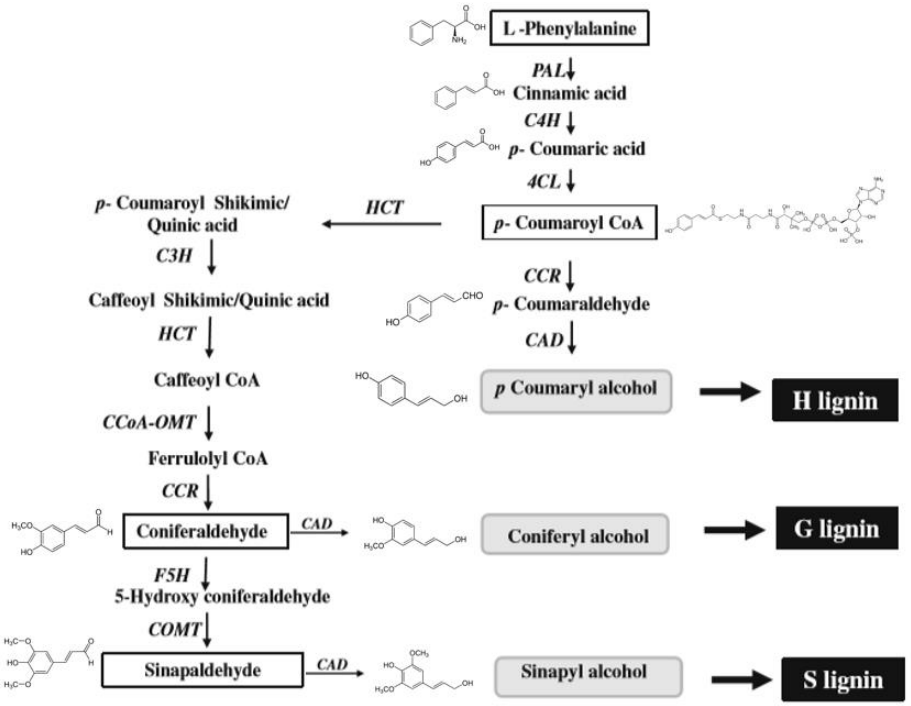

Figure 2-9. Schematic representation of the monolignol biosynthesis. H lignin, grass lignin; G lignin, softwood lignin; S lignin, hardwood lignin. *PAL* phenylammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* hydroxycinnamate-CoA/5hydroxyferuloyl-CoA ligase, *HCT* hydroxycinnamoyl-CoA shikimate/quinatehydroxycinnamoyl transferase, *CCR* hydroxycinnamoylCoA:NADPHoxidoreductase, *CAD* hydroxycinnamyl alcohol dehydrogenase, *C3′ H p*-coumaroyl shikimate 3′ hydroxylase, *CCoAOMT S* adenosyl-methioninecaffeoylCoA/5-hydroxyferuloyl-CoA *O*-methyltransferase, *COMT* caffeate *O*-methyltransferase, *F5H* ferulic acid 5-hydroxylase. Adapted from Ayyachamy et al. (2013).

Lignins can be classified in three major groups (Evert, 2006), softwood or guaiacyl (formed mostly from G structures), hardwood or guaiacyl-syringyl (formed by a mixture of S and G structures), and grass or syringyl-*p*-hydroxyphenyl lignin (formed from all three monomers). The rigidity of the structure, one of the main factors determining the overall physicochemical properties of lignin and therefore potential applications (Lora et al., 2002), will depend on the degree of cross-linking of each lignin group (Doherty et al., 2011). For example, lignin from soft wood is branched and cross-linked, whereas lignin

obtained from hard wood is more linear due to the S unit, which facilitates the retention of the linear structure (Thakur et al., 2014).

Technical Lignins

The extraction and isolation of lignin from lignocellulosic materials is conducted under diverse conditions and multiple reactions (e.g. catalyzed biomass hydrolysis and condensation of lignin fragments) resulting in products with different physicochemical properties relative to native lignin (Doherty et al., 2011). Therefore, besides lignin source, the method of extraction will have a substantial effect on structure and properties of the technical lignin (Lora, 2008). Approximately 50 million Mg of lignin are separated from wood pulp and paper-making industries via chemical processes each year (Wang et al., 2009). However, due to its highly complex structure, only a small portion (1 - 2%) of this material is processed into valuable byproducts (Lora et al., 2002); (Gosselink et al., 2004) with the rest being used as a fuel source for the pulping process (Ayyachamy et al., 2013).

Among the processes used to extract lignin, Kraft pulping is the most important pulping process globally, as well as in the United States (North American Pulp and Paper Fact Book, 1997). Other major processes include sulfite, soda, and organosolv (Espinoza-Acosta et al., 2016).

Kraft lignin

In the Kraft process, an aqueous solution of sodium hydroxide and sodium sulfide is used to obtain cellulose pulp under a strong alkaline environment in a large pressure vessel or digester (Fig. 2-10) followed by a final acidification process (Lange et al., 2013). This digestion causes the lignin polymer to fragment due to an extensive cleavage of β-aryl links, and consequently the generation of free hydroxyl groups

(Vishtal and Kraslawski, 2011). The Kraft delignification process occurs in three stages at temperatures of 150°C, between 150 and 170°C, and >170°C, respectively. Then, lignin is recovered from the black liquor by decreasing the pH to 5 with sulfuric acid (Koljonen et al., 2004). Kraft lignins are hydrophobic, therefore they need to be modified to improve solubility (Doherty et al., 2011). Molecular weights ranging from 200 to 200,000 g/mol (Niemela, 1992), and ash content of < 3% DM (Lora, 2008) have been described for this type of technical lignin. The production of Kraft lignin is reported to be 95% (47 million Mg) of all lignin produced worldwide (Gosselink et al., 2004). However, Kraft lignin is mostly used in low added-value applications (e.g. power generation; Mohan et al. (2006), and only about 100,000 Mg are commercially used in other products and applications (El Mansouri et al., 2006). These include binders and resins (Tejado et al., 2007), carriers for fertilizers and pesticides (Zhang et al., 2007), and production of low molecular weight compounds such as vanillin, aliphatic acids, and hydroxylated aromatics (Holladay et al., 2007).

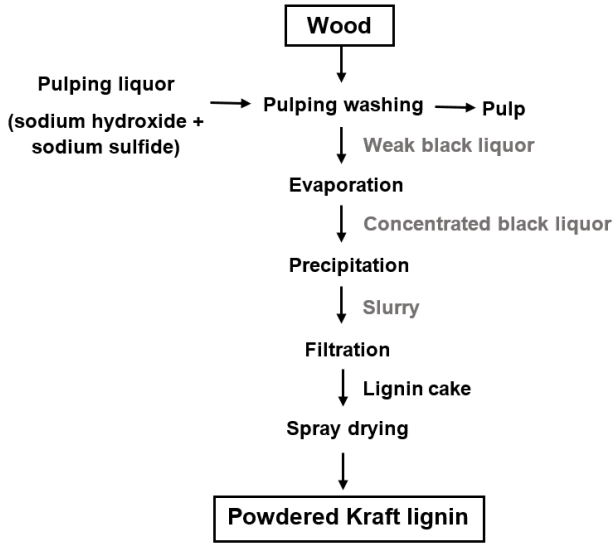

Figure 2-10. Production pathway of Kraft lignin. Adapted from Lora (2008).

Lignosulfonates

In the sulfite chemical pulping process, delignification of wood is carried out using sulfur dioxide and a base (Lora, 2008). Thus, lignosulfonates contain a high concentration of sulfur in the form of sulfonated groups on the aliphatic chains (Laurichesse and Averous 2014). The type of base used, typically, calcium, ammonium, magnesium, or sodium, and its solubility and dissociation properties influence the pH of the digestion (Doherty et al., 2011). Lignosulfonates can be obtained by diverse methods including alcoholic fermentation followed by distillation, ultrafiltration, or precipitation, which simultaneously can yield co-products such as vanillin (Fig. 2-11; Lora, 2008). This technical lignin is characterized for being soluble in water, having relatively high molecular weight ranging from 1,000 to 150,000 g/mol (Lora, 2008), and high ash content ranging from 9.3% DM for calcium lignosulfonate (Lignotech Iberica S.A., Spain) to 29.4% DM for a commercial mixed hardwood lignosulfonate (LRV, France). Also, total sugar content has been reported to be as high as 10.1% DM for a commercial mixed hardwood lignosulfonate (LRV, France).

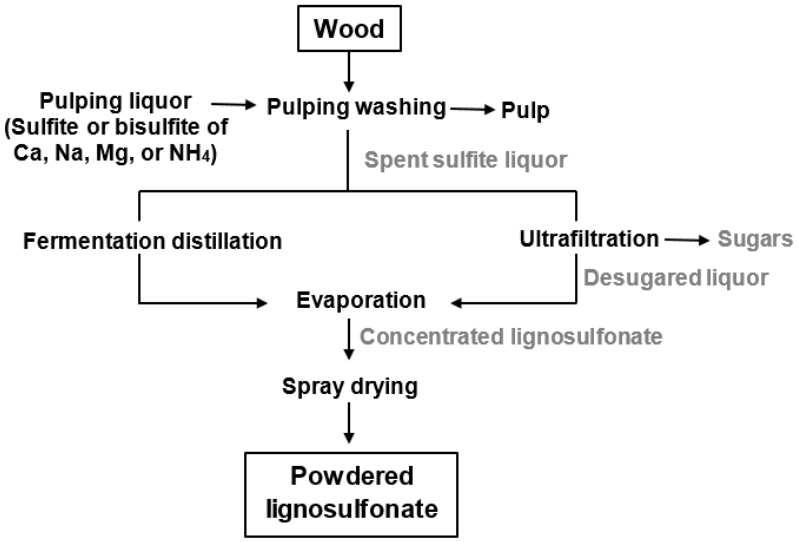

Figure 2-11. Production pathways for lignosulfonates. Adapted from Lora (2008).

The production of lignosulfonates has been reported as one million Mg per year (Belgacem and Gandini, 2008), constituting the bulk of technical lignins commercially used for materials and chemical applications. The variety of existing functional groups (hydroxyl, carboxylic, and sulfur containing groups) provides this lignin with distinctive colloidal properties, including superior wettability, dispersive ability, and absorptivity (Areskogh and Henriksson, 2011). Consequently, lignosulfonates are commercially used as dispersing agents, binders, adhesives, and stabilizers (Ghaffar and Fan, 2014). However, at present they are predominantly used as concrete dispersing agents as well as binders in animal feed pellets and agricultural fertilizers (Lora, 2008). Because of the hydrosoluble nature of lignosulfonates, the fertilizers can break down without difficulty in the environment or, in the case of feed pellets, in the animal digestive tract (Pye, 2008).

Lignosulfonates have shown a capacity to decrease the ruminal degradation of proteins (Wright et al., 2005; Borucki Castro et al., 2007; Wang et al., 2009). High producing dairy cattle need to supplement microbial protein with significant amounts of high quality dietary protein that can escape rumen fermentation (rumen undegradable protein) in order to meet their amino acid requirements (Harstad and Prestløkken, 2000). Currently, lignosulfonates are commercially used (e.g. Soypass; Borregaard Lignotech, WI) to increase ruminal protein bypass of legume seeds (soybean and canola) by up to 173% (Petit et al., 1999) with no negative effects on performance (McAllister et al., 1993; Stanford et al., 1995). Similarly, they have been used as a precipitator for protein recovery (Cerbulis, 1978, Becker and Lebo, 2002). In non-ruminants, lignosulfonates have been shown to increase desirable cecal and colonic microbial populations such as *Lactobacillus* and *Bifidobacterium*, and positively affect

pH (decreased to 6.5), relative to the control group in adult mice (Flickinger et al., 1998). Other applications are outlined in Table 2-4.

Table 2-4. Lignosulfonate products in speciality markets. Adapted from Doherty et al. (2011).

Product	Reference
Vanillin	(Bjørsvik and Minisci, 1999; Gogotov, 2000)
Pesticides	(Lebo, 1996)
Water treatments	(Zhuang and Walsh, 2013)
Industrial cleaners	(Jones, 2008)
Emulsifiers	(Gundersen et al., 2001)
Wood preservatives	(Dumitrescu et al., 2002)
Battery expanders	(Pavlov et al., 2000)

Soda lignin

This type of lignin is produced by treating non-wood fibers such as bagasse, flax, straws, or sugarcane with highly alkaline solutions of sodium hydroxide, and unlike Kraft lignin, the cooking process is done in a sulfur-free medium (Duval and Lawoko, 2014). In the soda pulping process, lignin is extracted by acid precipitation, heating, and filtration (Doherty et al., 2011). The chemical properties of the soda lignin are considerably different from lignosulfonates, as these are hydrophobic lignins with lower molecular weights (ranging from 1,000 to 3,000 g/mol; Lora, 2008). Due to the absence of sulfur, it is suggested that the composition of soda lignin is closer to native lignin relative to other technical lignins (Nadif et al., 2002). Potential application in areas such as animal feed and nutrition have been reported, particularly for the treatment of enteric disturbances in ruminants (Cruz et al., 1997), and as alternatives for antibiotics (Lora, 2008).

Organosolv lignin

The organosolv process consists of solubilization of wood using a mixture of organic solvents, predominantly formic or acetic acid and ethanol, followed by filtration,

and drying (Xu et al., 2006). Organosolv lignin has a high lignin purity due to minimal carbohydrate and ash content (Doherty et al., 2011), is hydrophobic, and has a low molecular weight (500 to 5,000 mol/g; Lora and Glasser, 2002). Several organosolv pulping processes are commercially registered, among which organosolv lignin from the Alcell (extracted with ethanol) process has been the most studied to date (Lora, 2008). Alcell lignin has been reported to have *in vitro* (Nelson et al., 1994) (Phillip et al., 2000) and *in vivo* antibacterial activity (Baurhoo et al., 2007a), as well as prebiotic effects by improving intestinal morphology and supporting growth of beneficial bacteria in broiler chickens (Baurhoo et al., 2007b). Furthermore, Wang et al. (2009) reported linear reductions in 24 h methane emissions and ammonia-N accumulation when Alcell lignin was added to feedlot lamb diets and fermented *in vitro*. Methane production is negatively correlated with energy utilization in ruminants (Ørskov et al., 1968). Furthermore, reduced methane has a positive impact on the environment (Smith et al., 2010).

Antimicrobial activity of lignin

A limited number of studies have evaluated the antimicrobial activity of technical lignins, which will be discussed next. Interpretation of these results can be challenging due to the different technical lignin types and sources tested, microorganisms, and methodologies used. In many studies, a thorough description of the chemical properties of the lignins tested is often lacking, which impedes direct comparisons among studies.

Lignosulfonates

Lignosulfonates represent the most widely used type of technical lignins. Recent reports of antimicrobial properties could expand their potential applications. Jha and Kumar (2018) reported MIC values for sodium lignosulfonate (Sigma-Aldrich Corp, St.

Louis, MO) of 50, 62, 62, 60, and 80 µg/mL for *Candida dubliniensis, C. tropicalis, C. albicans, C. glabrata,* and *C. parasilopsis*, respectively. When these values were evaluated using the disc diffusion method, it was observed that relative to fluconazole, the inhibition of diameter growth was 6, 10.3, and 23% for *C. glabrata*, *C. tropicalis*, and *C. albicans*, respectively.Similarly, Núñez-Flores et al. (2012) evaluated a sodium lignosulfonate extracted from eucalyptus wood (LignoTech Ibérica, S.A. Torrelavega, Spain) with a molecular weight of 7,085 Da, 4% reducing sugar content, and antioxidant activity of 97 µg/mL (DPPH IC_{50}). An undisclosed dose of this byproduct showed a 9.9% growth inhibition for *D. hansenii* using the disk diffusion method. However, no antifungal activity was observed against *Aspergillus niger* or *Penicillium expansum*.

Regarding antibacterial activity, Kim et al. (2013) reported that lignosulfonate nanoparticles isolated from calcium lignosulfonate (Borregaard-LignoTech, Sarpsborg, Norway) had a bacteriostatic effect at a dose of 5×10^{10} particles/mL against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* with an inhibition of 95, 58, and 13%, respectively, using a turbidimetric method (Kim et al., 2013). Furthermore, it is noteworthy to mention that antiviral activity has also been reported for lignosulfonates (Suzuki et al., 1989). Sodium lignosulfonate was highlighted as a potential microbicide with anti-HSV (herpes simplex virus) and anti-HIV activity (human immunodeficiency virus; Gordts et al., 2015). It is hypothesized that the strong surfactant properties of lignosulfonates may explain their antimicrobial activity (Núñez-Flores et al., 2012). Surfactants interact with different cellular constituents, especially lipids and proteins, causing adverse effects on the growth and viability of cells by disrupting normal microbial cellular functions (Merianos, 1991; Hugo, 1992).

