J. Dairy Sci. 103:6114–6134
https://doi.org/10.3168/jds.2019-17764

In vitro screening of technical lignins to determine their potential as hay preservatives

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ABSTRACT

Our objectives were to evaluate technical lignins for their antifungal properties against 3 molds and 1 yeast causing hay spoilage, and their ability to preserve ground high-moisture alfalfa hay nutritive value in vitro. 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 the ADV on yeast were evaluated separately with a RCBD. Sodium lignosulfonate (NaL) and PRP were the only treatments with 100 ± 2.8% inhibition of fungi. In experiment 2, the minimum inhibitory concentration (MIC) for selected lignins and PRP were determined. At pH 4, NaL had the lowest MIC across the molds (20–33.3 mg/mL) and magnesium lignosulfonate (MgL) for the yeast (26.7) among the lignins. However, PRP had MIC values that were several-fold lower across all fungi (1.25–3.33). In experiment 3, a RCBD (5 blocks) with a 3 (ADV; NaL, MgL, and PRP) × 4 (doses: 0, 0.5, 1, and 3% wt/wt fresh basis) factorial arrangement of treatments was used to evaluate the preservative effects of ADV in ground high-moisture alfalfa hay inoculated with a mixture of the fungi previously tested and incubated under aerobic conditions in vitro. After 15 d, relative to untreated hay (14.9), dry matter (DM) losses were lessened by doses as low as 1% for NaL (3.39) and 0.5% for PRP (0.81 ± 0.77%). The mold count was reduced in both NaL at 3% (3.92) and PRP as low as 0.5% (3.94) relative to untreated hay (7.76 ± 0.55 log cfu/fresh g). Consequently, sugars were best preserved by NaL at 3% (10.1) and PRP as low as 0.5% (10.5) versus untreated (7.99 ± 0.283% DM), while keeping neutral detergent fiber values lower in NaL (45.9) and PRP-treated (45.1) hays at the same doses, respectively, relative to untreated (49.7 ± 0.66% DM). Hay DM digestibility was increased by doses as low as 3% for NaL (67.5), 1% MgL (67.0), and 0.5% PRP (68.5) versus untreated hay (61.8 ± 0.77%). The lowest doses increasing neutral detergent fiber digestibility relative to untreated hay (23.3) were 0.5% for MgL and PRP (30.5 and 30.1, respectively) and 1% for NaL (30.7 ± 1.09% DM). Across technical lignins, NaL showed the most promise as a potential hay preservative. However, its effects were limited compared with PRP at equivalent doses. Despite not having an effect on preservation, MgL improved DM digestibility by stimulating neutral detergent fiber digestibility. This study warrants further development of NaL under field conditions.

Key words: hay preservation, technical lignin, ruminal digestibility

INTRODUCTION

In the United States, hay is the predominant forage conservation method (NASS, 2019a), the third most valuable crop ($17 billion/yr), and second in harvested acres (53 million acres, NASS, 2019b). The main goal in haymaking is to decrease the moisture concentration to less than 15 to 20% in no more than 3 to 5 d so most of the forage crop nutrient yield can be stored long term (Rees, 1982). Nutrient losses during hay harvest and storage are interdependent. During harvest, field losses can occur due to increased leaf fragility as moisture decreases, especially below 20%. However, if hay is baled above 15 to 20% moisture, extensive microbial spoilage will occur during storage and result in

Received October 17, 2019.
Accepted March 2, 2020.
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a significant decline in nutritive value (Coblentz and Hoffman, 2009) and increased DM losses (up to 30%; Ball et al., 1998). Baleage has been considered as an alternative for producers to store wet hay (Shinners, 2010). However, baled hay is more marketable, needs less equipment, and has fewer plastic disposal issues. 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 are the most used hay preservatives, but their efficacy in preventing spontaneous heating is limited to 6 mo (Coblentz et al., 2013). Therefore, more effective and inexpensive hay preservatives are needed to improve the efficiency of hay production.

Technical lignins are byproducts of paper mills and approximately 50 million Mg/yr 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), antiviral (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, potentially resulting in higher nutritive value of the forage and subsequent increase of animal performance.

The first objective of this study was to screen a set of technical lignins for their antifungal properties against 4 fungi isolated from spoiled alfalfa (*Medicago sativa* L.) hay (experiment 1). The second objective was to determine the MIC and minimum fungicidal concentration (**MFC**) 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 ground high-moisture alfalfa hay as measured by DM losses, microbial counts, nutritional composition, ruminal digestibility, and fermentation profile, using an in vitro aerobic incubation assay. We hypothesized that technical lignins can reduce DM losses and preserve the nutritive value of ground high-moisture alfalfa hay in vitro.

MATERIALS AND METHODS

Fungal Isolates

Fungi were isolated from moldy alfalfa hay (*Medicago sativa*, Pioneer 54QR04) harvested from a field in Exeter, Maine. Bales were stored under cover in high-humidity conditions, and moldiness was determined visually. Isolates were extracted as outlined by Müller et al. (2011) and plated on malt extract agar (**MEA**; BD Difco, Franklin Lakes, NJ). Identification to the species level was accomplished by a combination of morphological characters (Malloch, 1981) and DNA sequencing. Molecular identification used the internal transcribed spacer regions (**ITS1** and **ITS2**) of the rRNA genes, the β-tubulin, and the 28S large-subunit ribosomal RNA genes.

Molecular Identification

Fungal isolates were grown for 7 d at 25°C on MEA covered with sterile transparent cellophane (Bio-Rad, Hercules, CA). 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). The DNA was extracted using an E.Z.N.A. fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA) and quality and quantity were evaluated by absorbance spectroscopy at 260 and 280 nm with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA).