Kraft lignins

Fewer studies are available for Kraft lignins. Dong et al. (2011) reported a MIC of 0.01 and 0.0025 µg/mL using alkali Kraft lignin (Sigma-Aldrich Corp, St. Louis, MO) against *Candida lipolytica* and *S. aureus*. However, no antibacterial activity was reported against *Listeria monocytogenes*. The lignin tested by Dong et al. (2011) had an antioxidant activity of 3.5 µmol TE/g (TE, Trolox equivalent) and 165.5 mg/g total soluble phenolics. Similarly, Durmaz et al. (2015) evaluated the antifungal activity of Kraft black liquor extracted from Scots pine, and reported that a concentration of 5% liquor protected wood samples from fungal degradation by two species of brown-rot fungi, *Coniophora puteana*, and *Poria placenta*. Although the mode of action of Kraft lignins against fungi is unknown, Dizhbite et al., 2004 suggested that for bacteria it is associated with the inhibition of radical processes of bacterial cells. Hence, a correlation between radical scavenging (antioxidant) and antimicrobial activities was suggested (Dizhbite et al., 2004). Similarly, Dong et al. (2011) reported a positive association between antimicrobial and antioxidant activities of lignins. Conversely, Núñez-Flores et al. (2012) did not find such a relationship.

Lignin phenolic monomers

Lignin is a natural source of phenolic compounds (Fig. 2-12) with well-recognized antimicrobial properties (Baurhoo et al., 2008). Phenolic monomers, such as carvacrol and cinnamaldehyde, have shown antimicrobial effects when tested in fresh fruits and vegetables and meat (Ultee et al., 2000; Roller and Seedhar, 2002). Early studies have reported that lignin constituents (i.e. phenolic monomeric fragments) such as isoeugenol and ferulic acid can inhibit the growth of *S. cerevisiae*, *C. albicans*, and *A. niger* at

doses of 100 and 187; 100 and 375; and 250 and 700 µg/mL, respectively (Zemek et al., 1979).

Similarly, when vanillin, eugenol, and cinnamaldehyde were extracted from lignin by alkaline oxidation with benzene, these were found to be fungicidal at doses of 0.01% against *Fusarium* sp. (Telysheva et al., 1968). De Greef and van Sumere (1966) found that ferulic acid at 2.5 mM had antifungal activity against *S. cerevisiae*. Likewise, Baranowski et al. (1980) reported antimicrobial activity for ferulic acid against the same organism at a dose of 0.23 mM. The difference among these two studies was attributed to the lower pH of the medium in the second study (6.0 vs. 3.5, respectively), given that at lower pH the efficacy of ferulic acid is boosted due to an enhanced membrane permeability in the undissociated state (Baranowski et al., 1980).

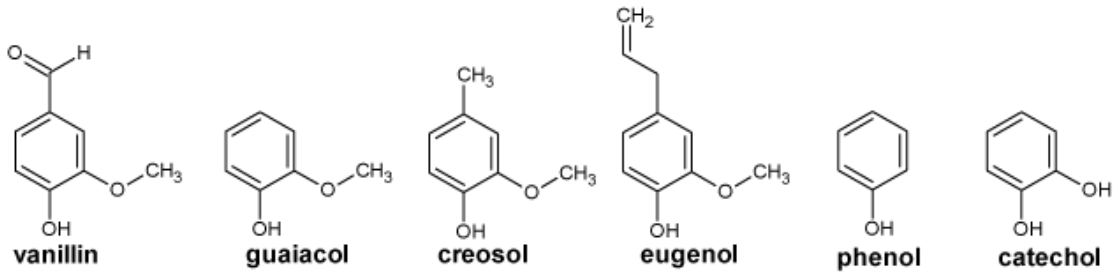

Figure 2-12. Structure of representative phenolic lignin model compounds. Adapted from Stanzione et al. (2016).

The MIC for the three main classes of intermediates of the lignin specific pathway (Hydroxycinnamaldehydes, hydroxycinnamic acids, and hydroxycinnamyl alcohols) were reported for *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Sporobolomyces roseus*, and *B. subtilis*, *E. coli*, and *Pseudomonas syringae (*Barber et al., 2000). Hydroxycinnamaldehydes were the most antimicrobial compounds, with coniferaldehyde being the most antifungal (MIC 1.2 mM) and *p*-coumaraldehyde, the most antibacterial (MIC 2.0 mM). In the case of the hydroxycinnamic acids (*p*-coumaric,

cafeic, ferulic, and sinapic acid; Fig. 2-13), a higher inhibitory effect against bacteria relative to fungi was observed (MIC 3.0 vs. > 8 mM), except for ferulic acid, which inhibit *S. cerevisiae* at a concentration of 4.0 mM. The hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohol) had the lowest antimicrobial properties (MIC ≥ 8.0 mM; Barber et al., 2000).

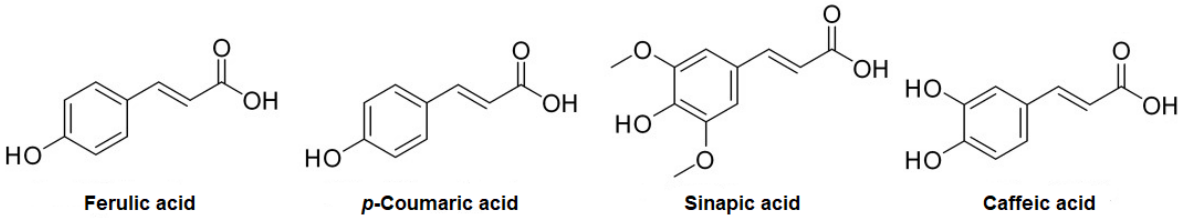

Figure 2-13. Structure of hydroxycinnamic acids. Adapted from Aguilar-Hernández et al. (2017).

Lignin-carbohydrate complexes

LCCs have been reported to have antimicrobial, antiparasitic, antitumor, and antiviral properties (Sakagami et al., 2010; Abe et al., 1989; Lee et al., 1993; Sakagami et al., 2008). LCC extracted from pine trees had an inhibitory effect against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *C. albicans*, but no antibacterial activity was reported against *Salmonella enteriditis* in mice at an undisclosed dose (Harada et al., 1988; Oh-hara et al., 1990). In that study, it was suggested that the sugar moiety of LCC had a significant influence on the induction of antimicrobial activity, because when the sugar fraction was removed with sulfuric acid, the antimicrobial activity decreased significantly (Sakagami et al., 2010). Moreover, LCC extracted from a pine species (*Pinus parviflora*) with an alkaline solution, evaluated at an undisclosed dose, showed a high anti-tumor activity in mice, which was increased when LCC was acidified (Sakagami et al., 2010). In addition, the same LCC extract applied subcutaneously at a dose of 10 mg/kg live weight, protected mice from infection

caused by the cestode *Hymenolepis nana* (Sagakami et al., 2010). Moreover, LCCs extracted from pine cone (Lai et al., 1990), *Theobroma cacao* (Sakagami et al., 2008), mulberry juice (Sakagami and Watanabe, 2010) were reported for having unique antiviral activity *in vivo* against HIV, HSV and influenza virus.

Lignin-based biopolymers

The current interest and demand for novel biopolymers have led to the exploration of lignin's antibacterial and antifungal properties as new materials, including lignin-based polymers (films, fibers, and hydrogels), as well as lignin nanoparticles (LNP). The risk of bacterial colonization is a frequent complication associated with the use of biomedical devices (Kai et al., 2016). Thus, Larrañeta et al. (2018) investigated the potential of lignin-based hydrogels for biomedical applications as material coatings. A substantial superior resistance to bacterial adherence from hydrogels containing 38 % (w/w) dealkaline lignin relative to a commonly employed medical material was observed against *S. aureus* and *Proteus mirabilis* (Larrañeta et al., 2018). Moreover, in the same study, lignin-based hydrogels were evaluated as hydrophobic drug delivery systems and concluded that those same hydrogels were able of sustaining the release of curcumin for up to 4 d (Larrañeta et al., 2018). In addition, nanocomposite fibers made from Alkali Kraft lignin with low sulfonate content (Sigma-Aldrich Corp, St. Louis, MO) were tested against *S. aureus* and *E. coli*. Fibers with 29% (w/w) lignin showed a 99.9% reduction rate of *S. aureus* populations, however no inhibition was observed for *E. coli* (Lee et al., 2017). Regarding nanotechnology assays, LNPs synthetized by dissolving alkali Kraft lignin into ethylene glycol followed by acidolysis were tested against plant pathogens including *Pseudomonas syringae*, *Xanthomonas axonopodis*, and *Xanthomonas arboricola* (Yang et al., 2018). LNP at a dose of 4% had the highest antibacterial activity

against *X. arboricola* with a 3-log reduction (1×10^8 to 5×10^4 CFU/mL) after 24 h of incubation using a broth susceptibility assay (Yang et al., 2018). These results had an important implication considering the economic losses caused by this pathogen on plum, peach, apricot, and cherry trees (Yang et al., 2018). The authors suggested that there are three possible antimicrobial mechanisms of action of LNPs (Fig. 2-14; Yang et al., 2018). First, lignin polyphenols cause lysis, damage to the cell wall, and consequently leakage of bacterial cell internal fluid. Alternatively, reactive oxygen species (ROS) in the surface of LNP induce oxidative stress by altering the normal redox physiological processes of bacteria. Lastly, because of their small size LNPs are able to penetrate the bacterial cell evading the cell membrane (i.e., Trojan horse approach), decrease the intracellular pH and consequently ATP levels, and lastly lead to the death of the cell (Yang et al., 2018).

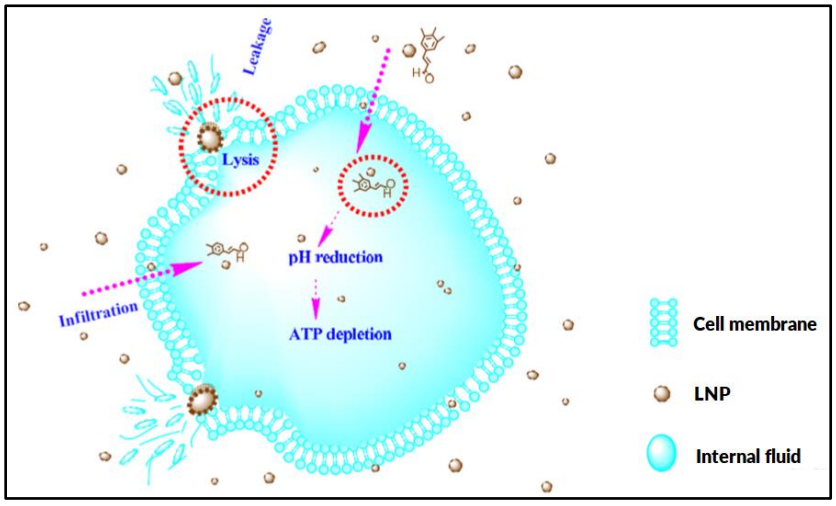

Figure 2-14. Involved mechanisms for antibacterial behavior of extracted LNP. Adapted from Yang et al. (2018).

Non-conventional lignins

Lignocellulosic materials such as crop residues are abundant, readily available, and low-cost (Doherty et al., 2011). The antibacterial properties of a lignin extracted

from sugarcane bagasse with alkaline solutions, and different modified lignins i.e. acetylated, epoxy, and hydroxymethyl lignin, were evaluated against *Bacillus aryabhattai* and *Klebsiella* sp. using the disk diffusion method (Kaur et al., 2017). Epoxy lignin (lignin extracted using a chlorinated cyclic ether) was the most effective among unmodified and modified lignins with MIC values of 90 and 200 μg/disc for each bacterium, respectively. The presence of methoxyl and epoxy groups in lignin was responsible for the enhanced antibacterial activity of the lignin modified by epoxidation (Kaur et al., 2017). Similarly, the antifungal properties of different lignin fractions from apple tree pruning waste obtained by autohydrolysis, organosolv treatment with acid, or with ethanol, and soda hydrolysis were investigated against *A. niger*, and *S. cerevisiae* (García et al., 2017). None of the lignins tested exhibited antifungal activity against *A. niger* for any lignin at doses of 500 and 5,000 ppm, and indeed the lower dose enhanced mold growth, which was explained by the presence of minerals and hemicelluloses of the lignin fractions (García et al., 2017). On the other hand, all of the tested lignin fractions decreased the growth of *S. cerevisia*e at 5,000 and 10,000 ppm, with autohydrolysis lignin at 10,000 ppm being the most antifungal fraction as observed by a 78.7% decrease in growth vs. control, using a spectrophotometric method. In addition, the pigmentation of *A. niger* was affected by lignin fractions at doses of 5,000 ppm with the colonies exhibiting pale blue, green or yellow pigmentation, compared to colonies growing on the control plates (García et al., 2017). These results agree with Rahouti et al. (1999), who studied seven phenolic lignin model compounds against various fungi, and observed that guaiacol and syringic acid induce production of atypical pigments and viscous compounds. Likewise, Coral Medina et al. (2016) evaluated the

antimicrobial activity of one of six lignins isolated from oil palm empty fruit bunches using a sequential acid-alkaline pretreatment. The lignin tested (L_3) was selected for having the highest total phenolic content (181.21 mg GAE/mg). However, L_3 did not have an effect against *C. albicans* or *A. niger* at 2000 µg/mL using the disk diffusion method. Conversely, using a spectrophotometric technique, L_3 exhibited antibacterial activity, with the greatest inhibition observed at 250 µg/mL for *B. subtilis* (39%), 1000 µg/mL for *S. enterica* (31%), and 2000 µg/mL for *E. coli* (50%), and *S. aureus* (67%), using a broth antimicrobial assay (Coral Medina et al., 2016).

Summary

This first portion of this review summarized hay production practices, and the DM and nutritional losses that characterize each hay production phase. The second part reviewed the microbiology of hay production and spoilage related to changes in nutritional value. The third revised the state of the art in hay preservatives, which relies heavily on organic chemicals, remaining the same since the 1970s, with limited recent progress observed with the use of hay inoculants. Consequently, in the fourth part, technical lignins are proposed as novel low cost-antimicrobials that could be used to prevent microbial spoilage. Therefore, the objective of this study was to evaluate the effect of technical lignins on the preservation of high moisture alfalfa (*Medicago sativa*) relative to propionic acid effects.

CHAPTER 3

IMPROVING HAY NUTRITIVE VALUE AND REDUCING DRY MATTER LOSSES BY USING TECHNICAL LIGNINS AS ADDITIVES TO PREVENT MICROBIAL SPOILAGE

Introduction

In the U.S., hay is the predominant forage conservation method, the third most valuable crop ($16 billion/y), and the second in harvested acres (54 million acres, NASS, 2017). The main goal in haymaking is to decrease the moisture concentration to less than 15-20% in no more than 3-5 d so most of the forage crop nutrient yield can be stored long-term (Rees, 1982). However, nutrient losses during hay harvest and storage are interdependent. During harvest, field losses occur due to increased leaf fragility as moisture decreases, especially below 20%. However, if hay is baled above 15-20% moisture, extensive microbial spoilage will occur during storage resulting in a significant decline in nutritive value (Coblentz and Hoffman, 2009) and increased DM losses (up to 30%; Ball et al., 1998). Thus, there is a great need for preservatives that can allow for baling hay above 20% moisture so both field and storage losses can be reduced. Currently, propionic acid-based products (~$4,000/Mg of preservative) are the most used hay preservatives, but their efficacy in preventing spontaneous heating is limited to 6 months (Coblentz et al., 2013). Therefore, more effective an inexpensive hay preservatives are needed to improve the efficiency of hay production.

Technical lignins are byproducts of paper mills, approximately 50 Mg/y are produced worldwide but only 2% are commercialized with the rest being incinerated (Gosselink et al., 2004). Certain technical lignin types, such as Kraft lignins and lignosulfonates, have reported antibacterial (Dong et al., 2011), antifungal (Jha and

Kumar, 2018), and antiviral activities (Gordts et al., 2015), and prebiotic effects (Flickinger et al., 1998). In fact, lignosulfonates have been long used to increase soybean protein bypass in the rumen (Borucki Castro et al., 2007) and as feed binders (Corey et al., 2014). These properties could be adapted to prevent hay spoilage and consequently increase animal performance.