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, ITS1 primer 5′-TCCGTAGGTGAACCTGCGG3′ and ITS4 primer 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990); for the 28S rRNA, NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTTCAAGACGG-3′ (O'Donnell, 1993); and for β-tubulin, Bt2a primer 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ and Bt2b primer 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′ (Glass and Donaldson, 1995). The PCR amplifications were conducted in 25-μL reactions using a C1000 Touch Thermal Cycler (Bio-Rad). The reaction contained 0.2 m*M* dNTP (Promega, Madison, WI), 0.2 μ*M* of each primer (Integrated DNA Technologies, Coralville, IA), 0.75 units of One*Taq* DNA polymerase (New England BioLabs, Ipswich, MA), 1 × of One*Taq* Standard Reaction Buffer (New England BioLabs), 10 ng of DNA template adjusted to be in a volume of 5 μL, and DNase-free water to make up 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. Last, reactions with primers Bt2a and Bt2b 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

Table 1. Chemical composition of technical lignins[1]

Lignin	Total soluble phenolics[2]	ORAC[3] (mmol of Trolox equivalents/g of DM)	DPPH scavenging effect[4] (%)	% of DM				
				WSC[5]	Ash[6]	Magnesium[7]	Sodium	Sulfur
AKL	219.1	10.53	−4.8	18.05	19.1	0.02	6.86	4.80
LBKL	222.5	935.0	40.8	0.045	2.95	0.01	0.777	2.20
AIF	241.9	886.6	65.2	0.037	0.62	0.02	0.082	1.49
HEX	265.8	834.9	79.9	0.027	0.45	<0.01	0.009	1.49
PI	382.6	888	69.4	0.025	0.07	<0.01	0.004	1.44
NaL	184.3	12.1	14.2	22.8	33.9	0.05	12.8	8.01
MgL	142.5	10.1	10.5	15.7	13.6	6.21	0.04	8.25
AMOL	132.9	8.79	25.9	24.8	2.16	0.07	0.517	7.93
Pooled SD	9.14	34.08	12.7	0.45	0.27	0.034	0.084	0.142

[1]AKL = alkali kraft lignin (Sigma-Aldrich, St. Louis, MO); LBKL = southern pine softwood kraft lignin; delignified at an H factor of about 1,600 via the kraft process and precipitated using the Lignoboost process with CO_2 as the acid (Tomani, 2010); AIF = LBKL acetone insoluble fraction; HEX = LBKL acetone soluble/hexane soluble fraction; PI = LBKL acetone soluble/hexane insoluble fraction; NaL = sodium lignosulfonate (Sappi North America, Skowhegan, ME); MgL = magnesium lignosulfonate lignin (Sappi North America); and AMOL = ammonium lignosulfonate lignin. The LBKL was donated by D. S. Argyropoulos (North Carolina State University, Raleigh) and LBKL fractions were prepared following the protocol outlined by Cui et al. (2014).

[2]Singleton and Rossi (1965).

[3]Hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC). LBKL, AIF, HEX, and PI were tested by lipophilic ORAC, and AKL, NaL, MgL, and AMOL were tested by hydrophilic ORAC (Dong et al., 2011).

[4]Wu et al. (2006) and method 2012.04 (AOAC International, 2012). DPPH = 2,2-diphenyl-1-picrylhydrazyl.

[5]WSC = water-soluble carbohydrates; DuBois et al. (1956).

[6]FAO (2008).

[7]Beliciu et al. (2012).

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.

The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in the University of Maine DNA Sequencing laboratory (Sanger et al., 1977). Sequence data were edited, assembled, and aligned using the CAP3 sequence assembly program (Huang and Madan, 1999) to obtain high-quality consensus sequences. Consensus sequence homologies were compared with those referenced in the National Center for Biotechnology Information database using the BLASTN search with default parameters (Altschul et al., 1990). *Aspergillus amoenus*, *Mucor circinelloides*, *Penicillium solitum*, and *Debaryomyces 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. Table 1 summarizes the set of technical lignins evaluated in this study. We also included in our evaluation propionic acid [positive control (**PRP**); 99.8%, MP Biomedicals, Solon, OH] and a control (untreated). Ash (FAO, 2008), water-soluble carbohydrates (**WSC**; DuBois et al., 1956), minerals (Beliciu et al., 2012), and total soluble phenolics concentrations, oxygen radical absorbance capacity (Dong et al., 2011), and 2,2-diphenyl-1-picrylhydrazyl antioxidant activity (Wu et al., 2006) of the lignins are listed in Table 1.

Antifungal Assay. The antifungal activity of additives (**ADV**) against the isolated fungi was determined using the poisoned food technique according to the method outlined by Balouiri et al. (2016). The assay was done in duplicate in each of 4 runs for each fungus. A randomized complete block design (**RCBD**) with a 10 (ADV) × 3 (molds) factorial arrangement of treatments and 4 blocks (runs) was used to analyze ADV effects on molds. A RCBD also was used to test the effect of the 10 ADV on yeast (*D. hansenii*).

Medium Preparation. Sterile MEA was mixed with each ADV as follows. Solutions of lignin and PRP were prepared in sterile nanopure water (20 and 32% wt/vol, respectively) in 50-mL polypropylene tubes. Dimethyl sulfoxide (**DMSO**; Fisher Scientific, Pittsburgh, PA) was added at 8% (vol/vol) to all treatments because there was a need to increase the solubility of kraft lignins. Solutions were sonicated for 60 min in an 8510 Series Ultrasonic Cleaning Bath (Emerson, St. Louis, MO) containing water at 40°C to ensure microbial inactivation with minimal effect on the lignin chemical structure (Piyasena et al., 2003). Subsequently, enough ADV stock solution was added to sterilized agar (40°C) under stirring to achieve a final concentration of 40 mg/

mL ADV and 1% DMSO (vol/vol; for all ADV), and the agar concentration recommended by the manufacturer. 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 medium pH to 4 for all treatments, including the control.

Molds Antifungal Assay. After 14 (*A. amoenus* and *P. solitum*) or 3 d of incubation (*M. circinelloides*), the border of single fungal colonies was 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, 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 following 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 Antifungal Assay. The antifungal activity of ADV against *D. hansenii* was determined using the method outlined by Li et al. (2016) with modifications. The ADV-containing and control plates of MEA were inoculated with 100 μL of yeast inoculum containing approximately 1×10^3 cfu/mL, which was spread around the plate. Plates were incubated at 25 ± 1°C for 72 h before yeast colonies were enumerated. The antifungal effect was estimated with the following formula: antifungal activity (%) = [(Cc − Cs)/Cc] × 100, where Cc is the number of cfu on the 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 MIC and MFC were determined for sodium lignosulfonate (**NaL**), magnesium lignosulfonate (**MgL**), and alkali kraft lignin (**AKL**), which were the most promising technical lignins, and PRP (positive control) for each of the fungal isolates previously evaluated. Macrodilution assays were carried out independently 3 times in duplicate and values are reported as mean concentrations (mg/mL ± SD).

Antifungal Assay for Molds. After 14 (*A. amoenus* and *P. solitum*) or 3 d of incubation (*M. circinelloides*), fungal spores were washed from the surface of MEA by adding sterile 0.05% (vol/vol) Tween 20 (Fisher Scientific, Pittsburgh, PA), and then the surface was gently rubbed 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 hemocytometer chamber, diluted, and dispensed to obtain a final concentration of 5×10^4 conidia/mL in the treatment medium (Rex et al., 2008).

Antifungal Assay for Yeast. *Debaryomyces hansenii* was grown on MEA for 72 h. The inoculum was prepared by picking 5 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 (CLSI, 2002). The suspension was adjusted with a V-1200 spectrophotometer (VWR, Radnor, PA) to an optical density at 600 nm of 0.2, diluted, and dispensed to yield a final concentration of 1×10^4 cfu/mL in the treatment medium.

Assay. The MIC was defined as the lowest concentration of ADV that prevented visible growth when compared with untreated controls. The macrodilution testing was performed according to CLSI (2002). Lignins and PRP stock solutions were prepared in sterile malt extract broth (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 malt extract broth were dispensed into 50-mL Erlenmeyer flasks to produce final volumes of 5 mL of medium. To achieve a final pH of 4 or 6 in the medium, HCl or NaOH was added, respectively. Flasks were inoculated with each fungal 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. Plates were incubated at 25°C for 48 h to determine if there were still live cells and therefore 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 8, 2018, 5 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), allowed to wilt in the field for 5 d to an 80% DM concentration, and

then safely stored in a room protected from rain and soil moisture. Afterward, 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 (94% DM), and ground to pass through a 3-mm screen of a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) for later use in the antifungal activity assay.

The effects of 2 dose-optimized ADV (NaL and MgL), selected due to their antifungal activity in aforementioned experiments, and PRP (positive control) on the spoilage of ground high-moisture alfalfa hay were evaluated in vitro using a RCBD with a 3 (ADV: NaL, MgL, and PRP) × 4 (dose: 0, 0.5, 1, and 3% wt/wt, fresh basis) factorial arrangement of treatments and 5 blocks (alfalfa stand plots).

Antifungal Activity. The antifungal activity of ADV on ground high-moisture alfalfa hay (30% moisture concentration) was evaluated using an in vitro aerobic incubation according to the jar method outlined by Lacey and Lord (1977) with modifications to reach target moisture, pH, and inoculation levels as shown in Figure 1. An incubation period of 15 d was chosen because undesirable heating of hay caused by aerobic spoilage occurs during the first 2 to 5 wk of storage (Collins and Coblentz, 2007).

Sampling Procedure. 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 60°C until constant weight in a forced-air oven. Dried samples were ground to pass a 2-mm screen using a Foss Cyclotec mill (Foss, Hillerød, Denmark). Ground samples were analyzed for ash (FAO, 2008). Concentrations of NDF (Van Soest et al., 1991) and ADF (AOAC International, 2000) were measured sequentially using an Ankom 200 Fiber Analyzer (Ankom, Macedon, NY). Heat-stable α-amylase was used for the NDF assay, but sodium sulfite was not used. Hemicellulose 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 400°C 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 (Thermo Fisher Scientific). 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 (NH_3-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). Carbon dioxide emissions due to hay spoilage (g of CO_2/kg of hay DM) were estimated using the method proposed by Magan and Aldred (2007) considering that for every 1% loss of DM, 14.7 g of CO_2/kg of substrate DM will be produced.

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 medium (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. After the 15-d incubation period, all treatments residual alfalfa were evaluated with a 24-h in vitro ruminal digestibility assay (Hall, 2015). 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 timothy grass silage (*Phleum pretense* L.; 6 kg), corn silage (*Zea mays* L.; 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 of the University of Maine. Ruminal fluid was filtered through 2 layers of cheesecloth and flushed with CO_2, and 26 mL of medium containing rumen fluid inoculum and Goering and Van Soest (1970) medium were added to each tube and the suspension was incubated for 24 h at 39°C. The fermentations were terminated by placing tubes at 5°C. Tubes were centrifuged at 900 × *g* for 20 min at 4°C and filtered through pre-weighed F57 Ankom bags (Ankom). 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 (−30°C) 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