The first objective of this study was to screen a set of technical lignins for their antifungal properties against four fungi isolated from spoiled alfalfa (*Medicago sativa*) hay. A second objective was to find the minimum inhibitory and minimum fungicidal concentration of the most promising technical lignins from Experiment 1. The third objective was to evaluate the dose-optimized technical lignins from Experiment 2 for their potential preservation properties in high moisture alfalfa hay on dry matter losses, microbial counts, nutritional composition, ruminal digestibility and fermentation profile. We hypothesized that certain technical lignins can reduce the DM losses and preserve the nutritive value of high moisture alfalfa hay during the storage phase.

Materials and Methods

Fungal isolates

Colonies of fungi were isolated from spoiled alfalfa (*Medicago sativa,* Pioneer 54QR04) harvested in Exeter, Maine, in the fall of 2016. Isolates were extracted as outlined by Queiroz et al. (2012). Identification to the species level was accomplished by a combination of morphological (Malloch, 1981) and genetic sequencing evaluations. Molecular identification used the internal transcribed spacer regions (ITS 1 and 2) of the rRNA genes, the β-tubulin (BenA), and the 28S large-subunit ribosomal RNA (rDNA) genes.

Genetic identification

DNA isolation

Fungal isolates were grown for 7 d at 25°C on malt extract agar (MEA; BD Difco, Franklin Lakes, NJ) covered with sterile transparent cellophane (Flexel, Covington, IN). The mycelia were carefully removed with the aid of a scalpel, transferred to a sterile microcentrifuge tube, and ground to a fine powder under liquid N_2 with a pestle (Goodwin and Annis, 1991). DNA was extracted using an E.Z.N.A. fungal DNA Mini Kit (Omega biotek, Norcross, GA). DNA quality and quantity were evaluated by absorbance spectroscopy at 260 and 280 nm with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA).

Amplification of DNA

The amplification of the ITS regions, 28S rRNA, and β-tubulin genes of these isolates was performed using the following primer pairs: for ITS1 and ITS2 regions, 5'-TCCGTAGGTGAACCTGCGG3' ITS1 and 5'-TCCTCCGCTTATTGATATGC-3' ITS4 (White et al., 1990); for the 28S rRNA, NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4 5'-GGTCCGTGTTTCAAGACGG-3' (O'donnell, 1993); and for β-tubulin (BenA), BenA 5'-GGTAACCAAATCGGTGCTGCTTTC-3' and BenB 5'-ACCCTCAGTGTAGTGACCCTTGGC-3' (Glass et al., 1995). PCR amplifications were conducted with 25 μl of reaction mix in 0.5 mL PCR tubes using a C1000 Touch Thermal Cycler (Biorad, Hercules, CA). The reaction contained 0.2 mM dNTPs (Promega, Madison, WI), 0.2 μM of each primer (Integrated DNA technologies, Coraville, IA), 0.75 units of One*Taq* DNA polymerase (New England Biolabs, Ipswich, MA), 1× of One*Taq* Standard Reaction Buffer (New England Biolabs, Ipswich, MA), 10 ng of DNA template adjusted to be in a volume of 5 μl, and DNase-free water to makeup

the final volume. For ITS primers, conditions for amplification were: 5 min at 94°C, then 30 cycles of 60 s at 95°C, 60 s at 55°C, 60 s at 72°C, and a final step of 10 min at 72°C. For NL1 and NL4, amplification was performed with a slightly different protocol with 5 min at 94°C, then 35 cycles of 60 s at 95°C, 60 s at 53°C, 2 min at 72°C, followed by a final cycle of 7 min at 72°C. Lastly, reactions with primers BenA and BenB, were carried out with 5 min at 94°C, followed by 35 cycles of 60 s at 95°C, 60 s at 58°C, 60 s at 72°C, and a final cycle of 10 min at 72°C. Amplification products were separated by electrophoresis in 1.2% agarose gel (Cambrex Bio Science, Rockland, ME) with 1×TBE (0.089 M Tris-borate, 0.002 M EDTA), stained with GelStar (Lonza, Rockland, ME), and viewed under 280 nm UV to see band sizes.

DNA sequencing and identification of isolates

The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in the University of Maine DNA Sequencing laboratory by the double-strand dideoxynucleotide sequencing method from (Sanger et al., 1977). Sequence data was edited, assembled and aligned using the CAP sequence assembly program (Huang and Madan, 1999) to obtain high-quality consensus sequences. Consensus sequence homologies were compared to those referenced in the NCBI database BLASTN search using default parameters (Altschul et al., 1990). *Aspergillus amoenus, Mucor circinelloides*, *Penicillium solitum*, and *Debaromyces hansenii* were each identified by ≥ 99% identity match to published sequences in Genbank and E-value = 0.0. Fungal isolates were preserved as spores (molds) and cells (yeast) in a 30% glycerol solution at −80°C in cryogenic vials (Corning Inc., Corning, NY).

Experiment 1

Additives

A set of commercially available technical lignins and their fractions were screened for antifungal properties to identify antimicrobial candidates that could be evaluated in the field. The following additives (ADV) were evaluated: 1) Southern pine softwood Kraft lignin (UW; Delignified at an H factor of about 1600 via the Kraft process and precipitated using the Lignoboost process with CO_2 as the acid; Tomani, 2010), 2) ADV 1 acetone insoluble fraction (AI), 3) ADV 1 acetone soluble fraction 1 (Hexane insoluble; HEX), 4) ADV 1 acetone soluble fraction 2 (Hexane soluble; PI), 5) alkali Kraft lignin (AKL; Sigma-Aldrich Corp, St. Louis, MO), 6) sodium lignosulfonate (NaL; Sappi North America, Boston, MA), 7) magnesium lignosulfonate (MgL; Sappi North America, Boston, MA), 8) ammonium lignosulfonate (AMOL; Sappi North America, Boston, MA), 9) propionic acid (positive control PRP; MP Biomedicals, Solon, OH, 99.8%), and 10) control (untreated). Kraft lignin fractions (ADV 2 - 4) were extracted following the procedures outlined by Cui et al. (2014). The ash (AOAC International, 2000; method 942.05), water soluble carbohydrates (WSC) (Dubois et al., 1956), mineral (Beliciu et al., 2012), and total soluble phenolics concentrations, and DDPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of the technical lignins are listed in Table 3-1.

Antifungal assay

The antifungal activity of ADV against the isolated fungi was determined using the poisoned food technique according to the method outlined by Balouiri et al. (2016). Analysis was done in duplicate in each of four runs for all fungi. The experiment had a randomized complete block design (RCBD) with a 10 (ADV) × 3 (molds) factorial

arrangement of treatments and 4 blocks (runs). A RCBD also was used to test the effect of ADV on yeast (*D. hansenii*).

Media preparation

Sterile MEA was mixed with each ADV (technical lignins or PRP) as follows. Solutions of lignin and PRP in sterile nanopure water (20 and 32% w/v, respectively) were prepared in 50 mL polypropylene tubes. Dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA) was supplemented at 8% (v/v) to increase the solubility of Kraft lignins. Solutions were sonicated (non-thermal sterilization technology) for 60 min in an 8510 Series Ultrasonic Cleaning Bath (Emerson, St. Louis, MO) containing water at 40°C, to ensure sterility with minimal impact on the lignins chemical structure (Piyasena et al., 2003). Subsequently, enough ADV stock solution was dispensed to sterilized agar (40°C) under stirring to achieve a final concentration of 40 mg/mL of ADV, 1% DMSO (v/v; for all ADV), and the manufacturer recommended agar concentration. Previous studies indicated that the antimicrobial activity of technical lignins increased when pH was modified from 6.0 to 3.5 (Baranowski et al., 1980). Furthermore, our preliminary tests across a pH gradient showed that lignins were more antifungal at pH 4. Thus, to properly evaluate antimicrobial effects of ADV, enough HCl was added to set the initial media pH to 4 for all treatments.

Molds

After 14 (*A. amoenus* and *P. solitum*) or 3 d of incubation period (*M. circinelloides*), the border of single fungal colonies were punched aseptically with a sterile cork borer (7 mm diameter), and discs were inoculated on the center of ADV containing and untreated MEA. Plates were incubated at 25 ± 1°C for 7 d. At the end of the incubation period, the diameters (long and short dimension) of mold growth in control and treated

plates were measured using a digital caliper (Beckman Coulter, Pasadena, CA), and the antifungal effect was estimated with the formula: Antifungal activity (%) = ((Dc − Ds)/Dc) × 100. Where Dc is the average diameter of growth in control plate and Ds is the average diameter of growth in the plate containing the ADV (Balouiri, et al., 2016).

Yeast

The antifungal activity of ADV against *D. hansenii* was determined using the method outlined by Li et al. (2016) with modifications. MEA plates were inoculated with 100 µl of yeast inoculum containing approximately 1×10^3 cfu/mL. Plates were incubated at 25 ± 1°C for 72 h before colonies were enumerated. The antifungal effect was estimated with the formula: Antifungal activity (%) = ((Cc − Cs)/Cc) × 100, where Cc is the number of cfu on control plate, and Cs is the number of cfu on the plate containing the ADV.

Experiment 2

Additives

Following the results from experiment 1, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) was determined for the most promising technical lignins (NaL, MgL, and AKL) and PRP (positive control) on each of the fungal isolates previously evaluated. Macrodilution assays were carried out independently three times in duplicate and values are reported as mean concentrations (mg/ml ± standard deviation; SD).

Antifungal assay

Molds

After 14 (*A. amoenus* and *P. solitum*) or 3 d of incubation period (*M. circinelloides*), fungal spores were washed from the surface of MEA by adding sterile 0.05% (v/v)

tween 20 (Fisher Scientific, Pittsburgh, PA), and then the surface was gently probed with a sterile glass hockey stick to loosen spores. This solution was pipetted off into a sterile 15 mL tube, heavy particles were allowed to settle for 3 to 5 min, and the upper homogeneous suspension was transferred to another sterile tube. Subsequently, the spore concentration of this suspension was enumerated with a haemocytometer chamber, diluted, and dispensed to obtain a final concentration of 5×10^4 conidia/mL of media, according to the M38-A Broth dilution antifungal susceptibility testing of filamentous fungi (Rex et al., 2008).

Yeast

D*ebaromyces hansenii* was grown on MEA for 72 h until clear colonies could be identified. The inoculum was prepared by picking five yeast colonies of approximately 1 mm diameter and suspending them in 5 mL of sterile 0.145 M saline solution (8.5 g/L NaCl) by shaking on a vortex mixer for 15 s, according to the M27-A2 Broth Dilution Antifungal Susceptibility Testing of Yeast (NCCLS, 2002). The suspension was adjusted with a spectrophotometer (VWR, Radnor, PA) to an OD_{600} of 0.2, diluted, and dispensed to yield a final concentration of 1×10^4 cfu/mL of media.

Assay

MIC was defined as the lowest concentration of ADV that prevents visible growth when compared to untreated controls. The macrodilution testing was performed according to the National Committee for Clinical Laboratory Standards (NCCLS). Lignins and PRP stock solutions were prepared in sterile malt extract broth (MEB; BD Difco, Franklin Lakes, NJ) and sonicated as described in experiment 1, without using DMSO. According to the concentration tested (ranging from 1.5 to 60 mg/mL), different proportions of stock ADV and sterile MEB were dispensed into 50 mL Erlenmeyer flasks

to complete a final volume of 5 mL of media, and enough HCl or NaOH were added to achieve a final pH of 4 or 6 in the media, respectively. Flasks were inoculated with each test inoculum, covered with a double layer of sterile aluminum foil, and incubated at 25°C for 5 d with shaking (60 rpm). After this period, an aliquot (100 µl) was taken from each flask lacking visible growth and inoculated on fresh MEA. Plates were incubated at 25°C for 48 h to find the MFC, which was defined as the lowest concentration of ADV that decreases 99.8% of the initial fungal concentration.

Experiment 3

Substrate, Additives, and Design

An established 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). On June 8th, 2018, five randomly located plots in the alfalfa stand (first cut, bud stage) were mowed to 7.6-cm stubble height with a BCS 725 sickle bar mower (Portland, OR) and allowed to wilt in the field for 5 d to an 80% DM concentration. Afterwards, the alfalfa hay 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).

The effects of two dose-optimized ADV (NaL and MgL), selected due to their antifungal activity in aforementioned experiments, and PRP (positive control) on the spoilage of high moisture alfalfa hay were evaluated using a RCBD with a 3 (ADV: NaL, MgL, and PRP) × 4 (dose: 0, 0.5, 1, and 3% 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) was evaluated according to the jar method outlined by Lacey and Lord (1977) with modifications as shown in Fig. 3-1.

Sampling and analysis

At d 0 and 15, samples were taken from each replicate for the determination of nutritional value (10 g, fresh basis), and microbial counts (10 g, fresh basis). In the case of d 0, samples were obtained immediately after inoculation.

Nutritional analysis

From samples taken at d 0 and 15, subsamples were processed for the determination of DM concentration by drying at 60ºC until constant weight in a forced-air oven. Dried samples were ground to pass a 2-mm screen using a Foss Cyclotec mill (Foss, Denmark). 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 NDF assay, but sodium sulfite was not used. Hemicellulose concentration (NDF minus ADF) was calculated. Hay 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 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 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 were kept for further analysis. 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).

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 orchardgrass silage (*Dactylis glomerata*; 6 kg), corn silage (6.8 kg), and concentrate (9.5 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 (−30ºC) and subsequently analyzed for concentration of VFA 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 DAD detector set to 210 nm (Siegfried et al., 1984). 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 weights and their DM and NDF concentrations.

Statistical Analysis

For experiment 1, a RCBD with a 10 (ADV) × 3 (MOLD) factorial arrangement of additives and 4 blocks (runs) was used to determine the effects of ADV on mold inhibition.

The model used to analyze mold inhibition data was:

$Y_{ijkl} = \mu + MOLD_i + ADV_j + \beta_k + MOLDADV_{ij} + \varepsilon_{ijk}$

Where:

μ = general mean

$MOLD_i$ = effect of mold i

ADV_j = effect of additive j

β_k = effect of run k

$MOLDADV_{ij}$ = effect of the $MOLD_i \times ADV_j$ interaction

ε_{ijk} = experimental error

A similar model that included only the ADV effect was used to analyze the yeast inhibition data. In the case of experiment 2, MIC and MFC assays were carried out independently three times in duplicate and values are reported as mean concentrations (mg/mL ± standard deviation; SD).

For experiment 3, a RCBD with a 3 (ADV) × 4 (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 these data was:

$Yijkl = \mu + ADVi + DOSEj + \beta k + ADVDOSEij + \varepsilon ijk$

Where:

μ = general mean

ADVi = effect of additive i

DOSEj = effect of dose j

βk = effect of block k

ADVDOSEij = effect of the ADVi × DOSEj interaction

εijk = 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. In experiment 1, mean separation was based on the PDIFF procedure of LSMEANS. For

experiment 3, polynomial contrasts were used to determine dose effects and the Tukey's test was used to compare least squares means within dose and ADV. Both of these mean characterization and separation tests are considered necessary to properly interpret the results because they depict the polynomial trend and the optimal dose, respectively. Data were tested for normality using the Shapiro-Wilk test. Significance was declared at $P \leq 0.05$.

Results

Experiment 1

We found an interaction effect of mold × ADV on antifungal activity ($P < 0.001$). For *A. amoenus*, *M. circinelloides*, and *P. solitum* we observed that PRP and NaL had the highest antifungal activity (100 ± 2.77%), followed by MgL (40.9, 73, and 28.1%, Fig. 3-2, 3-3 and 3-4, respectively). For NaL, PRP, and HEX there were not significant differences across molds. However, for MgL, different antifungal activities were observed across *M. circinelloides, A. amoenus,* and *P. solitum* (72.9, 40.9, and 28.1 ± 2.77%, respectively; $P \leq 0.05$) and a similar trend was observed for AKL (49.7, 12.1, and -8.0 ± 2.77 %, respectively; $P \leq 0.05$). Overall, *M. circinelloides* was the most sensitive mold, followed by *A. amoenus*, and *P. solitum*. For *D. hansenii*, we identified PRP, NaL, and MgL as the most effective treatments with 100 ± 3% antifungal activity (Fig. 3-5).

Experiment 2

Table 3-2 shows the MIC and MFC of technical lignins and PRP against the fungi previously described. Among technical lignins at pH 4, NaL had the lowest MIC across molds, with values of 20.0, 25.0, and 33.3 mg/mL for *A. amoenus*, *M. circinelloides*, and *P. solitum*, respectively. In the case of MgL, MIC values of 33.3, 36.7, and 46.7 mg/mL

were found for *A. amoenus*, *M. circinelloides*, and *P. solitum*, respectively. None of the technical lignins tested inhibited the molds at a pH of 6. The PRP (positive control) was an effective fungistatic agent at both pH levels tested, but had lower MIC at pH 4, with values as low as 1.25 for *A. amoenus* and *P. solitum*, and 3.33 mg/mL for *M. circinelloides*. For the yeast *D. hansenii*, we found that MgL had a lower MIC compared to NaL (26.7 vs. 40 mg/mL, respectively) but both had a lower inhibitory activity relative to PRP (1.25). No MIC was observed at either pH for AKL.