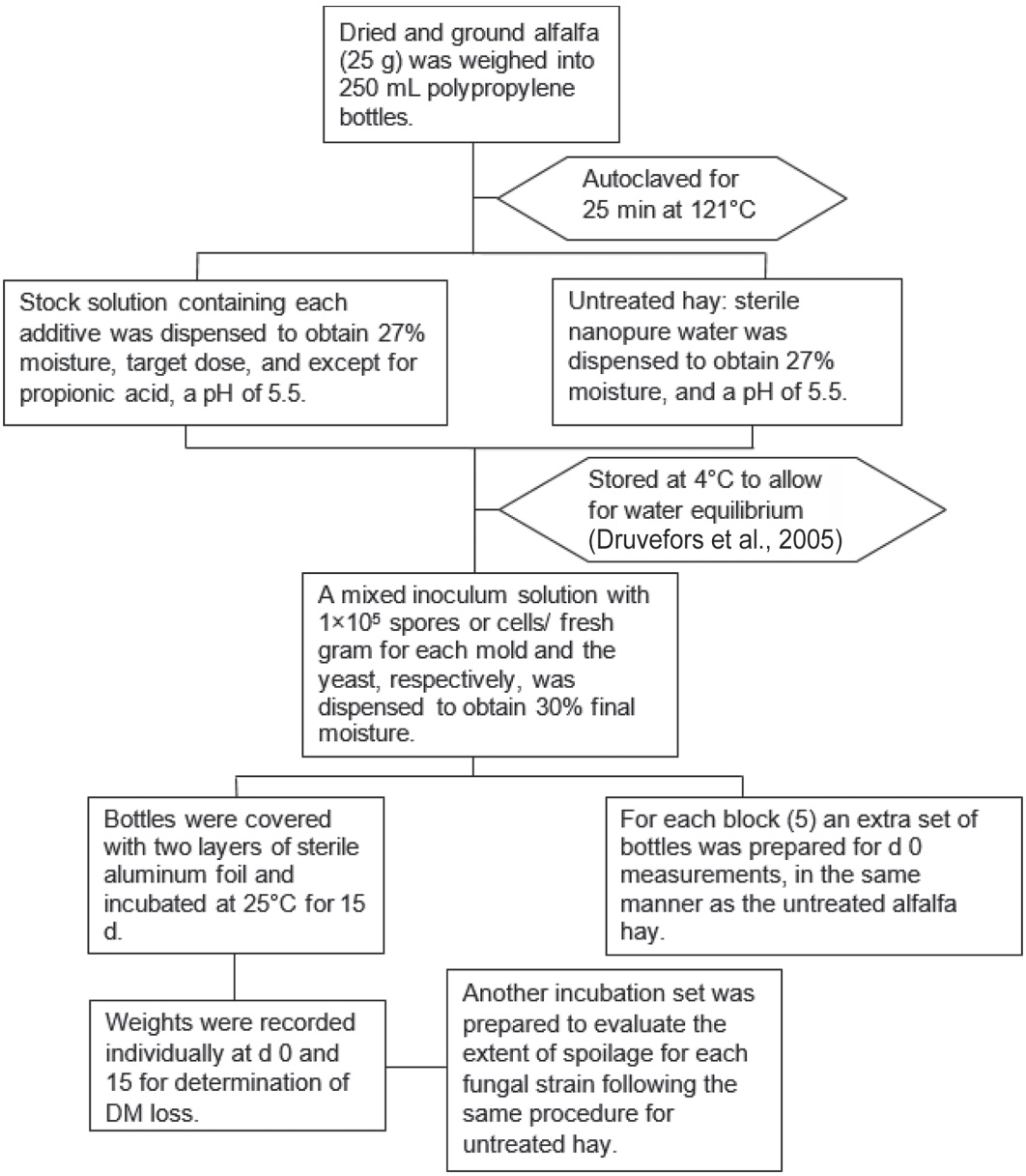

Figure 1. Flowchart for the in vitro aerobic evaluation method of hay spoilage.

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 dry matter digestibility (**DMD**) and neutral detergent fiber digestibility (**NDFD**) were calculated from the residue and original sample weights and their DM and NDF concentrations. Digestible DM recovery (%) was determined by multiplying DM recovery (%; final hay weight/initial hay weight on a DM basis × 100) by true DMD (%).

Statistical Analyses

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

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

where μ = the general mean, $MOLD_i$ = the effect of mold i, ADV_j = the effect of additive j, β_k = the effect of run k, $MOLDADV_{ij}$ = the effect of the MOLD i × ADV j interaction, and E_{ijk} = the 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 3 times in duplicate and values are reported as mean concentrations (mg/mL ± 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

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

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

The GLM procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC) was used to analyze the data. When an interaction was present the SLICE option was used. 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 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, followed by MgL (Table 2). For NaL, PRP, and LBKL acetone soluble/hexane soluble fraction (**HEX**), no significant differences were observed 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; $P \leq 0.05$). Under the conditions of this test *P. solitum* was the most sensitive mold, followed by *A. amoenus*, and *M. circinelloides*. For *D. hansenii*, we identified PRP, NaL, and MgL as the most effective treatments (Table 3).

Experiment 2

Table 4 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 (Table 4). 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

Table 2. Antifungal activity (%) of additives (ADV) as a function of spoiled forage isolated molds (mold) and ADV[1]

	Antifungal activity[2]					*P*-value		
ADV	*A. amoenus*	*M. circinelloides*	*P. solitum*	Mean	SEM	Mold	ADV	Mold × ADV
AKL	$12.1^{C,b}$	$-8.0^{D,c}$	$49.7^{C,a}$	17.9^{C}	2.77	<0.001	<0.001	<0.001
LBKL	$4.9^{D,a}$	$-3.0^{D,b}$	$-17.4^{G,c}$	-5.2^{F}				
AIF	$-2.6^{DE,b}$	$-3.8^{D,b}$	$9.1^{E,a}$	1.1^{E}				
HEX	-7.1^{E}	-4.0^{D}	1.5^{F}	-3.2^{F}				
PI	$4.8^{D,b}$	$8.1^{C,b}$	$23.2^{D,a}$	12.1^{D}				
NaL	100^{A}	100^{A}	100^{A}	100^{A}				
MgL	$40.9^{B,b}$	$28.1^{B,c}$	$72.9^{B,a}$	47.3^{B}				
AMOL	$13.2^{C,b}$	$-8.5^{D,c}$	$50.6^{C,a}$	18.4^{C}				
PRP	100^{A}	100^{A}	100^{A}	100^{A}				
Mean	29.7^{b}	23.2^{c}	43.3^{a}					

[A–G,a–c]Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]AKL = alkali kraft lignin; LBKL = southern pine softwood kraft lignin; AIF = LBKL acetone insoluble fraction; HEX = LBKL acetone soluble/hexane soluble fraction; PI = LBKL acetone soluble/hexane insoluble fraction; NaL = sodium lignosulfonate; MgL = magnesium lignosulfonate lignin; AMOL = ammonium lignosulfonate lignin; and PRP = positive control (propionic acid).

[2]*Aspergillus amoenus*, *Mucor circinelloides*, and *Penicillium solitum*. Antifungal activity (%) is estimated by the formula [(diameter of growth in control plate − diameter of growth in plate containing tested additive)/diameter of growth in control plate] × 100.

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 with NaL (26.7 vs. 40, respectively) but both had less inhibitory activity relative to PRP (1.25 mg/mL). No MIC was observed at either pH for AKL.

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

Experiment 3

DM Losses and Microbial Populations. Effects of treatments on DM loss, CO_2 emissions, hay pH, and microbial counts of alfalfa hay at d 15 are shown in Table 5. We found an interaction effect of ADV × dose on all of the above variables ($P < 0.001$), except *D. hansenii* counts ($P = 0.1$). For DM loss (Figure 2),

Table 3. Antifungal activity (%) of additives (ADV) on *Debaryomyces hansenii*[1,2]

ADV	Antifungal activity (%)
AKL	9.8[bc]
LBKL	8.4[bc]
AIF	−2.2[c]
HEX	1.4[c]
PI	10.6[b]
NaL	100[a]
MgL	100[a]
AMOL	9.9[bc]
PRP	100[a]
SEM	3.0
P-value	<0.001

[a–c]Means with different lowercase letters within a column are different, $P \leq 0.05$.

[1]AKL = alkali kraft lignin; LBKL = southern pine softwood kraft lignin; AIF = LBKL acetone insoluble fraction; HEX = LBKL acetone soluble/hexane soluble fraction; PI = LBKL acetone soluble/hexane insoluble fraction; NaL = sodium lignosulfonate; MgL = magnesium lignosulfonate lignin; AMOL = ammonium lignosulfonate lignin; and PRP = positive control (propionic acid).

[2]Antifungal activity (%) is estimated by the formula [(colony counts in control plate − colony counts in plate containing tested additive)/colony counts in control plate] × 100.

Table 4. Minimal inhibitory concentration (mg/mL) and minimum fungicidal concentration (MFC, mg/mL) of additives (ADV) against fungi isolated from spoiled hay as a function of medium 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	NC[3]	>60	NC	>60	NC	>60	NC
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	NC	>60	NC	>60	NC	>60	NC
AKL	4	>60	NC	>60	NC	>60	NC	>60	NC
	6	>60	NC	>60	NC	>60	NC	>60	NC
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 lignosulfonate; MgL = magnesium lignosulfonate; AKL = alkali kraft lignin; PRP = propionic acid (positive control).

[2]Mean ± SD.

[3]NC = not calculated.

relative to untreated hay (14.9), 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 ± 0.773%; $P < 0.001$). At a dose of 0.5%, PRP reduced DM losses to a greater extent compared with NaL and MgL, which were not different. However, at 1% both NaL and PRP had similar results, and at 3% all ADV were similar. A decrease in CO_2 emissions relative to untreated hay was observed for NaL at 1%, MgL at 3%, and PRP at 0.5% ($P < 0.001$) and above (Table 5).

Propionic acid had a higher antifungal effect (1.96) against *D. hansenii* compared with NaL and MgL, which were similar (3.54 and 4.67 ± 0.914 log cfu/fresh g, respectively; $P < 0.001$). Across all ADV, a 0.5% dose decreased *D. hansenii* counts to the greatest extent versus untreated hay ($P < 0.001$). Total mold counts were reduced by 3% NaL and 0.5% PRP, relative to untreated hay ($P < 0.001$). At both 0.5% and 1%, PRP decreased total mold counts further compared with both NaL and MgL, which showed no decrease; at 3% all ADV were similar.

Nutritional Composition. We found an interaction between ADV × dose on all nutritive value estimates at d 15 ($P < 0.001$) except for CP, ADF, and hemicellulose ($P > 0.07$; Table 6). The untreated hay DM (62.4) was lower than the values obtained for at least 1% NaL (68.7), 3% MgL (69.1), and 0.5% PRP (69.2 ± 0.491%; $P < 0.001$), which were numerically similar to the original value observed at d 0 (69.3%, Table 7). At a dose of 0.5%, PRP-treated hay had a higher DM % than 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, ADV and dose had no effects. However, a decrease in hay NH_3-N was observed for NaL and PRP at 0.5% (0.071 and 0.061, respectively) and above, and for MgL at 3% (0.062), compared with untreated hay (0.249 ± 0.007% DM; $P < 0.001$). When comparing ADV within doses tested, at 0.5 and 1% both NaL and PRP resulted in a lower NH_3-N compared with MgL, whereas at 3% PRP decreased NH_3-N to a greater extent than MgL. At 3%, NaL NH_3-N values were not different from either PRP or MgL. Compared with WSC in untreated hay, the doses preserving WSC to the greatest extent were 3% for NaL and MgL and 0.5% for PRP ($P < 0.001$). At 0.5 and 1%, PRP preserved WSC to greater extent compared with NaL and MgL, and at 3% all ADV were comparable (Figure 3). Therefore, NaL at 3% and PRP at a dose as low as 0.5% impeded an increase in NDF concentration relative to untreated hay ($P = 0.001$). At 0.5 and 1%, a lower NDF was observed for PRP-treated hay compared with NaL and MgL. However, at 3% PRP resulted in a lower NDF compared with MgL, but NaL was similar to both PRP and MgL.

In Vitro Ruminal Digestibility. We found an interaction effect of ADV × dose on all ruminal in vitro fermentation measures ($P < 0.001$; Table 8), except for ruminal pH and isovalerate concentration. An increased true DMD was observed for 3% NaL (67.5), 1% MgL (67.0), and 0.5% PRP (68.5) versus untreated hay (61.8 ± 0.771%). At a dose of 0.5%, MgL and PRP increased DMD to the same level, which was higher than that of NaL; at 1%, PRP resulted in higher DMD than NaL, but MgL was similar to both PRP and NaL; and at 3% all ADV were comparable. Magnesium lignosulfonate and PRP at a dose as low as 0.5% (56.4 and 68.0, respectively) and NaL at 1% (63.3) had increased digestible DM recovery compared with untreated hay (52.6 ± 0.747%; $P < 0.001$). Across ADV, at 0.5 and 1%, PRP improved digestible DM recovery to a greater extent compared with both lignosulfonates, but at 3% all ADV were similar. Similarly, in the case of NDFD (Figure 4), MgL and PRP at 0.5% (30.5 and 30.1, respectively) and NaL at 1% (30.7) increased NDFD compared with untreated hay (23.3 ± 1.09%; $P < 0.003$). At 0.5%, MgL increased NDFD to a larger extent compared with 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 versus untreated hay ($P = 0.009$). In contrast, NaL at 3% increased total VFA (**TVFA**) concentration to the greatest extent relative to untreated hay ($P < 0.001$). However, MgL decreased TVFA at 0.5% ($P = 0.01$), whereas other MgL doses were similar to untreated. No dose of PRP was different than untreated. At 0.5, 1, and 3%, NaL increased TVFA to a greater extent than PRP or MgL. At 0.5%, VFA for PRP was higher than MgL, and both were comparable at 1 and 3%.

DISCUSSION

Experiments 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 variation in the types 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

Table 5. The CO_2 emissions, pH, and microbial counts of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation[1]

	Dose (%, wt/wt)						*P*-value			
Item	0	0.5	1	3	Mean	SEM	ADV	Dose	ADV × dose	Contrast[2]
CO_2 emissions (g of CO_2/kg of hay DM)										
MgL	219.0^{a}	202.0^{A,ab}	153.7^{A,b}	5.41^{c}	145.0^{A}	11.4	<0.001	<0.001	<0.001	L**
NaL	219.0^{a}	223.8^{A,a}	49.9^{B,b}	6.33^{b}	124.7^{B}					CU**
PRP	219.0^{a}	11.9^{B,b}	13.8^{B,b}	6.92^{b}	62.9^{C}					CU**
Mean	219.0^{a}	145.9^{b}	72.5^{c}	6.22^{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}						
Debaryomyces 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}						
Mold (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}						
Aspergillus 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^{B,b}	4.74^{C}					CU**
Mean	7.64^{a}	6.19^{b}	5.99^{b}	4.71^{c}						
Mucor 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}						
Penicillium 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}						

$^{A–C,a–d}$Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; and PRP = propionic acid.