Across technical lignins, NaL at pH 4 had a higher fungicidal activity for *P. solitum* (60.0 mg/mL) and lower for *D. hansenii* (40.0) compared to MgL (> 60.0 and 30.0, respectively). For *A. amoenus*, NaL and MgL had similar fungicidal activity (40.0). Across all fungi, PRP (positive control) had a lower MFC at both pH levels compared to the technical lignins tested. However, its fungicidal activity was higher at a pH of 4 relative to 6 for *A. amoenus* (5 vs. 10 mg/mL), *D. hansenii* (5 vs. 15), *P. solitum* (10 vs. 16.7), and *M. circinelloides* (20 vs. 40).

Experiment 3

Day 15

DM losses and microbial populations

Effects of treatments on DM loss and microbial counts are shown in Table 3-3. We found an interaction effect of ADV × dose on DM losses, hay pH, and total molds, *A. amoenus*, *M. circinelloides*, and *P. solitum* counts ($P < 0.001$). For DM loss, relative to untreated hay (14.9 ± 0.773%), 1% was the lowest dose that resulted in the minimum DM losses for NaL (3.39); 3% for MgL (0.37); and 0.5% for PRP (0.47; $P < 0.001$). At a dose of 0.5%, PRP reduced DM losses to a greater extent compared to NaL and MgL, which were not different. However, at 1% NaL and PRP had similar results, and at 3%

all ADV were similar. Compared to untreated (7.99 ± 0.156), NaL and MgL at 3% (5.37 and 5.24, respectively), and PRP at a dose as low as 0.5% (5.25) decreased hay pH (P < 0.001). At 0.5% and 1%, PRP resulted in a lower pH compared to NaL and MgL, while at 3% no differences were observed across ADV.

Propionic acid had a higher antifungal effect (1.96 ± 0.914 log cfu/fresh g) against *D. hansenii* compared to NaL and MgL, which were similar (3.54 and 4.67, respectively; P < 0.001). Across all ADV, a 0.5% dose decreased *D. hansenii* counts to the greatest extent vs. untreated (3.02 vs. 7.0 log cfu/fresh g, respectively; P < 0.001). Total mold counts were reduced by 3% NaL (3.92 ± 0.549 log cfu/fresh g) and 0.5% PRP (3.94), relative to untreated (7.76; P < 0.001). At both 0.5% and 1%, PRP decreased total mold counts further compared to NaL and MgL, which were not different; at 3% all ADV were similar.

Nutritional composition

Except for CP, ADF, and hemicellulose (P > 0.07), we found an interaction between ADV × dose on all other nutritive value estimates at d 15 (P < 0.001; Table 3-4). Relative to untreated hay (62.4 ± 0.491%), the lowest dose that preserved hay DM % at d 0 (69.3%; Table 3-5) was 1% for NaL (68.7); 3% for MgL (69.1), and 0.5% for PRP (69.2; P < 0.001). At a dose of 0.5%, PRP-treated hay had a higher DM % vs. NaL and MgL, which were similar. However, at 1% no difference was observed between NaL and PRP, and at 3% all ADV were comparable. For CP concentration, there were no effects of ADV and dose. However, a decrease in hay NH_3-N was observed for NaL and MgL at 3% (0.043 and 0.062 ± 0.007% DM, respectively) and PRP at 0.5% (0.061) and above, compared to untreated hay (0.249; P < 0.001). Across ADV, at 0.5% and 1%

NaL and PRP resulted in a lower NH_3-N compared to MgL, while at 3% PRP decreased NH_3-N to a greater extent than MgL, but both were similar to NaL.

Compared to untreated hay (7.99 ± 0.283% of DM), the doses preserving WSC to the greatest extent were 3% for NaL and MgL (10.1 and 10.3, respectively) and 0.5% for PRP (10.5; $P < 0.001$). At 0.5% and 1%, PRP preserved WSC further vs. NaL and MgL, and at 3% all ADV were comparable. Consequently, NaL at 3% (45.9 ± 0.663% DM) and PRP at a dose as low as 0.5% (45.1), impeded an increase in NDF concentration relative to untreated (49.7; $P = 0.001$). At 0.5% and 1%, a lower NDF was observed for PRP-treated hay compared to NaL and MgL; as for 3%, PRP resulted in a lower NDF compared to MgL, but both were similar to NaL.

In vitro ruminal digestibility

We found an interaction effect of ADV × dose on all ruminal *in vitro* fermentation measures ($P < 0.001$; Table 3-6), except for ruminal pH and isovalerate concentration. An increased DMD was observed for 3% NaL (67.5 ± 0.771%), 1% MgL (67.0), and 0.5% PRP (68.5) vs. untreated hay (61.8 ± 0.771%). At a dose of 0.5%, MgL and PRP increased DMD to the same level but to a greater extent than NaL; at 1%, PRP resulted in higher DMD than NaL, but both were similar to MgL; and at 3% all ADV were comparable. In the case of NDFD, MgL and PRP at a dose as low as 0.5% (30.5 and 30.1 ± 1.09%, respectively) and NaL at 1% increased NDFD (30.7) compared to untreated hay (23.3; $P < 0.003$). At 0.5%, MgL increased NDFD to a larger extent relative to NaL, but both were not different from PRP, and all ADV were similar at 1% and 3%.

Sodium lignosulfonate at 3% decreased ruminal NH_3-N concentration to the greatest extent (49.6 ± 1.50 mg/dL) vs. untreated hay (58.2; $P = 0.009$). In contrast,

relative to untreated (86.7 ± 1.30 mM), NaL at 3% increased TVFA concentration to the greatest extent (111.9; $P < 0.001$). However, MgL decreased TVFA at 0.5% (78.0 mM; $P = 0.01$) while other doses were similar to untreated. No dose of PRP was different than untreated. At 0.5, 1, and 3%, NaL increased TVFA to the greatest extent, followed by PRP and MgL, which were comparable at 1% and 3%.

Acetate concentration was increased by NaL at 3% (64.1 ± 0.805 mM) vs. untreated hay (48.0; $P < 0.001$). At 0.5% there was no difference between NaL and PRP, both of which resulted in higher acetate than MgL; at 1% NaL resulted in higher acetate than MgL, but both were similar to PRP; at 3%, NaL had the highest acetate concentration, followed by MgL and PRP, which were comparable. Likewise, a dose of 1% for NaL and PRP, and 3% for MgL increased propionate concentration to the largest extent (21.8, 20.5 and 20.6 ± 0.352 mM, respectively) relative to untreated (18.0; $P < 0.001$). At 0.5 and 1%, MgL resulted in lower propionate compared to NaL and PRP, which were not different. At 3%, NaL had the highest propionate concentration vs. MgL and PRP, which were similar.

Relative to untreated (10.9 ± 0.418 mM), only 3% NaL increased butyrate concentration (13.5). In contrast, 0.5% was the lowest dose decreasing butyrate to the greatest extent for MgL and PRP (8.79 and 8.32 mM, respectively; $P = 0.001$). Within dose, NaL had the highest concentration of butyrate compared to MgL and PRP, which were not different at 0.5%, 1%, and 3%. Sodium lignosulfonate had more isobutyrate compared to MgL and PRP, which were not different at 0.5% (1.71 vs. 1.33 and 1.31 mM ± 0.06 mM) and 1% (1.68 vs. 1.25 and 1.36, respectively; $P < 0.01$); at 3%, NaL

had more isobutyrate than MgL (1.66 vs. 1.29 mM, respectively), but both were not different from PRP (1.43; $P = 0.001$).

Discussion

Experiment 1 and 2

A limited number of studies have evaluated the antimicrobial activity of technical lignins (Kim et al., 2013; Kaur et al., 2017; Jha and Kumar, 2018). Interpretation of these results can be challenging due to the type of technical lignin (García et al., 2017), microorganisms (Dong et al., 2011), and methodologies used (Yang et al., 2018). In most articles, a thorough description of the lignin chemical properties is often lacking, which impedes direct comparisons among studies. In our study, NaL and MgL were found to have the strongest inhibitory properties among all lignins tested when evaluated against *A. amoenus*, *M. circinelloides*, *P. solitum* (molds) and *D. hansenii* (yeast) at a pH of 4. Jha and Kumar (2018) reported MIC values for NaL (Sigma-Aldrich Corp, St. Louis, MO) of 50, 62, 62, and 80 µg/mL for *Candida dubliniensis, C. tropicalis, C. albicans, C. glabrata,* and *C. parasilopsis*, respectively. When these values were evaluated using the disc diffusion method, they observed that relative to fluconazole (undisclosed source) the inhibition of diameter growth was 6, 10.3, and 23% for *C. glabrata*, *C. tropicalis*, and *C. albicans*, respectively. Similarly, Núñez-Flores et al. (2012) reported that an undisclosed dose of NaL (4% reducing sugar content; 7085 Da; DPPH IC_{50} 97 µg/mL) extracted from eucalyptus wood (LignoTech Ibérica, S.A. Torrelavega, Spain) showed a 9.9% growth inhibition for *D. hansenii* using the disk diffusion method. Our results obtained with NaL were comparable to those reported by Jha and Kumar (2018) with *Candida* spp. and Núñez-Flores et al. (2012) with *D. hansenii*. However, Núñez-Flores et al. (2012) did not observe an inhibitory activity for

sodium lignosulfonate against *P. expansum* and *A. niger*. This discrepancy with our study maybe due to the different *Penicillium* and *Aspergillus* species tested, as well as NaL sources and doses used across studies. Furthermore, these studies did not report media pH values. In our study, we observed that media pH plays a major role in the extent of the antifungal activity of technical lignins, with a lower pH (4 vs. 6) resulting in greater inhibition. Baranowski et al. (1980) hypothesized that at a lower pH the efficacy of ferulic acid increases due to an enhanced membrane permeability in the undissociated state. In that study, ferulic acid at 0.23 mM had antifungal activity against *S. cerevisiae* at a pH of 3.5 in the medium. However, (De Greef and Van Sumere, 1966) reported an antimicrobial activity against the same organism for ferulic acid at a dose of 2.5 mM and a medium pH of 6.0.

Although the antimicrobial mechanism of lignosulfonates has not been yet elucidated, it is hypothesized that is linked with the strong surfactant properties of lignosulfonates (Núñez-Flores et al., 2012). Surfactants interact with different cellular constituents, especially lipids and proteins, causing adverse effects on the growth and viability of cells by disrupting normal microbial cellular functions (Merianos, 1991; (Hugo, 1992). Hugo and Russell, 1992). For *B. subtillis*, surfactants such as triton X-100 and palmityltrimethylammonium iodide have been shown to cause cell autolysis at doses of 150 μM and 10 μM, respectively (Tsuchido et al. 1990). Similarly, bile salts (anionic surfactants) resulting from the metabolism of cholesterol inhibit bacterial growth, by affecting cell morphology, leading to the breakdown of the bacterial cell wall and cell death (Ronsin et al., 2002). Evidently, the mechanism of action of lignosulfonates needs to be further investigated.

Fewer studies have been conducted using Kraft lignins. Dong et al. (2011) reported a MIC of 0.01 and 0.0025 µg/mL using alkali Kraft lignin (Sigma-Aldrich Corp, St. Louis, MO) against *Candida lipolytica* and *S. aureus*. However, no antibacterial activity was reported against *L. monocytogenes*. The lignin tested in that study had an antioxidant activity of 3,517 µmol TE/g (TE, Trolox equivalent) and 165.5 mg/g total soluble phenolics. We believe that the absence of activity against yeast for AKL in our study compared to Dong et al. (2011) is mostly a consequence of the different species evaluated, considering that the lignin source was the same and the methodologies comparable. Although the mode of action of Kraft lignins against fungi is unknown, Dizhbite et al., 2004 suggested that for bacteria it is associated with the inhibition of radical processes of bacterial cells. Hence, a correlation between radical scavenging (antioxidant) and antimicrobial activities was suggested (Dizhbite et al., 2004). Similarly, Dong et al. (2011) reported a positive association between antimicrobial and antioxidant activities of lignins. Conversely, Núñez-Flores et al. (2012) did not find such a relationship, which agrees with our findings as the technical lignins with higher radical scavenging activity were less antifungal (Table 3-1).

In the current study, PRP had fungistatic and fungicidal activity against fungi isolated from spoiled hay at much lower doses compared to NaL and MgL. The antimicrobial mechanism of action of PRP consists of the disruption of the electrochemical proton gradient when undissociated acid molecules penetrate the cell wall and dissociate internally. This process depletes cellular energy and therefore, limits cellular growth and metabolic functions (Davidson et al., 2013). In fungi, recent research suggested that PRP induces the generation of reactive oxygen species and

mitochondrial dysfunction, leading to oxidative stress and apoptosis (Yun and Lee, 2016). In addition, because the content of undissociated acid declines as pH increases, PRP is more effective at a lower pH (< 4.5; Woolford, 1984; Lück and Jager, 1997). The lowest doses that were selected for further testing in experiment 3 (0.5-1%, w/w; fresh basis) corresponded to the actual concentrations typically used in the field for propionic acid (~0.67-1.34%, w/w; fresh basis), considering the application rate of commercially available propionic acid-based preservatives (1-2%, w/w; fresh basis; Rotz, 2003) and the typical concentration of propionic acid in those commercial products (~67%, v/v; EFSA, 2011).

Experiment 3

Hay baled above recommended moisture levels (15-20%) results in spoilage during the storage phase, with DM losses being as high as 40% (Ball et al., 1998) caused by proliferating fungal populations (Roberts, 1995) that preferably oxidize WSC (Turner et al., 2002) releasing moisture and CO_2 (Rees, 1982) and reducing OM concentrations (Coblentz and Hoffman, 2009). Plant proteins are also decomposed in this spoilage process (i.e. proteolysis), releasing NH_3-N in the process (Rotz and Muck, 1994). The breakdown and oxidation of rapidly digestible fractions by the spoilage microbial community leaves behind the most recalcitrant and slowly degradable fractions such as NDF and ADF (Coblentz et al., 2012), which impacts not only the nutritional composition of spoiled hay but also its digestibility (Montgomery et al., 1986) (McBeth et al., 2001) (Coblentz and Hoffman, 2010) and the extent and composition of volatile fatty acids being produced during ruminal fermentation (Mohanty et al., 1969). This was evident when the nutritional values of untreated hay at d 15 (Table 3-4) were compared to the ones obtained from untreated hay at d 0 (Table 3-5).The overall

nutritional value is severely compromised along with the potential voluntary intake, if we were to consider the NDF % increase in the spoiled alfalfa hay (Mertens, 1977). Both, the decrease in nutritional value and voluntary intake can explain the decrease animal performance that has been reported in spoiled vs. well preserved hays (Deetz et al., 1989; Ziemer et al., 1991).

At d 15, alfalfa hay DM losses were mitigated to the same extent by NaL and PRP at a dose of 1%, with no further benefit observed at a higher dose for both. However, PRP was the only ADV that was able to impede DM losses at a dose 0.5%, due its more potent antifungal activity relative to the other ADV, as reported in experiment 1 and 2. The mold and yeast counts closely followed the DM loss results, with decreasing counts being observed as doses were increased to different extent across ADV. It is interesting to note that at a dose of 1%, NaL decreased the yeast but not the total mold counts in spite of a reduction in DM loss. We believe that this apparent discrepancy is explained by the differences in metabolic activity between fungal communities exposed (or not) to antifungal compounds. For instance, Vale-Silva et al. (2012) observed that essential oils from oregano decreased metabolic activity (viability) of fungi without affecting their sporulation.

In this study, *A. amoenus* and *P. solitum* seemed to have caused most of the spoilage observed for alfalfa hay, compared to *M. circinelloides* and *D. hansenii* (Table A-4). Furthermore, these two molds, also appeared to be the most resistant fungi across experiment 1 (Fig. 3-2 and 3-4) and 2 (Table 3-2). More research needs to be conducted in order to understand the relative contribution to hay spoilage across fungal taxa and their relative resistance to preservatives. (Magan and Aldred, 2007) reported

that *Penicillium verrucosum* and *Aspergillus ochraceus* caused DM losses of 7.5 and 4%, respectively, in high moisture wheat grain after 14 d of incubation.

A positive relationship between spoilage extent and fiber concentration has been reported in hay (Coblentz and Hoffman, 2009; Coblentz et al., 2013). This is a consequence of the preferential oxidation of rapidly degradable fractions, such as WSC (Coblentz et al., 1996), which leaves recalcitrant fiber behind, causing a relative increase in fiber concentration (Coblentz and Hoffman, 2009). In our study, NaL and MgL at a dose of 3% prevented the increase of NDF and the decrease of OM observed in untreated hay, and preserved WSC to the same extent as PRP at 0.5%. These effects were attributed to the antifungal properties of lignosulfonates (Jha and Kumar, 2018) and PRP (Lacey et al., 1978). Numerous studies have reported PRP as a successful ADV preserving WSC (Knapp et al., 1976; Davies and Warboys, 1982), OM (Coblentz and Hoffman, 2009), and NDF concentrations (Coblentz et al., 2000; Coblentz et al., 2013) found at the time of hay baling.

Sodium lignosulfonate and PRP at 0.5% showed a protective effect against proteolysis, which was observed as a reduction in NH_3-N relative to the spoiled hay. These results confirm that preservatives can prevent the degradation of plant proteins, preserving their biological value (Rotz and Muck, 1994). Unfortunately, CP is a measurement with a limited ability to describe proteolysis because it only measures N concentration. Coblentz et al. (2013) reported minimal changes in CP concentration during the first 60 d of hay storage. However, after 6 months, losses of 0.25% DM of CP per month were observed due to ammonia volatilization (Rotz and Muck, 1994). The incubations of hay and molds in the current study only lasted for 15 d. Other important

protein quality changes result from the formation of insoluble N components through Maillard reactions, measured as acid detergent insoluble nitrogen (ADIN; Guerrero and Shenvood, 1997). These compounds are essentially indigestible in ruminants (E Schroeder et al., 1996) . However, in our study the amount of hay biomass in the incubation (25 g, DM basis), which was kept at 25°C, was not enough to accumulate heat as observed in hay bales (McDonald et al., 1991). These conditions limited the formation of ADIN as a temperature > 50°C is required for the Maillard reaction to occur (Guerrero and Shenvood, 1997). Thus, our laboratory model is not suited for evaluating the effect of spoilage on ADIN formation (Lacey and Lord, 1977).

Sodium lignosulfonate at 3% had an effect preventing the increase in pH observed in the untreated alfalfa hay at d 15, which was caused by spoilage. (Chancharoonpong et al., 2012) observed that *Aspergillus oryzae* increased the pH of a soybean incubation due to the production of metabolites that included undescribed extracellular proteins. Furthermore, we hypothesize that the increasing NH_3-N concentration due to spoilage contributed to the pH increase in untreated hay at d 15. Table A-4 results seem to indicate that there may be a difference among fungi relative to their effect on pH during spoilage. *Aspergillus amoenus* and *P. solitum* seemed to have increased the pH to a greater extent compared to *D. hansenii* and *M. circinelloides* (7.7 and 7.0 vs. 5.1 and 5.0, respectively), and relative to the pH of the untreated hay at d 0 (5.51).

Despite not having an effect on the preservation of most nutrient constituents compared to PRP and NaL, MgL applied at 1 and 3% increased DMD relative to untreated hay at d 15 to the same extent as PRP at the same doses and NaL at 3%. In the case of PRP and NaL it is evident that the increment in DMD was partially the result

of halting the increase in NDF observed with greater spoilage, as their DMD results were similar to untreated at d 0. However, MgL did not prevent the increase in NDF due to spoilage and yet it seemed to have stimulated rumen fibrolytic bacteria activity as observed by an increased NDFD for all doses ≥ 0.5% relative to untreated (32.4 vs. 23.3%), and a numerical increase relative to NaL and PRP at 1% (34.7 vs. 30.7 and 30.4, respectively) and 3% (32.0 vs. 29.1 and 28.9, respectively). We hypothesize that the surfactant properties of MgL explained this stimulatory effect on NDFD. Surfactants have been previously reported to improve the adsorption of microbial enzymes onto feed particles, which results in an increase in the rate of digestion of cellulose (Kamande et al., 2000). Also, it is unlikely that a Mg deficiency may explain the increase in *in vitro* digestibility since the Van Soest medium is supplemented with this micromineral (Goering, 1970). The reasons why NaL did not have the same effects even though it is also a surfactant, remain unclear.

Several studies evaluating the positive effects of lignosulfonates on rumen undegradable protein have reported increases in NDFD of ruminant diets. Stanford et al. (1995) reported that a barley-based diet supplemented with soybean and canola meal treated with calcium lignosulfonate, increased *in vivo* NDFD relative to control in lambs. Similarly, (Hussein et al., 1991) found that in grass hay-based diets supplemented with barley treated with calcium lignosulfonate, *in vitro* NDFD increased relative to control. Furthermore, Wang et al. (2009) reported that diets containing Alcell lignin increased gas production relative to control diets during a 24 h *in vitro* ruminal fermentation. Conversely, Windschitl and Stern (1988) found a decrease in ruminal in situ ADFD but no effect on NDFD with a corn silage-based diet supplemented with

soybean meal treated with calcium lignosulfonate in dairy cattle. Overall, for PRP and NaL, the increase in DMD is explained by a decrease in NDF concentration and an increase in NDFD (Mertens, 2003). However, stimulatory effects of MgL on DMD and NDFD remain unclear due to its null impact on NDF concentration relative to untreated hay at d 15. It is important to note that a 1% unit increase in *in vitro* NDFD is associated with a 0.25 kg/d increase in 4% FCM (Oba and Allen, 1999). Therefore, feeding hay treated with 1% MgL can potentially increase milk production by 2.9 kg/d, respectively, relative to spoiled alfalfa hay, which in a medium-size dairy operation with 200 cows, could represent an increase in 580 kg/d 4% FCM. Further research is needed to understand the effects of lignosulfonates on *in vitro* ruminal NDFD.

In our study, the increased DMD observed with MgL and PRP relative to untreated at d 15 did not result in a TVFA increment. It seems that for these treatments more OM was used for microbial growth or gas production (Owens and Basalan, 2016). Conversely, NaL had greater TFVA at all doses with the highest concentration observed at a dose of 3% relative to untreated at d 15 (111.9 vs. 86.7 mM, respectively). Volatile fatty acids contribute with 70% of the caloric requirements in ruminants (Bergman, 1990), therefore an increase in TVFA could potentially provide lactating cows with a higher energy supply for maintenance, gain, and lactation requirements. Furthermore, NaL increased acetate concentration at all doses relative to untreated at d 15. This increased acetate availability may result in additional building blocks for de novo fat synthesis in the mammary gland (Mohammed et al., 2011). Similarly, NaL at 1 and 3% increased propionate relative to untreated at d 15. Propionate is an important VFA used for glucose synthesis, which is crucial for dairy cattle, especially at early lactation

(Drackley, 1999). Butyrate is known to have a stimulatory effect on cell proliferation and ruminal epithelial growth (Górka et al., 2009). Sodium lignosulfonate at 3% increased butyrate concentration, which could have enhanced VFA absorption. Conversely, MgL (0.5-3%) and PRP (0.5 and 1%) had less butyrate concentration. Across doses, NaL increased isobutyrate compared to MgL and PRP. Isobutyrate is required by ruminal cellulolytic bacteria for optimal growth (Liu et al., 2009). Hence, it may have contributed to the improvement in NDFD for NaL at a dose of 3%.

High producing dairy cattle need to supplement microbial protein with significant amounts of high quality dietary protein that can escape rumen fermentation (rumen undegradable protein) in order to meet their amino acid requirements (Harstad and Prestløkken, 2000). Lignosulfonates have shown a capacity to increase the ruminally undegradable protein fraction (Wright et al., 2005; Borucki Castro et al., 2007; Wang et al., 2009) since they seem to bind and precipitate proteins, as observed in other applications (Cerbulis, 1978; Becker, 2002). Therefore, a decrease in ruminal NH_3-N seems to indicate that NaL at a dose of 3% reduced ruminal proteolysis and could increase rumen undegradable protein *in vivo*. Further research is needed to confirm these effects.

Overall, NaL was the most promising technical lignin preventing spoilage in high moisture alfalfa hay. We observed that the antifungal properties of lignosulfonates were underestimated when evaluated using an artificial media vs. a hay substrate (~5 fold difference). This was most likely due to the limited availability of nutrients and moisture in the hay. However, before its implementation in the field, NaL antifungal activity needs to be increased further in order to match PRP effects. Since the cost of lignosulfonates

is around $400/Mg and PRP-based products are $4,000/Mg, there is enough economical margin for the removal of impurities in lignosulfonate products and the isolation of the active antimicrobial fraction, which will allow for a much lower application rate. Few studies have fractionated lignosulfonates into fractions with unique physicochemical properties (Ringena et al., 2005; Duval et al., 2015). Unfortunately, none of these studies have evaluated the antimicrobial activity of these fractions. For instance, the NaL evaluated in our study had high ash and sugar impurities that if removed should significantly increase the antifungal effect. García et al. (2017) reported that the presence of impurities such as minerals and hemicelluloses in different lignin fractions from apple tree pruning waste, increased the growth of *A. niger*, and *S. cerevisiae*. Further studies should be conducted to compare the antifungal activities across a wide range of sodium and magnesium lignosulfonates from different sources.

Conclusion

Sodium lignosulfonate was found to be the most antifungal technical lignin due its promising fungistatic activity against *A. amoenus*, *M. circinelloides*, *P. solitum* (molds) and *D. hansenii* (yeast) strains isolated from spoiled alfalfa hay. When evaluated using hay as substrate, NaL had superior preservation properties measured as decreased DM losses, NDF, fungal counts, and increased WSC, OM, DMD, and NDFD. Furthermore, its antiproteolytic properties were confirmed with a decrease in hay and ruminal *in vitro* NH_3-N. Also, *in vitro* ruminal VFA concentration was greatly increased by NaL relative to all the other ADV tested. However, before its field implementation, NaL preservation effects need to be increased 3-fold in order to match all the nutritional benefits obtained with PRP hay treatment. Considering the high level of ash and WSC impurities

lignosulfonates have and their low cost, it should be cost-effective and feasible to isolate the antimicrobial fraction and increase the antifungal activity several fold.

It is interesting to note that even though MgL did not preserve hay nutritional composition as extensively as NaL, it improved DMD and NDFD despite the increase in NDF concentration due to unrestricted spoilage. Previous research seems to point out lignosulfonate stimulatory effects on NDFD due to its surfactant properties, but more research needs to be conducted to the understand the mechanisms behind its stimulatory effects on ruminal digestibility.

Table 3-1. Chemical composition of technical lignins

Lignin	Total soluble phenolics[1] (mg/g DM)	DDPH Scavenging effect[2]	WSC[3]	Ash[4]	Magnesium[5]	Sodium	Sulfur
		% of DM					
AKL	219.1	-4.8	18.05	61.9	0.02	6.86	4.80
UW	222.5	40.8	0.045	2.23	0.01	0.777	2.20
AI	241.9	65.2	0.037	0.69	0.02	0.082	1.49
HEX 250	265.8	79.9	0.027	0.69	< 0.01	0.009	1.49
PI	382.6	69.4	0.025	0.022	< 0.01	0.004	1.44
NaL	184.3	14.2	22.8	61.0	0.05	12.8	8.01
MgL	142.5	10.5	15.7	15.1	6.21	0.04	8.25
AMOL	132.9	25.9	24.8	1.95	0.07	0.517	7.93
Pooled SD	9.14	12.7	0.45	4.5	0.034	0.084	0.142

[1]Singleton and Rossi, 1965.
[2]Wu et al., 2006 and AOAC 2012.04.
[3]Water soluble carbohydrates; Dubois et al., 1956.
[4]AOAC, 2000.
[5]Beliciu et al., 2012.

Table 3-2. 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[1]

		A. amoenus		*P. solitum*		*M. circinelloides*		*D. hansenii*	
ADV	pH	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
NaL	4	20.0 ± 0[2]	40.0 ± 0	33.3 ± 5.77	60.0 ± 0	25.0 ± 0	> 60	40.0 ± 0	40.0 ± 0
	6	>60	n.c.[3]	>60	n.c.	> 60	n.c.	> 60	n.c.
MgL	4	33.3 ± 5.77	40.0 ± 0	46.7 ± 5.77	> 60	36.7 ± 5.77	>60	26.7 ± 2.89	30.0 ± 0
	6	> 60	n.c.	> 60	n.c.	> 60	n.c.	> 60	n.c.
AKL	4	> 60	n.c.	> 60	n.c.	> 60	n.c.	> 60	n.c.
	6	> 60	n.c.	> 60	n.c.	> 60	n.c.	> 60	n.c.
PRP	4	1.25 ± 0	5.0 ± 0	1.25 ± 0	10.0 ± 0	3.33 ± 1.44	20.0 ± 0	1.25 ± 0	5.0 ± 0
	6	5.0 ± 0	10.0 ± 0	5.0 ± 0	16.7 ± 5.77	10.0	40.0 ± 0	4.17 ± 1.44	15.0 ± 0

[1]*Aspergillus amoenus*, *Penicillium solitum*, *Mucor circinelloides* (molds), and *Debaryomyces hansenii* (yeast). NaL= Sodium lignosulphonate, MgL= Magnesium lignosulphonate, AKL= Alkali Kraft lignin, PRP= Propionic Acid (positive control).
[2]Mean ± standard deviation.
[3]Not calculated.

Table 3-3. Dry matter losses (%), pH, and microbial counts of alfalfa hay as a function of additive (ADV) and dose at d 15

Item	Dose (%, w/w)				Mean	SEM	*P*-value			Contrast[1]
	0	0.5	1	3			ADV	Dose	ADV × Dose	
DM loss, %										
MgL[2]	14.9^{a}	13.8^{A,a}	10.5^{A,b}	0.368^{c}	9.87^{A}	0.773	<0.001	<0.001	<0.001	L**
NaL[3]	14.9^{a}	15.2^{A,a}	3.39^{B,b}	0.428^{b}	8.49^{B}					CU**
PRP[4]	14.9^{a}	0.808^{B,b}	0.944^{B,b}	0.47^{b}	4.28^{C}					CU**
Mean	14.9^{a}	9.93^{b}	4.93^{c}	0.422^{d}						
Hay pH										
MgL	7.99^{ab}	8.24^{A,a}	7.39^{A,b}	5.37^{c}	7.25^{A}	0.156	<0.001	<0.001	<0.001	L**
NaL	7.99^{a}	8.47^{A,a}	6.52^{B,b}	5.24^{c}	7.05^{A}					CU**
PRP	7.99^{a}	5.25^{B,b}	5.14^{C,b}	4.80^{b}	5.79^{B}					CU**
Mean	7.99^{a}	7.32^{b}	6.35^{c}	5.14^{d}						
D. hansenii, log cfu/fresh g										
MgL	7.0	6.12	3.5	2.06	4.67^{A}	0.914	0.006	<0.001	0.1	L*
NaL	7.0	2.6	2.83	1.74	3.54^{A}					L*
PRP	7.0	0.34	0.5	0.0	1.96^{B}					CU**
Mean	7.0^{a}	3.02^{b}	2.28^{b}	1.27^{b}						
Molds, log cfu/fresh g										
MgL	7.76	7.6^{A}	7.52^{A}	5.42	7.08^{A}	0.549	<0.001	<0.001	0.007	L*
NaL	7.76^{a}	7.4^{A,a}	7.18^{A,a}	3.92^{b}	6.57^{A}					L**
PRP	7.76^{a}	3.94^{Bb}	4.2^{Bb}	3.5^{b}	4.85^{B}					CU**
Mean	7.76^{a}	6.31^{b}	6.3^{b}	4.28^{c}						
A. amoenus, log cfu/fresh g										
MgL	7.64	7.42^{A}	7.04^{A}	6.51^{A}	7.15^{A}	0.265	<0.001	<0.001	<0.001	L**
NaL	7.64^{a}	7.28 ^{A,a}	6.86^{A,a}	4.21^{B,b}	6.50^{B}					L**
PRP	7.64^{a}	3.86^{Bb}	4.06^{Bb}	3.4^{Bb}	4.74^{C}					CU**
Mean	7.64^{a}	6.19^{b}	5.99^{b}	4.71^{c}						

Table 3-3. Continued

M. circinelloides, log cfu/fresh g										
MgL	6.92^{a}	6.12^{A,a}	2.12^{b}	2.12^{b}	4.32^{A}	0.701	0.003	<0.001	<0.001	QU*
NaL	6.92^{a}	6.26^{A,a}	0.0^{b}	0.52^{b}	3.42^{AB}					CU**
PRP	6.92^{a}	1.42^{B,b}	1.08^{b}	0.0^{b}	2.36^{B}					CU**
Mean	6.92^{a}	4.60^{b}	1.07^{c}	0.88^{c}						
P. solitum, log cfu/fresh g										
MgL	6.66	7.0^{A}	7.22^{A}	6.01^{A}	6.72^{A}	0.485	<0.001	<0.001	<0.001	L**
NaL	6.66^{a}	6.56^{A,a}	6.96^{A,a}	2.82^{B,b}	5.75^{B}					QU**
PRP	6.66^{a}	2.62^{B,b}	3.34^{B,b}	2.52^{B,b}	3.79^{C}					CU**
Mean	6.66^{a}	5.39^{ab}	5.84^{b}	3.78^{c}						

^{A,B,C}Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$).
^{a,b,c}Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$).
[1]Linear (L), quadratic (QU) and cubic (CU) effect ($P < 0.05$). *$P < 0.05$; **$P < 0.01$.
[2]Magnesium lignosulfonate.
[3]Sodium lignosulfonate.
[4]Propionic acid.