[2]Linear (L), quadratic (QU), and cubic (CU) effect ($P < 0.05$).

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

NaL (Sigma-Aldrich Corp., St. Louis, MO) of 50, 62, 62, and 80 μg/mL for the yeasts *Candida dubliniensis*, *Candida tropicalis*, *Candida albicans*, *Candida glabrata*, and *Candida 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; 7,085 Da) 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 *Aspergillus niger*. This discrepancy with our study may be due to the different *Penicillium* and *Aspergillus* species tested, as well as the NaL sources and doses used across studies. Furthermore, these studies did not report medium pH values. In our study, we observed that medium 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 m*M* had antifungal activity against the yeast *Saccharomyces 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 m*M* and a medium pH of 6.0.

Although the antimicrobial mechanism of lignosulfonates has not yet been 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). Anionic surfactants synthetized from alkyl ester of phosphoric acid and polyethylene glycol-400 had inhibitory activities of 23 to 26 mm/mg and 18 to 25 mm/mg for *A. niger* and *C. albicans*, respectively, using an inhibition zone diameter test (Negm and Tawfik, 2014). Similarly, Tawfik et al. (2015) reported that synthetized anionic surfactants at doses ranging from 125 to 8,000 ppm decreased the mycelial growth of 8 phytopathogenic fungi from the genera *Colletotrichum*, *Fusarium*, *Humicola*, *Pestalotia*, *Phoma*, and *Phytophthora*, using the poison food technique, by disrupting the native membrane-associated function of integral

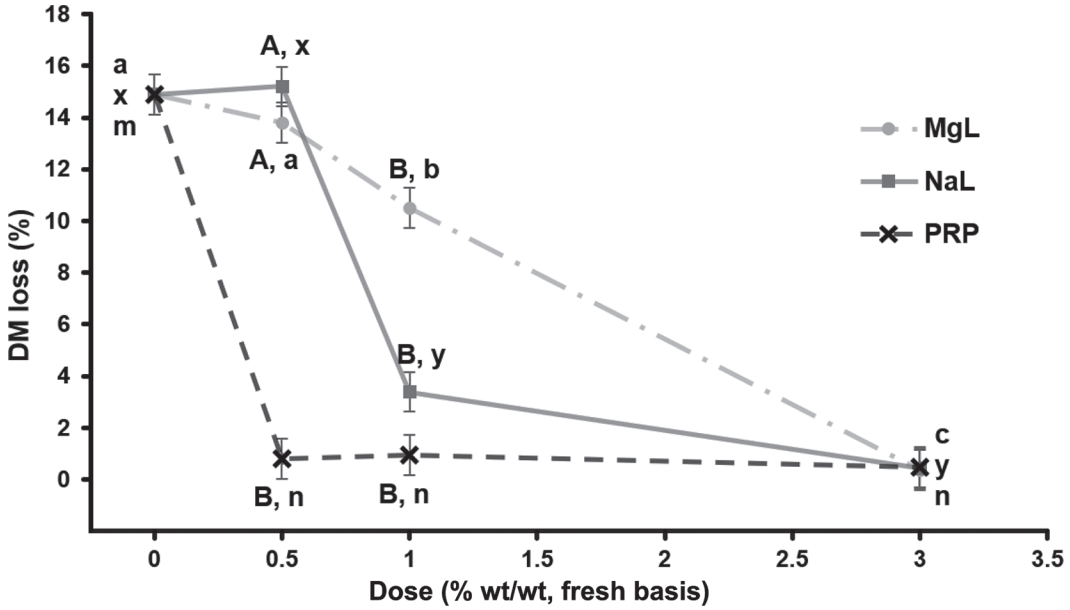

Figure 2. Dry matter loss (%) of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation. MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; PRP = propionic acid. Bars represent means ± SEM. Uppercase letters depict differences across ADV within dose ($P \leq 0.05$) and lowercase letters depict differences across dose within ADV (MgL: a, b, c, NaL: x, y, and PRP: m, n; $P \leq 0.05$).

Table 6. Nutritional composition of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation[1]

	Dose (%, wt/wt)						*P*-value			
Item	0	0.5	1	3	Mean	SEM	ADV	Dose	ADV × dose	Contrast[2]
DM (%)										
MgL	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	62.4^{b}	62.5^{A,b}	68.7^{A,a}	69.5^{a}	65.8^{B}					CU**
PRP	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}						
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}	45.1^{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						

$^{A–C,a–d}$Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; and PRP = propionic acid.

[2]Linear (L), quadratic (QU), and cubic (CU) effect ($P < 0.05$).

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

proteins. Lignosulfonates are also anionic surfactants, but their mechanism of action against microbes needs to be 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 AKL (Sigma-Aldrich Corp.) against *Candida lipolytica* (yeast) and *Staphylococcus aureus* (bacteria). However, no antibacterial activity was reported against *Listeria monocytogenes*. The lignin tested in that study had an antioxidant activity of 3,517 μmol Trolox equivalents/g and 165.5 mg/g total soluble phenolics. We believe that the absence of activity against yeast for AKL in our study compared with 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 1).

In the current study, PRP had fungistatic and fungicidal activity against fungi isolated from spoiled hay at much lower doses compared with 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 concentration of undissociated acid declines as pH increases, PRP is more effective at a lower pH (Lück and Jager, 1997). The lowest doses that were selected for further testing in experiment 3 (0.5–1%, wt/wt; fresh basis) corresponded to the actual concentrations typically used in the field for propionic acid (~0.67–1.34%, wt/wt; fresh basis) when calculated from the application rate of commercially available propionic acid-based preservatives (1–2%, wt/wt; fresh basis; Rotz, 2003)

Table 7. Microbial counts, nutritional composition, and 24-h in vitro digestibility and rumen fermentation measurements of untreated ground alfalfa hay at d 0