Table 3-4. Nutritional composition of alfalfa hay as a function of additive (ADV) and dose at d 15

Item	Dose (%, w/w)				Mean	SEM	*P*-value			Contrast[1]
	0	0.5	1	3			ADV	Dose	ADV × Dose	
DM, %										
MgL[2]	62.4^{c}	62.4^{A,bc}	64.8^{B,b}	69.1^{a}	64.7^{C}	0.491	<0.001	<0.001	<0.001	L**
NaL[3]	62.4^{b}	62.5^{A,b}	68.7^{A,a}	69.5^{a}	65.8^{B}					CU**
PRP[4]	62.4^{b}	69.2^{B,a}	69.2^{A,a}	69.0^{a}	67.4^{A}					CU**
Mean	62.4^{d}	64.7^{c}	67.6^{b}	69.2^{a}						
OM, % of DM										
MgL	90.3^{c}	91.0^{B,bc}	91.2^{ab}	91.6^{a}	91.0^{B}	0.174	<0.001	<0.001	0.009	QU*
NaL	90.3^{c}	90.6^{B,bc}	91.6^{a}	91.1^{ab}	90.9^{B}					QU**
PRP	90.3^{b}	91.8^{A,a}	91.9^{a}	91.9^{a}	91.5^{A}					CU*
Mean	90.3^{c}	91.1^{b}	91.5^{a}	91.6^{a}						
CP, % of DM										
MgL	16.8	18.1	17.7	16.6	17.3	0.497	0.460	0.137	0.790	NS
NaL	16.8	17.5	17.3	17.1	17.2					NS
PRP	16.8	17.4	16.5	16.7	16.9					NS
Mean	16.8	17.6	17.2	16.8						
NH_3-N, % of DM										
MgL	0.249^{a}	0.238^{A,a}	0.174^{A,b}	0.062^{A,c}	0.159^{A}	0.007	<0.001	<0.001	<0.001	CU*
NaL	0.249^{a}	0.071^{B,b}	0.063^{B,b}	0.043^{AB,c}	0.083^{B}					CU**
PRP	0.249^{a}	0.061^{B,b}	0.062^{B,b}	0.013^{B,b}	0.059^{C}					CU**
Mean	0.249^{a}	0.101^{b}	0.088^{c}	0.033^{d}						
WSC, % of DM										
MgL	7.99^{b}	7.73^{B,b}	8.86^{B,b}	10.3^{a}	8.73^{B}	0.283	<0.001	<0.001	<0.001	L**
NaL	7.99^{b}	8.07^{B,b}	8.93^{B,ab}	10.1^{a}	8.78^{B}					L**
PRP	7.99^{b}	10.5^{A,a}	10.9^{A,a}	10.8^{a}	10.0^{A}					CU*
Mean	7.99^{d}	8.77^{c}	9.56^{b}	10.4^{a}						

Table 3-4. Continued

NDF, % of DM										
MgL	49.7	49.8^{A}	50.5^{A}	49.01^{A}	49.8^{A}	0.663	<0.001	<0.001	<0.001	NS
NaL	49.7^{a}	51.9^{A,a}	49.8^{A,a}	45.9^{AB,b}	49.3^{A}					CU*
PRP	49.7^{a}	45.05^{B,b}	44.0^{B,b}	44.6^{B,b}	45.8^{B}					QU**
Mean	49.7^{a}	48.9^{ab}	48.1^{b}	46.5^{c}						
ADF, % of DM										
MgL	35.9	36.7	37.7	34.0	36.09^{A}	1.06	0.047	0.021	0.07	NS
NaL	35.9	37.6	36.3	33.2	35.8^{AB}					L*
PRP	35.9	34.6	32.9	34.4	34.4^{B}					NS
Mean	35.9^{ab}	36.3^{a}	35.6^{ab}	33.9^{b}						
Hemicellulose, % of DM										
MgL	13.8	13.1	12.8	15.0	13.7^{B}	0.818	0.004	0.223	0.121	NS
NaL	13.8	14.3	13.5	12.7	13.6^{B}					NS
PRP	13.8	10.5	11.1	12.0	11.8^{A}					NS
Mean	13.8	12.6	12.5	13.2						

^{A,B,C}Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$).
^{a,b,c}Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$).
[1]Linear (L), quadratic (QU) and cubic (CU) effect ($P < 0.05$). *$P < 0.05$; **$P < 0.01$.
[2]Magnesium lignosulfonate.
[3]Sodium lignosulfonate.
[4]Propionic acid.

Table 3-5. 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	5.4 ± 0.1
Debaromyces hansenii counts	4.8 ± 0.2
Aspergillus amoenus counts	4.9 ± 0.19
Mucor circinelloides counts	4.9 ± 0.36
Penicillium solitum counts	4.7 ± 0.34
Nutritional value	
DM, %	69.3 ± 0.6
Hay pH	5.52 ± 0.2
OM, % DM	92.4 ± 0.6
NDF, % DM	47.8 ± 1.2
ADF, % DM	34.8 ± 1.5
CP, % DM	16.7 ± 0.81
Hay ammonia nitrogen (NH_3-N), % DM	0.065 ± 0.005
WSC (water soluble carbohydrates), % DM	11.1 ± 0.59
In vitro digestibility and rumen fermentation parameters	
24 h IVDMD, %	66.2 ± 1.5
24 h NDFD, % DM	30.0 ± 1.2
Total VFA, mM	96.8 ± 0.9
Acetate, mM	52.7 ± 0.6
Propionate, mM	22.5 ± 0.4
Butyrate, mM	12.5 ± 0.4
Isobutyrate, mM	1.5 ± 0.2
Isovalerate, mM	3.13 ±0.3
Valerate, mM	5.45 ± 0.4
Acetate to propionate ratio	2.34 ± 0.03
Ruminal pH	6.59 ± 0.05
Ruminal NH_3-N, mg/dL	54.96 ± 4.11

Table 3-6. 24 h *In vitro* DM (DMD), NDF digestibility (NDFD), and rumen fermentation parameters of alfalfa hay as a function of additive (ADV) and dose at d 15

	Dose (%, w/w)						*P*-value			
Item	0	0.5	1	3	Mean	SEM	ADV	Dose	ADV × Dose	Contrast[1]
DMD, %										
MgL[2]	61.8^{b}	65.4^{A,ab}	67.0^{AB,a}	66.6^{a}	65.2^{B}	0.771	<0.001	<0.001	<0.001	QU**
NaL[3]	61.8^{bc}	61.1^{B,c}	65.5^{B,ab}	67.5^{a}	64.0^{B}					CU**
PRP[4]	61.8^{b}	68.5^{A,a}	69.4^{A,a}	68.3^{a}	67.0^{A}					CU*
Mean	61.8^{c}	65.0^{b}	67.3^{a}	67.5^{a}						
NDFD, % of DM										
MgL	23.3^{b}	30.5^{A,a}	34.7^{a}	32.0^{a}	30.1^{A}	1.09	<0.001	<0.001	0.043	QU**
NaL	23.3^{c}	25.0^{B,bc}	30.7^{a}	29.1^{ab}	27.0^{B}					CU*
PRP	23.3^{b}	30.1^{AB,a}	30.4^{a}	28.9^{a}	28.2^{B}					QU**
Mean	23.3^{c}	28.6^{b}	31.9^{a}	30.0^{ab}						
pH										
MgL	6.79	6.79	6.77	6.71	6.77	0.021	0.192	0.036	0.1	L*
NaL	6.79	6.75	6.76	6.77	6.77					NS
PRP	6.79	6.71	6.72	6.75	6.74					QU*
Mean	6.79^{a}	6.75^{ab}	6.75^{ab}	6.74^{b}						
NH_3-N, mg/dL										
MgL	58.2	53.7	56.7	53.5^{AB}	55.5	1.50	0.120	0.005	0.009	NS
NaL	58.2^{a}	55.8^{ab}	54.5^{ab}	49.6^{B,b}	56.8					L**
PRP	58.2	53.1	56.4	59.3^{A}	54.5					NS
Mean	58.2^{a}	54.2^{b}	55.9^{ab}	54.1^{b}						
TVFA, mM										
MgL	86.7^{a}	78.0^{C,b}	83.2^{B,ab}	88.1^{B,a}	83.5^{C}	1.30	<0.001	<0.001	<0.001	CU**
NaL	86.7^{c}	91.8^{A,b}	96.0^{A,b}	111.9^{A,a}	96.1^{A}					L**
PRP	86.7	85.0^{B}	87.9^{B}	89.9^{B}	86.9^{B}					L**
Mean	86.7^{c}	85.0^{c}	89.0^{b}	96.7^{a}						

Table 3-6. Continued

Acetate, mM										
MgL	48.0^{ab}	45.3^{B,b}	48.0^{B,ab}	50.1^{B,a}	47.8^{C}	0.805	<0.001	<0.001	<0.001	CU*
NaL	48.0^{c}	52.2^{A,b}	54.8^{A,b}	64.1^{A,a}	54.8^{A}					L**
PRP	48.0	49.1^{A}	50.0^{AB}	50.6^{B}	49.5^{B}					L**
Mean	48.0^{c}	48.9^{c}	51.0^{b}	54.9^{a}						
Propionate, mM										
MgL	18.0^{bc}	16.5^{B,c}	18.5^{B,b}	20.6^{B,a}	18.4^{C}	0.352	<0.001	<0.001	<0.001	CU**
NaL	18.0^{c}	19.3^{A,c}	21.8^{A,b}	24.5^{A,a}	20.9^{A}					QU**
PRP	18.0^{b}	19.4^{A,ab}	20.5^{A,a}	20.8^{B,a}	19.7^{B}					QU**
Mean	18.0^{c}	18.4^{c}	20.3^{b}	22.0^{a}						
A:P ratio[5]										
MgL	2.67^{a}	2.68^{a}	2.63^{ab}	2.44^{b}	2.61^{A}	0.046	<0.001	<0.001	0.031	L**
NaL	2.67	2.70	2.59	2.62	2.65^{A}					NS
PRP	2.67^{a}	2.54^{ab}	2.44^{b}	2.43^{b}	2.52^{B}					QU**
Mean	2.67^{a}	2.64^{ab}	2.55^{bc}	2.50^{c}						
Butyrate, mM										
MgL	10.9^{a}	8.79^{B,b}	8.43^{B,b}	8.84^{B,b}	9.24^{B}	0.418	<0.001	<0.001	<0.001	QU**
NaL	10.9^{b}	12.1^{A,ab}	11.1^{A,b}	13.5^{A,a}	11.9^{A}					L**
PRP	10.9^{a}	8.32^{B,b}	8.80^{B,b}	9.30^{B,ab}	9.33^{B}					CU**
Mean	10.9^{a}	9.74^{b}	9.45^{b}	10.6^{a}						
(A+B):P ratio[6]										
MgL	3.27^{a}	3.20^{AB,a}	3.11^{ab}	2.87^{B,b}	3.11^{B}	0.061	<0.001	<0.001	0.004	L**
NaL	3.27	3.33^{A}	3.06	3.18^{A}	3.21^{A}					CU*
PRP	3.27^{a}	2.97^{B,b}	2.87^{b}	2.88^{B,b}	3.00^{C}					QU**
Mean	3.27^{a}	3.17^{a}	3.01^{b}	2.97^{b}						

Table 3-7. Continued

Isobutyrate, mM										
MgL	1.47	1.33^{B}	1.25^{B}	1.29^{B}	1.33^{B}	0.063	<0.001	0.862	0.003	QU**
NaL	1.47	1.71^{A}	1.68^{A}	1.66^{A}	1.63^{A}					NS
PRP	1.47	1.31^{B}	1.36^{B}	1.43^{AB}	1.39^{B}					QU*
Mean	1.47	1.45	1.43	1.46						
Isovalerate, mM										
MgL	3.03	3.55	3.52	3.61	3.42^{A}	0.145	0.034	0.003	0.605	QU*
NaL	3.03	3.36	3.08	3.19	3.16^{B}					NS
PRP	3.03	3.42	3.54	3.51	3.38^{AB}					QU**
Mean	3.03^{b}	3.44^{a}	3.38^{a}	3.43^{a}						
Valerate, mM										
MgL	3.23^{ab}	2.55^{B,b}	3.23^{ab}	3.72^{B,a}	3.18^{B}	0.174	<0.001	<0.001	0.004	CU**
NaL	3.23^{bc}	3.04^{AB,c}	4.03^{b}	4.93^{A,a}	3.81^{A}					CU*
PRP	3.23^{b}	3.55^{A,ab}	3.62^{ab}	4.26^{AB,a}	3.66^{A}					L**
Mean	3.23^{c}	3.05^{c}	3.63^{b}	4.30^{a}						

^{A,B,C}Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$).
^{a,b,c}Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$).
[1]Linear (L), quadratic (QU) and cubic (CU) effect ($P < 0.05$). *$P < 0.05$; **$P < 0.01$.
[2]Magnesium lignosulfonate.
[3]Sodium lignosulfonate.
[4]Propionic acid.
[5]A = acetic acid; P = propionic acid.
[6]A = acetic acid; P = propionic acid; B = butyric acid.

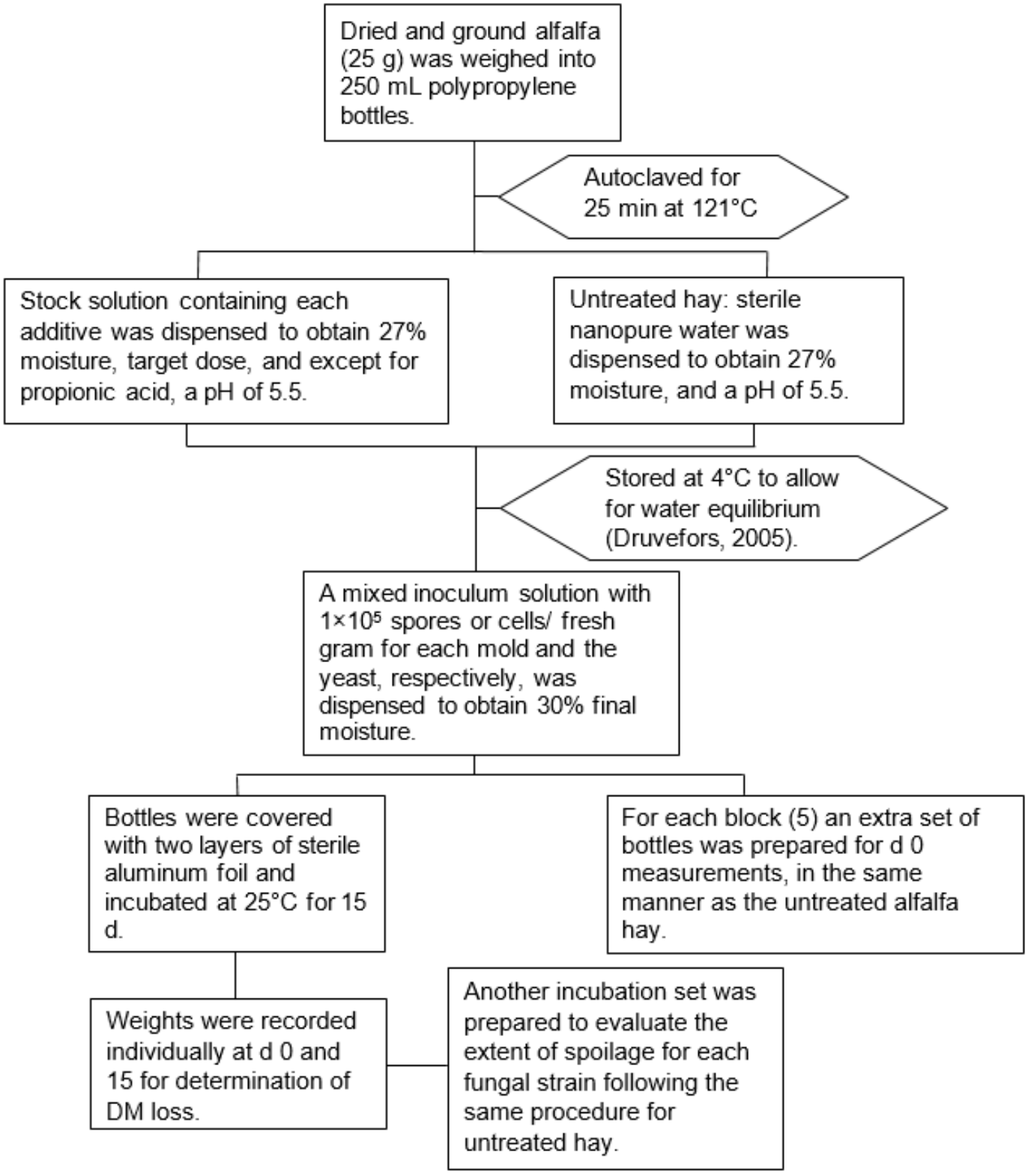

Figure 3-1. Flowchart for the *in vitro* evaluation method of hay spoilage.