Item	Value (mean ± SD)
Microbial count (log cfu/fresh g)	
Total mold counts	5.4 ± 0.1
Debaryomyces 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 (% of DM)	92.4 ± 0.6
NDF (% of DM)	47.8 ± 1.2
ADF (% of DM)	34.8 ± 1.5
CP (% of DM)	16.7 ± 0.81
Hay NH_3-N (% of DM)	0.065 ± 0.005
Water-soluble carbohydrates (% of DM)	11.1 ± 0.59
In vitro digestibility and rumen fermentation measurement	
24-h in vitro DM digestibility (%)	66.2 ± 1.5
24-h NDF digestibility (% of DM)	30.0 ± 1.2
Total VFA (m*M*)	96.8 ± 0.9
Acetate (m*M*)	52.7 ± 0.6
Propionate (m*M*)	22.5 ± 0.4
Butyrate (m*M*)	12.5 ± 0.4
Isobutyrate (m*M*)	1.5 ± 0.2
Isovalerate (m*M*)	3.13 ± 0.3
Valerate (m*M*)	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

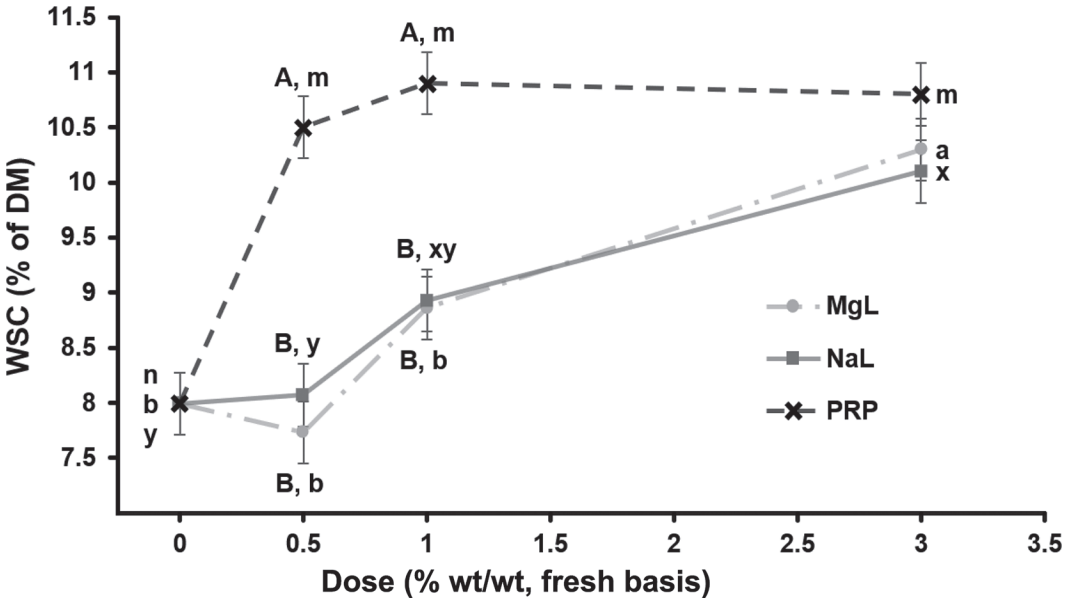

Figure 3. Water-soluble carbohydrates (WSC; % of DM) of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation. MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; PRP = propionic acid. Bars represent means ± SEM. Uppercase letters depict differences across ADV within dose ($P \leq 0.05$) and lowercase letters depict differences across dose within ADV (MgL: a, b, NaL: x, y, and PRP: m, n; $P \leq 0.05$).

and the typical concentration of propionic acid in those commercial products (~67%, vol/vol; 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 30% (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 and increasing pH (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 and Bertram, 2012), which affects 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 VFA being produced during ruminal fermentation (Mohanty et al., 1969). Furthermore, a significant decrease is present in digestible energy from the recovered DM (Atwal et al., 1984; Russell et al., 1990). This was evident when the nutritional values of untreated hay at d 15 (Table 6) are compared with the ones obtained from untreated hay at d 0 (Table 7). The overall nutritional value is severely compromised along with the potential voluntary intake, if we were to consider the NDF percent increase in the spoiled alfalfa hay (Mertens, 1977). The decrease in both nutritional value and voluntary intake can explain the decrease in animal performance that has been reported in spoiled versus 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 experiments 1 and 2. To the best of our knowledge, no studies have measured CO_2 emissions during hay storage. A relationship between DM losses and CO_2 production has been described for grain crops (Magan and Aldred, 2007). Approximately 14.7 g of CO_2 per kg of grain is released for every 1% loss of grain DM during aerobic spoilage. If we were to apply this relationship to hay, a typical 800-kg (DM basis) round bale with a 14.9% DM loss during storage could release 175 kg of CO_2. In this study, we observed that NaL and PRP at 1% decreased theoretical CO_2 emissions to the same level. Consequently, if the same 800-kg DM bale would be treated with 1% NaL, that could represent a potential reduction in CO_2 emissions from 175 to 40 kg of CO_2 (77% decrease). More research needs to be

Table 8. The 24-h in vitro DM digestibility (DMD), digestible DM recovery, and rumen fermentation measurements of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation[1]

	Dose (%, wt/wt)						*P*-value			
Item[2]	0	0.5	1	3	Mean	SEM	ADV	Dose	ADV × dose	Contrast[3]
DMD (%)										
MgL	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	61.8^{bc}	61.1^{B,c}	65.5^{B,ab}	67.5^{a}	64.0^{B}					CU**
PRP	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}						
Digestible DM recovery (%)										
MgL	52.6^{c}	56.4^{B,b}	60.0^{B,b}	66.4^{a}	58.8^{B}	0.747	<0.001	<0.001	<0.001	QU*
NaL	52.6^{c}	51.8^{C,c}	63.3^{B,b}	67.2^{a}	58.7^{B}					CU**
PRP	52.6^{b}	68.0^{A,a}	68.7^{A,a}	67.9^{a}	64.3^{A}					CU**
Mean	52.6^{c}	58.7^{b}	64.0^{a}	67.2^{a}						
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}						
Total VFA (m*M*)										
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}						
Acetate (m*M*)										
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 (m*M*)										
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										
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 (m*M*)										
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										
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}						
Isobutyrate (m*M*)										
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 (m*M*)										
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}						

Continued

Table 8 (Continued). The 24-h in vitro DM digestibility (DMD), digestible DM recovery, and rumen fermentation measurements of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation[1]

	Dose (%, wt/wt)						*P*-value			
Item[2]	0	0.5	1	3	Mean	SEM	ADV	Dose	ADV × dose	Contrast[3]
Valerate (m*M*)										
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–C,a–c]Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; and PRP = propionic acid.