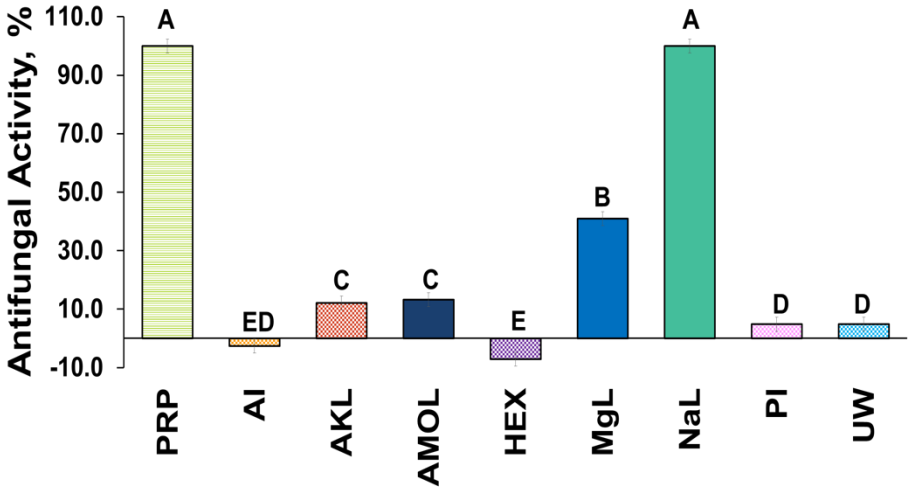

Figure 3-2. Antifungal activity of technical lignins on *A. amoenus*. SEM: 2.77; P ≤ 0.05.

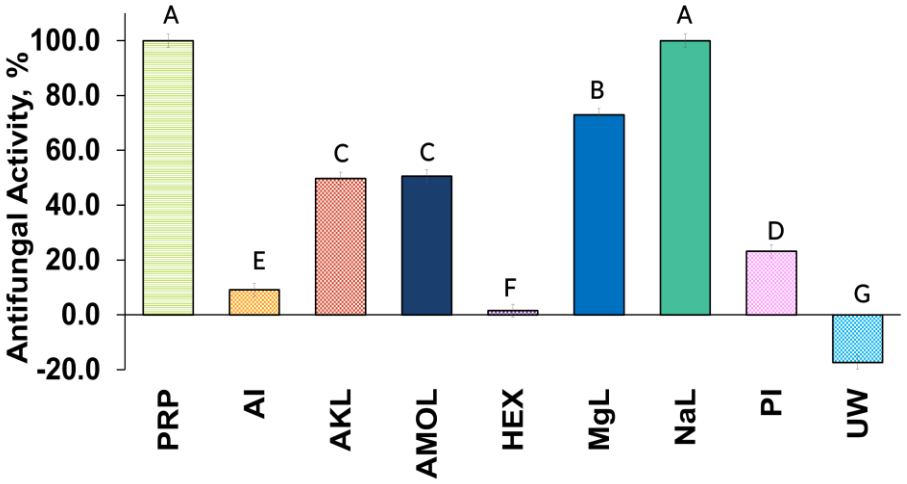

Figure 3-3. Antifungal activity of technical lignins on *M. circinelloides*. SEM: 2.77; P ≤ 0.05.

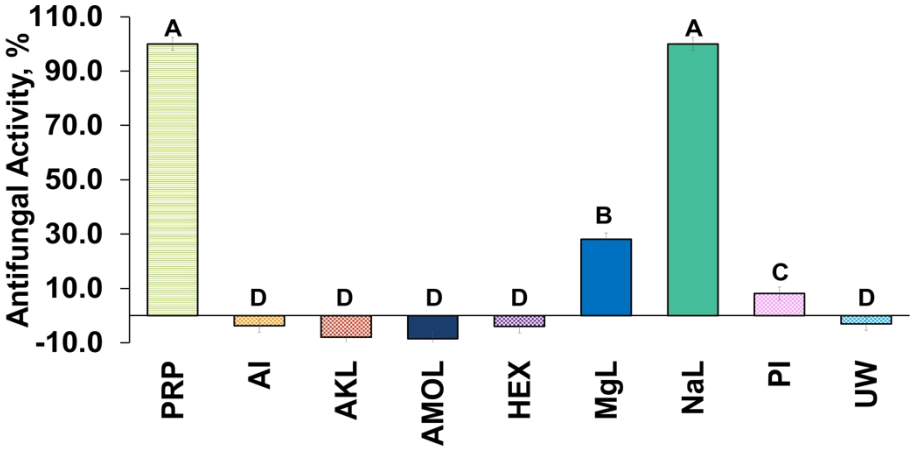

Figure 3-4. Antifungal activity of technical lignins on *P. solitum*. SEM: 2.77; P ≤ 0.05.

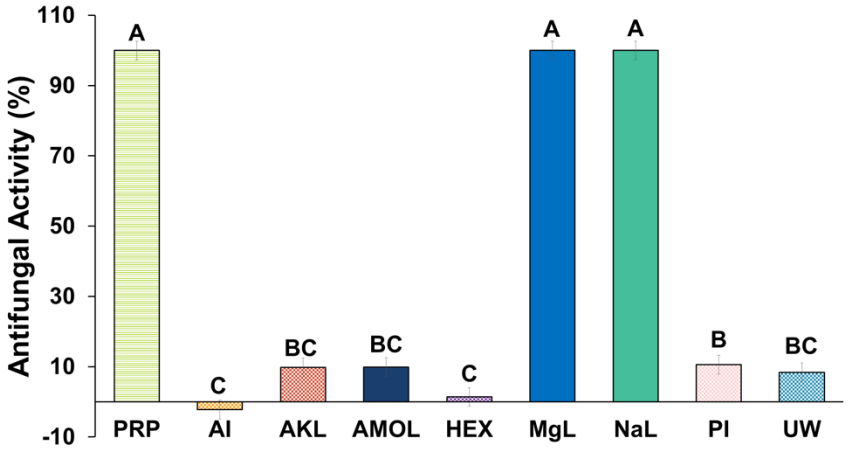

Figure 3-5. Antifungal activity of technical lignins on *D. hansenii*. SEM: 2.77; P ≤ 0.05.

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APPENDIX A

Table A-1. Effects of organic acids on hay quality, preservation, and animal performance measurements.

Source	ADV[1]	App. rate (%)[2]	Bale[3]	M (%)[4]	Relative Effect[5]								
					DM loss[6]	High T.[7]	Visual spoil.[8]	WSC[9]	CP[10]	NDF[11]	ADF[12]	DMD[13]	DMI[14]
Knapp et al. (1976)	Propionic acid	0.0198	S	32.4	10.6	3.92	33.3	-8.82	3.85	n.a.	n.a.	2.15	n.a.
	Propionic acid	0.198	S	32.4	-12.6	-9.80	-33.3	14.7	3.85	n.a.	n.a.	2.81	n.a.
	Propionic acid	0.495	S	32.4	-22.5	-21.6	-66.7	20.6	3.85	n.a.	n.a.	0.83	n.a.
	Propionic acid	0.99	S	32.4	-49.7	-43.2	-100	91.2	3.85	n.a.	n.a.	7.44	n.a.
Nash and Easson (1977)	Propionic acid	0.99	n.a.[15]	25	0	-37.8	-66.7	n.a.	n.a.	n.a.	n.a.	4.61	n.a.
	Propionic acid	1.98	n.a.	25	-36.5	-48.9	-100	n.a.	n.a.	n.a.	n.a.	6.09	n.a.
	Propionic acid	2.97	n.a.	25	-86.5	-48.9	-100	n.a.	n.a.	n.a.	n.a.	6.91	n.a.
	Propionic acid	3.96	n.a.	25	-51.4	-48.9	-100	n.a.	n.a.	n.a.	n.a.	9.21	n.a.
	Propionic acid	0.99	n.a.	35	-36.7	-14.5	-29.4	n.a.	n.a.	n.a.	n.a.	6.64	n.a.
	Propionic acid	1.98	n.a.	35	-10.8	-47.3	-58.8	n.a.	n.a.	n.a.	n.a.	11.8	n.a.
	Propionic acid	2.97	n.a.	35	-37.4	-45.5	-76.5	n.a.	n.a.	n.a.	n.a.	5.79	n.a.
	Propionic acid	3.96	n.a.	35	-81.3	-47.3	-76.5	n.a.	n.a.	n.a.	n.a.	4.94	n.a.
	Propionic acid	0.99	n.a.	45	-17.6	-29.2	-35	n.a.	n.a.	n.a.	n.a.	3.72	n.a.
	Propionic acid	1.98	n.a.	45	-40.9	-23.1	-40	n.a.	n.a.	n.a.	n.a.	5.84	n.a.
	Propionic acid	2.97	n.a.	45	-55.7	-38.5	-50	n.a.	n.a.	n.a.	n.a.	1.95	n.a.

Table A-1. Continued

	Propionic acid	3.96	n.a.	45	-49.4	-33.9	-65	n.a.	n.a.	n.a.	n.a.	5.84	n.a.
	Propionic acid	1.98	n.a.	30	-43.2	-19.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	2.97	n.a.	30	-58.1	-19.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	4.95	n.a.	30	-100	-15.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Easson and Nash (1978)	Propionic acid	0.99	S	41	-41.7	n.a.	-33.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.485	S	41	-98.7	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.98	S	41	-96.7	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	2.48	S	41	-92.7	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	2.97	S	41	-97.4	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	0.495	S	32	-12.6	n.a.	-33.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	0.99	S	32	-70.1	n.a.	-66.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.49	S	32	-99.1	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.98	S	32	-68.7	n.a.	-100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Davies and Warboys (1978)	Propionic acid	3.47	n.a.	35.9	-31.7	-33.3	n.a.	n.a.	n.a.	n.a.	n.a.	7.93	n.a.
	Propionic acid	2.08	n.a.	26.1	-4.65	-21.6	n.a.	n.a.	n.a.	n.a.	n.a.	-0.95	n.a.
Nehrir et al. (1978)	Acetic acid	0.8	R	29	74.4	n.a.	n.a.	n.a.	5.80	n.a.	2.86	0.49	n.a.
	Acetic acid	1.3	R	30	2.6	n.a.	n.a.	n.a.	7.25	n.a.	1.19	1.97	n.a.
	Propionic acid	0.99	R	31	21.4	n.a.	n.a.	n.a.	5.80	n.a.	2.86	0.49	n.a.

Table A-1. Continued

	Propionic acid	1.39	R	30	-28.6	n.a.	n.a.	n.a.	7.25	n.a.	1.19	1.97	n.a.
Lacey et al. (1978)	Propionic acid	0.99	R	41	112	-20.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.98	R	41	106	-32.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	0.99	R	28	-96.3	-23.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.98	R	28	-85.2	-39.1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	2.97	R	28	-32.1	-45.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	0.99	R	26	-100	-26.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	1.98	R	26	-100	-38.1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	2.97	R	26	3.2	-28.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Davies and Warboys (1982)	Propionic acid	4.65	n.a.	43.4	n.a.	n.a.	n.a.	n.a.	0	n.a.	n.a.	n.a.	4.18
	Propionic acid	4.26	n.a.	34.9	n.a.	n.a.	n.a.	n.a.	-8.3	n.a.	n.a.	n.a.	-5.1
	Propionic acid	3.47	n.a.	35.9	n.a.	n.a.	n.a.	n.a.	2.56	n.a.	n.a.	n.a.	21.0
Walgenbach and Massengill (1986)	Sodium Propionate	0.223	R	23	n.a.	-2.27	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Sodium Propionate	0.454	R	24	n.a.	0	6.90	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Sodium Propionate	0.68	R	21	n.a.	0	-3.45	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Propionic acid	0.36	R	21	n.a.	-12.3	-60	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Atwal and Erfle (1988)	Propionic acid	0.297	Ro	17.8	-20.9	-7.72	n.a.	n.a.	n.a.	-12.1	-11.3	9.2	n.a.

Table A-1. Continued

	Propionic acid	0.297	Ro	23.6	-9.30	-13.2	n.a.	n.a.	n.a.	-6.16	-5.96	2.78	n.a.
	Propionic acid	0.03	Ro	25	37.2	2.12	n.a.	n.a.	n.a.	-1	2.19	-0.52	n.a.
Baron and Greer (1988)	Propionic acid	0.075	n.a.	15	n.a.	-0.87	0	n.a.	n.a.	n.a.	2.79	-1.84	n.a.
	Propionic acid	0.057	n.a.	15	n.a.	-0.87	0	n.a.	n.a.	n.a.	2.44	-1.07	n.a.
	Propionic acid	0.075	n.a.	25	n.a.	-11.2	17.5	n.a.	n.a.	n.a.	-12.5	5.25	n.a.
	Propionic acid	0.057	n.a.	25	n.a.	4.13	-12.3	n.a.	n.a.	n.a.	-6.8	3.39	n.a.
	Propionic acid	0.075	n.a.	35	n.a.	0.955	10	n.a.	n.a.	n.a.	-10.6	10.5	n.a.
	Propionic acid	0.057	n.a.	35	n.a.	0.234	0	n.a.	n.a.	n.a.	-6.42	10.5	n.a.
	Propionic acid	0.075	n.a.	30	-13.1	4.19	-42.1	n.a.	0	n.a.	1.43	n.a.	n.a.
	Propionic acid	0.057	n.a.	30	-15.9	4.19	-7.02	n.a.	6.25	n.a.	-2.62	n.a.	n.a.
Rotz et al. (1988)	Propionic acid	1.17	n.a.	21	-25	0	91.3	n.a.	0.93	n.a.	-8.81	n.a.	n.a.
	Propionic acid	0.754	n.a.	17	n.a.	0	48.39	n.a.	4.38	n.a.	0.49	n.a.	n.a.
	Propionic acid	1.40	n.a.	22	n.a.	-30.0	-10.8	n.a.	-0.61	n.a.	2.91	n.a.	n.a.
	Propionic acid	0.718	n.a.	20	10.3	28.9	-3.23	n.a.	8.12	n.a.	-6.41	n.a.	n.a.
	Propionic acid	1.31	n.a.	27	3.26	-22.0	-10.2	n.a.	-1.46	n.a.	-4.59	n.a.	n.a.
	Propionic acid	0.683	n.a.	24	-70.6	-31.0	-33.3	n.a.	-13.6	n.a.	5.84	n.a.	n.a.
	Propionic acid	1.13	n.a.	n.a.	-27.6	-36.6	-32.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table A-1. Continued

Baron and Mathison (1990)	Propionate salt	1.25	n.a.	19.5	-23.5	-7.64	n.a.	n.a.	n.a.	-5.46	-5.44	n.a.	n.a.
Khalilian et al. (1990)	Propionic acid	0.396	R	20	n.a.	6.42	0	n.a.	2.06	-1.64	-2.66	n.a.	n.a.
	Propionic acid	0.792	R	20	n.a.	2.75	0	n.a.	-7.59	-0.21	n.a.	n.a.	n.a.
	Propionic acid	0.371	R	25	-11.8	3.35	-43.5	n.a.	-1.85	-5.31	-4.69	n.a.	n.a.
	Propionic acid	0.347	R	30	-12.5	-13.6	-11.5	n.a.	18.7	-0.65	-1.63	n.a.	n.a.
	Propionic acid	0.347	R	30	-15.6	-7.87	-13.0	n.a.	-5.15	4.65	8.12	n.a.	n.a.
	Propionic acid	0.693	R	30	-55.6	-28.1	-43.5	n.a.	-16.9	11.8	6.02	n.a.	n.a.
Rotz et al. (1990)	Propionic acid	0.368	R	26.5	-25.9	n.a.	-14.3	n.a.	0.518	n.a.	-1.52	n.a.	n.a.
	Propionic acid	0.362	R	27.6	-27.5	n.a.	-26.7	n.a.	-2.19	n.a.	5.67	n.a.	n.a.
	Propionic acid	0.73	R	27	-4.8	n.a.	12	n.a.	2.47	n.a.	-6.84	n.a.	n.a.
Baron et al. (1991)	Propionate salt	1	n.a.	24.1	-66.9	-46.6	150	n.a.	n.a.	-23.8	-14.5	6.17	n.a.
	Propionate salt	1.25	n.a.	24.1	-66.1	-43.1	150	n.a.	n.a.	-17.4	-7.47	5.29	n.a.
	Propionate salt	1	n.a.	28.3	-68.5	-45.7	n.a.	n.a.	n.a.	-19.5	-11.3	10.6	n.a.
	Propionate salt	1.25	n.a.	28.3	-68.5	-43.2	n.a.	n.a.	n.a.	-16.2	-8.50	9.17	n.a.
Wittenberg (1991)	Buffered Propionic acid	8.78	R	27.5	n.a.	-12.1	-38.9	n.a.	n.a.	n.a.	-2.4	n.a.	n.a.