[2]A = acetic acid; P = propionic acid; B = butyric acid.

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

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

conducted to assess the effects of hay spoilage on greenhouse gases emissions.

Mold and yeast counts were observed to decrease to different extents as doses of different ADV were increased. 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 hypothesize that this apparent discrepancy between DM loss and total mold counts 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 hyphal growth.

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

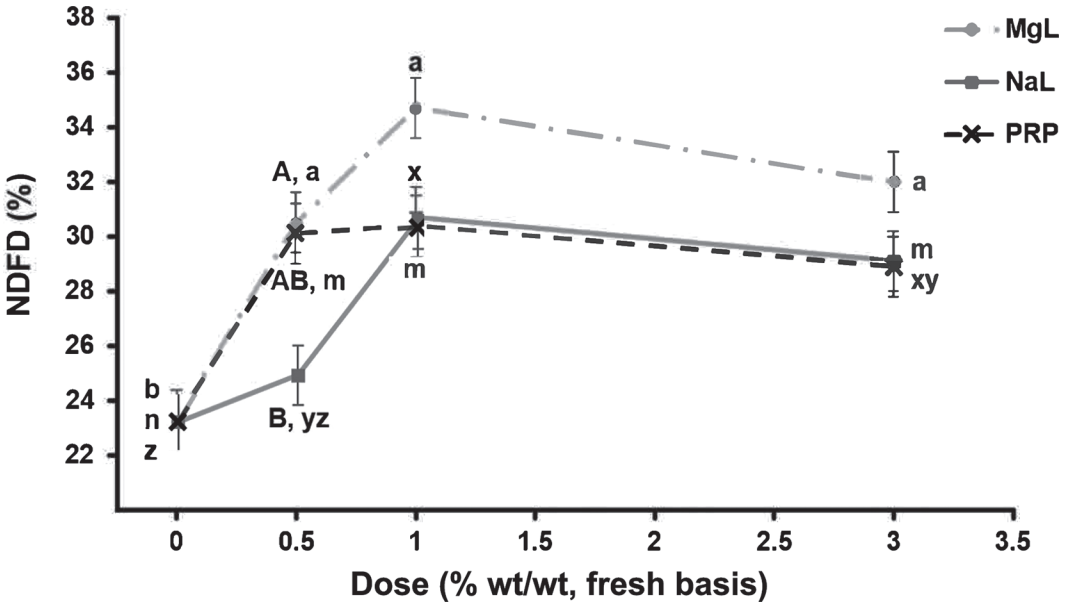

Figure 4. The 24-h in vitro NDF digestibility (NDFD; %) of ground alfalfa hay as a function of additive (ADV) and dose after a 15-d in vitro aerobic incubation. MgL = magnesium lignosulfonate; NaL = sodium lignosulfonate; PRP = propionic acid. Bars represent means ± SEM. Uppercase letters depict differences across ADV within dose ($P \leq 0.05$) and lowercase letters depict differences across dose within ADV (MgL: a, b, NaL: x, y, z, and PRP: m, n; $P \leq 0.05$).

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 hay 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 and preserve 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 mo, losses of 0.25% DM of CP per month were observed due to ammonia volatilization (Rotz and Muck, 1994). The incubations of hay with fungi in the current study only lasted for 15 d. Other important protein quality changes result from the formation of insoluble N components thanks to the Maillard reaction, measured as ADIN (Guerrero and Shenvood, 1997). These compounds are essentially indigestible in ruminants (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 limit the formation of ADIN as a temperature >50°C is required for the Maillard reaction to occur (Guerrero and Shenvood, 1997).

Sodium lignosulfonate at 3% prevented 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. Despite not having an effect on the preservation of most nutrient constituents compared with 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 hay 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 at all doses. We hypothesize that the surfactant properties of MgL explain 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). It also is unlikely that a Mg deficiency may explain the increase in in vitro digestibility because the Van Soest medium is supplemented with this micromineral (Goering and Van Soest, 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 may be explained by the decrease in NDF concentration and increase in NDFD (Mertens, 2003). However, stimulatory effects of MgL on DMD and NDFD remain unclear due to its null effect 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 and a 0.17 kg/d increase in DMI (Oba and Allen, 1999). Therefore, feeding hay treated with 1% MgL could potentially increase milk production by 2.9 and DMI by 1.9 kg/d, respectively, relative to spoiled alfalfa hay. 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 hay at d 15 did not result in a TVFA increase. We hypothesize 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 m*M*, respectively). Volatile fatty acids contribute 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 lower butyrate concentrations than those of NaL.

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) to meet their AA 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) because they seem to bind and precipitate proteins, as observed in other applications (Cerbulis, 1978; Becker and Lebo, 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 tested in preventing spoilage in ground high-moisture alfalfa hay in vitro. We observed that the antifungal properties of lignosulfonates were underestimated when evaluated using an artificial medium versus a ground hay substrate (~5 fold difference). This was most likely due to the limited availability of nutrients and moisture in the ground hay. However, before its implementation in the field, NaL antifungal activity needs to be increased further to match PRP effects. Because the cost of lignosulfonates is around $600/Mg and PRP-based products are $5,000/Mg, the economic margin is enough to cover the costs 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 (the cost estimates provided here should be used with caution as they were obtained from industry representatives). Few studies have separated 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 could 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.

CONCLUSIONS

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 in vitro using ground high-moisture 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 to match all the nutritional benefits obtained with PRP hay treatment. Considering the high level of ash and WSC impurities that 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 points out lignosulfonate stimulatory effects on NDFD due to its surfactant properties, but more research needs to be conducted to understand the mechanisms behind its stimulatory effects on ruminal digestibility.

ACKNOWLEDGMENTS

We acknowledge the contributions of D. S. Argyropoulos (North Carolina State University, Raleigh), R. Kersbergen (University of Maine Extension, Orono), D. Gomez, H. M. Dubuc, and D. N. DePippo (University of Maine, Orono), the Fogler family (Stonyvale Farm, ME), and the staff from the J. Franklin Witter Teaching and Research Center and Rogers Farm (University of Maine, Orono). This project was supported by the USDA National Institute of Food and Agriculture, Hatch Project number ME0-21717 and AFRI grant 2019-67016-29447 (Animal Nutrition, Growth and Lactation program) through the Maine Agricultural & For-

est Experiment Station. Maine Agricultural and Forest Experiment Publication Number 3747. The authors have not stated any conflicts of interest.

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