Table A-1. Continued

	Buffered Propionic acid	6.82	R	22.5	n.a.	4.22	0	n.a.	n.a.	n.a.	1.70	n.a.	n.a.
Shinners (2000)	Propionic acid	0.495	S	16.3	-6.67	n.a.	n.a.	n.a.	-1.29	-0.70	0.29	n.a.	n.a.
	Propionic acid	0.495	S	14.2	48	n.a.	n.a.	n.a.	-2.20	-1.12	-1.42	n.a.	n.a.
	Propionic acid	0.297	S	15.8	2.63	n.a.	n.a.	n.a.	0.60	-1.52	-1.6	n.a.	n.a.
	Propionic acid	0.792	S	27.6	9.02	n.a.	n.a.	n.a.	-2.68	-5.57	-7.02	n.a.	n.a.
Reboux et al. (2002)	Buffered Propionic acid	0.189	Ro	20	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Baah et al. (2005)	Buffered Propionic acid	8.22	Ro	17	n.a.	n.a.	n.a.	-46.3	16.6	-10.1	-11.3	n.a.	n.a.
	Buffered Propionic acid	8.02	Ro	19	n.a.	n.a.	n.a.	23.6	0	-5.48	3.90	n.a.	n.a.
	Buffered Propionic acid	7.92	Ro	20	n.a.	n.a.	n.a.	65.9	-1.02	-8.31	-7.49	n.a.	n.a.
	Buffered Propionic acid	8.22	Ro	17	n.a.	n.a.	n.a.	19.0	5.26	-4.39	-1.15	n.a.	n.a.
Coblentz et al. (2012)	Propionic acid	0.294	Ro	30	10.4	-0.75	n.a.	n.a.	2.1	-0.21	0.28	n.a.	n.a.
	Propionic acid	0.315	Ro	25	2.63	-10.1	n.a.	n.a.	-0.82	-2.23	-3.80	n.a.	n.a.
Coblentz et al. (2013)	Propionic acid	0.36	R	27.4	n.a.	-12.3	n.a.	n.a.	6.18	-3.03	-1.84	-1.13	-3.0
	Propionic acid	0.6	R	27.4	n.a.	-21.2	n.a.	n.a.	2.81	-5.58	-3.45	-2.26	-49

Table A-1. Continued

	Propionic acid	0.36	R	23.8	n.a.	-27.2	n.a.	n.a.	-4.30	-7.94	-14.8	3.44	0
	Propionic acid	0.6	R	23.8	n.a.	-25.6	n.a.	n.a.	-0.54	-6.48	-8.45	9.92	1.49
	Propionic acid	0.36	R	19.6	n.a.	-23.2	n.a.	n.a.	-4.3	-2.87	-3.32	3.66	0
	Propionic acid	0.6	R	19.6	n.a.	-20.8	n.a.	n.a.	1.08	-1.18	3.08	-0.55	0
Jin et al. (2018)	Propionic acid	0.272	Ro	25	n.a.	n.a.	n.a.	-11.9	10.5	6.23	13.4	n.a.	n.a.
	Propionic acid	0.272	Ro	25	n.a.	n.a.	n.a.	24.7	35.5	6.23	13.4	n.a.	n.a.
	Propionic acid	0.272	Ro	25	n.a.	n.a.	n.a.	-65.6	23.5	6.23	13.4	n.a.	n.a.
	Average			27.9	-32.2	-19.0	-18.9	8.89	2.42	-5.77	-2.79	3.99	-1.96

[1]Additive; [2]Application rate (% w/w fresh basis); [3]Bale type (S, squared; R, rectangular; RO, round); [4]Moisture concentration (%); [5]Effect or relative difference expressed as % calculated as treatment mean - untreated mean/treatment mean × 100; [6]Dry matter (DM) loss; [7]Maximum internal bale temperature; [8]Visible spoilage; [9]Water soluble carbohydrates; [10]Crude protein; [11]Neutral detergent fiber; [12]Acid detergent fiber; [13]DM digestibility; [14]DM intake; [15]Not available.

Table A-2. Effects of anhydrous ammonia on hay quality, preservation, and animal performance measurements.

Source	App. rate (%)[1]	Bale[2]	M (%)[3]	Relative Effect[4]							
				DM loss[5]	High T.[6]	Visual spoil.[7]	CP[8]	NDF[9]	ADF[10]	DMD[11]	DMI[12]
Weiss et al. (1982)	1.3	R	32	n.a.	n.a.	n.a.	26.6	4.65	-1.05	n.a.	-2.56
Thorlacius and Robertson (1984)	1.0	R	35	n.a.	n.a.	n.a.	16.0	-7.97	-5.63	n.a.	n.a.
	2.0	R	35	n.a.	n.a.	-100	32.9	-18.0	-7.50	13.8	n.a.
	1.0	R	35	n.a.	n.a.	-68.1	0.00	2.69	0.81	1.60	n.a.
	2.0	R	35	n.a.	n.a.	-100.0	3.73	5.55	-1.08	-3.39	n.a.
Woolford and Tetlow (1984)	1.6	R	20	n.a.	n.a.	n.a.	n.a.	-6.10	1.84	n.a.	n.a.
	3.2	R	20	n.a.	n.a.	n.a.	n.a.	-8.85	0.79	n.a.	n.a.
	6.4	R	20	n.a.	n.a.	n.a.	n.a.	-10.6	5.25	n.a.	n.a.
	1.2	R	40	n.a.	n.a.	n.a.	n.a.	-7.59	-8.78	n.a.	n.a.
	2.4	R	40	n.a.	n.a.	n.a.	n.a.	-9.31	-10.4	n.a.	n.a.
	4.8	R	40	n.a.	n.a.	n.a.	n.a.	-13.5	-15.2	n.a.	n.a.
Grotheer et al. (1985)	2.4	Ro	19.8	n.a.	n.a.	n.a.	76.9	-11.5	3.17	25.0	n.a.
	2.0	Ro	32.5	n.a.	n.a.	n.a.	56.0	-10.9	4.37	19.7	n.a.
L. Jones et al. (1985)	0.2	Ro	19	-26.5	n.a.	n.a.	n.a.	n.a.	n.a.	48.4	37.7
G. Koegel et al. (1985)	1.8	R	25.8	n.a.	-36.6	n.a.	16.1	n.a.	n.a.	n.a.	n.a.
	1.3	R	27.5	n.a.	-14.6	n.a.	27.5	n.a.	n.a.	n.a.	n.a.
	1.2	R	27.2	n.a.	-19.5	n.a.	25.0	n.a.	n.a.	n.a.	n.a.
	1.0	R	25.6	n.a.	-41.5	n.a.	21.50	n.a.	n.a.	n.a.	n.a.
	1.4	R	25	n.a.	0.00	n.a.	32.7	n.a.	n.a.	n.a.	n.a.
	1.2	R	19.9	n.a.	-15.6	n.a.	31.2	n.a.	n.a.	n.a.	n.a.
	1.4	R	25.1	n.a.	28.6	n.a.	17.7	n.a.	n.a.	n.a.	n.a.
	2.2	Ro	30.9	-91.5	-8.77	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2.3	Ro	33.8	-83.8	1.75	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	1.6	Ro	33.8	-100	-14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	1.3	Ro	30.8	-51.4	3.51	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table A-2. Continued

	1.9	Ro	28	-78.9	-14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	1.7	Ro	22.9	n.a.	-2.63	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2.0	Ro	31.1	91.8	2.17	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	1.8	Ro	34.1	-80.8	6.52	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2.3	Ro	30.3	60.3	-15.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	1.7	Ro	29.7	24.7	15.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
van de Riet et al. (1988)	3.0	n.a.[13]	30	n.a.	n.a.	n.a.	96.9	n.a.	n.a.	10.3	n.a.
	3.0	n.a.	15	n.a.	n.a.	n.a.	81.2	n.a.	n.a.	12.4	n.a.
Wylie and Steen (1988)	1.4	R	30.1	n.a.	n.a.	n.a.	n.a.	-6.69	2.15	n.a.	n.a.
	2.1	R	30.1	n.a.	n.a.	n.a.	n.a.	-6.69	3.59	n.a.	n.a.
	1.7	R	16.5	n.a.	n.a.	n.a.	n.a.	-4.65	-1.79	n.a.	n.a.
	2.5	R	16.5	n.a.	n.a.	n.a.	n.a.	-1.94	1.56	n.a.	n.a.
	3.3	R	16.5	n.a.	n.a.	n.a.	n.a.	-8.13	-1.34	n.a.	n.a.
Wittenberg (1991)	2.3	R	22.5	n.a.	-3.59	n.a.	22.3	16.5	9.73	n.a.	n.a.
	2.0	R	27.5	n.a.	-7.48	n.a.	n.a.	n.a.	2.40	n.a.	n.a.
	1.9	R	22.5	n.a.	26.2	40	n.a.	n.a.	-0.85	n.a.	n.a.
Mir et al. (1991)	2.0	Ro	19.5	-55.2	n.a.	n.a.	32.2	4.77	4.12	2.64	-4.40
	2.0	Ro	29.1	110	n.a.	n.a.	40.1	-6.36	0.55	3.16	-11.0
	2.0	Ro	18.1	-58.1	n.a.	n.a.	50	2.12	2.92	0.81	1.20
	2.0	Ro	30.9	-22.6	n.a.	n.a.	63.8	1.79	14.3	-4.38	6.02
de Freitas et al. (2002)	0.8	R	25	n.a.	n.a.	n.a.	2.01	2.66	1.42	n.a.	n.a.
Average			27.1	-25.8	0.50	-54.2	35.1	-4.08	0.21	10.4	4.49

[1]Application rate (CFU/ fresh g); [2]Bale type (S, squared; R, rectangular; RO, round); [3]Moisture concentration (%); [4]Effect or relative difference expressed as % calculated as treatment mean - untreated mean/treatment mean × 100; [5]Dry matter (DM) loss; [6]Maximum internal bale temperature; [7]Visible spoilage; [8]Crude protein; [9]Neutral detergent fiber; [10]Acid detergent fiber; [11]DM digestibility; [12]DM intake; [13]Not available.

Table A-3. Effects of microbial inoculants on hay quality, preservation, and animal performance measurements.

Source	App. rate (log CFU/fresh g)[1]	Bale[2]	M (%)[3]	Relative Effect[4]							
				DM loss[5]	High T.[6]	Visual spoil.[7]	CP[8]	NDF[9]	ADF[10]	DMD[11]	DMI[12]
Rotz et al. (1988)	5.0	n.a.[13]	21	-5.80	1.18	43	-10.2	n.a.	7.44	n.a.	n.a.
	5.0	n.a.	21	-48.6	-10.1	43	-0.51	n.a.	-14.9	n.a.	n.a.
Nelson et al. (1989)	5.05	S	43.4	3.27	n.a.	n.a.	-1.77	5.95	-2.09	3.16	-13.6
	5.17	S	26.5	4.44	n.a.	n.a.	7.14	-1.01	-0.96	-0.14	-8.5
	5.11	Ro	35.7	-12.9	n.a.	n.a.	-3.03	3.35	3.0	0.50	0
Wittenberg (1991)	5.42	R	22.5	n.a.	1.20	n.a.	17.8	13.8	2.79	0.469	-2.38
	5.38	R	27.5	n.a.	88.2	n.a.	-1.54	16.5	4.93	n.a.	n.a.
	5.65	R	27.5	n.a.	-1.43	56	n.a.	n.a.	-1.87	n.a.	n.a.
	5.65	R	22.5	n.a.	-6.44	10	n.a.	n.a.	-1.13	n.a.	n.a.
	5.45	R	27.5	n.a.	-6.69	48	n.a.	n.a.	1.87	n.a.	n.a.
	5.51	R	22.5	n.a.	-1.78	16	n.a.	n.a.	-5.09	n.a.	n.a.
	4.57	R	22.5	16.7	n.a.	20	3.29	n.a.	-6.07	8.11	-1.88
	6.07	R	22.5	66.7	n.a.	22	0.43	1.90	3.66	n.a.	n.a.
Shinners (2000)	4.92	S	15.8	-10.5	n.a.	n.a.	-1.81	0.65	1.87	n.a.	n.a.
	4.86	S	27.6	-10.7	n.a.	n.a.	-1.34	-3.48	-2.63	n.a.	n.a.
Bass et al. 2(012)	7.4	R	17.4	n.a.	-5.07	n.a.	n.a.	-0.84	1.48	n.a.	-13.7
	7.4	R	26.7	n.a.	-12.9	n.a.	n.a.	0.54	-0.28	n.a.	-13.2
Caldwell et al. (2013)	8.3	R	16.3	n.a.	-2.78	n.a.	-3.54	-0.59	2.16	n.a.	34.1
	8.3	R	25.1	n.a.	-7.41	n.a.	11.6	-2.63	-2.48	n.a.	-3.56
Average			24.8	0.28	2.99	32.25	1.27	2.85	-0.44	2.42	-2.5

[1]Application rate (CFU/ fresh g); [2]Bale type (S, squared; R, rectangular; RO, round); [3]Moisture concentration (%); [4]Effect or relative difference expressed as % calculated as treatment mean - untreated mean/treatment mean × 100; [5]Dry matter (DM) loss; [6]Maximum internal bale temperature; [7]Visible spoilage; [8]Crude protein; [9]Neutral detergent fiber; [10]Acid detergent fiber; [11]DM digestibility; [12]DM intake; [13]Not available.

Table A-4. Dry matter (DM), DM losses, pH, and fungal counts of alfalfa hay as a function of fungi at d 0

Item	*A. amoenus*	*D. Hansenii*	*M. Circinelloides*	*P. Solitum*	Pooled SD
DM, %	64.9	70.2	70.0	68.3	1.55
DM loss, %	12.3	2.36	2.77	7.04	1.52
Hay pH	7.65	5.02	5.1	7.02	1.24
Counts, log cfu/fresh g	8.5	7.3	7.2	8.3	0.18

BIOGRAPHY OF AUTHOR

Diana Reyes was born in Bogota, Colombia in 1993. She graduated from Nuestra Señora de la Sabiduria High School in 2009 with her favorite courses being biology and chemistry. She grew up with great affection for animals and owing a dairy farm encourage her to enroll in the animal science program at Universidad Nacional de Colombia. On her final year, she made her internship at Southern Illinois University under the supervision of Dr. Amer AbuGhazaleh. In this laboratory she enriched her knowledge in ruminant nutrition, and so she realized she wanted to get a deeper knowledge on those fields. At the end of 2016 she finished her studies and got her B.S. as Animal Scientist at the top of her class. Soon after her graduation, she was admitted to the University of Maine to pursue a M.S. in Animal Science under the supervision of Dr. Juan Romero. The fact that she was going to work on ruminal and conserved forages microbiology was particularly exciting to her. During her time at U. Maine, she presented her research findings on the 2018 American Dairy Science Association meeting in Tennessee. During the same year she also presented her research on the International Silage Conference in Bonn, Germany. She plans to continue understanding the ruminal microbiome using metagenomics in her PhD studies. Diana is a candidate for the Master of Science degree in Animal Science from the University of Maine in December 2018.