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Effect of Ensiling on Nutritional Properties of Sericea Lespedeza Alone or in Mixtures with Alfalfa

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Abstract: In this investigation, fresh sericea lespedeza (SL; *Lespedeza cuneata*) and alfalfa (*Medicago sativa*) were cut in the field, frozen, chopped and mixed into ratios of 100:0, 75:25, 50:50, 25:75 and 0:100, respectively, with each treatment combination packed into 12 mini-silos and sealed to be air-tight. Three mini-silos per treatment were opened after 1, 7, 21 and 84 d of ensiling and analyzed for pH, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), unbound and bound condensed tannins (CT), nitrogen (N), nitrate N (NO_3-N), and ammonia N (NH_{4+}N) content. All of the forage combinations ensiled well, with a rapid drop in pH (below 5.0 by Day 7). Fiber concentrations (NDF, ADF, ADL) were greater in 75% and 100% SL silages than in 0%, 25% and 50% SL samples by Day 84 of the study, possibly due to interference of CT in the detergent analysis system. Concentrations of N, NO_3-N and NH_{4+}N were decreased in silages as percentage SL in the mixture increased, while unbound, bound and total CT increased as percentage SL increased. In this study, there was reduced proteolysis during ensiling of combinations of SL and alfalfa, as indicated by reduced NO_3-N and NH_{4+}N production as percentage SL in the silage mixtures increased.

Key words: Alfalfa, condensed tannins, ensiling, sericea lespedeza.

Abbreviations

ADF	acid detergent fiber
ADL	acid detergent lignin
CP	crude protein
CT	condensed tannins
DM	dry matter
NDF	neutral detergent fiber
N	nitrogen
NH_{4+}N	ammonia N
NO_3-N	nitrate N

1. Introduction

Fresh forages are important feedstuffs for herbivorous animals as they provide most of the required nutrients, minerals and vitamins needed for good performance and welfare [1]. During rainy parts of the year (spring and fall), forage production usually exceeds animal consumption demands, and commonly, excess forage is baled as hay, which requires hot, sunny weather for rapid drying of plants after cutting to prevent spoilage during storage. Alternatively, green forage can be preserved at a higher moisture level by ensiling if weather is not appropriate for hay-making. The composition of well-ensiled feed remains stable for a long period (up to five years in enclosed silos), and during the fermentation process, ensiling lowers harmful nitrates gathered in plants during droughts and in over-fertilized crops [2].

A challenge with making silage from high-CP legumes, such as alfalfa (*Medicago sativa*), is excessive degradation of protein into non-protein nitrogen (NPN) during the ensiling process [3, 4]. On the other hand, presence of CT affects the ensiling properties of forages, including reducing proteolysis of CP [5-7]. Ensiling can also alter the form of CT in plants from more extractable to less extractable forms [8, 9]. According to a research in New Zealand, fresh birdsfoot trefoil (*Lotus corniculatus*) and sulla

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(*Hedysarum coronarium*) forage had 67% and 88% free CTs, but there were only 11% and 8% free CTs present in the ensiled forages, respectively, with the majority bound to protein or fiber [8].

Sericea lespedeza (SL; *Lespedeza cuneata*) is a perennial, erect, deep-rooted warm-season legume that is rich in CT [10]. It is a low-input forage with the ability to persist for several years under low maintenance. It is particularly resistant to drought and disease and can be grown on infertile, acidic soil with minimal inputs of lime or fertilizer [11]. Since the 1940's, SL has been utilized extensively as a pasture and hay crop, and more recently as leaf meal or feed pellets [12], but this forage has rarely been utilized as silage. Information on effects of ensiling on CT structure and proteolysis of CP in SL are currently non-existent.

There have also been few reports on ensiling of mixtures of non-CT forages with CT-containing forages to reduce degradation of CP in the non-tannin plants. Wang *et al*. [6] found that ensiling mixtures of alfalfa with sainfoin (*Onobrychis viciifolia*) decreased production of soluble N, NPN, and $NH_{4+}N$ compared with ensiled alfalfa alone. Kamalak *et al*. [13] reported the effects of added oak tannin extract (hydrolysable tannins) on composition of alfalfa silage. They reported that there was an increase in DM, but a decrease in production of ammonia, biogenic amine, and acetic acid, as well as lower pH in alfalfa ensiled with oak tannin [13]. However, there have been no reports on the effect of ensiling alfalfa together with SL on CP degradation.

The objectives of this investigation were to evaluate effects of ensiling SL alone or in mixtures with alfalfa on selecting nutrient quality components and concentration of unbound and bound CT.

2. Materials and Methods

2.1 Field Preparation, Planting and Crop Management

The experiments were conducted at the Fort Valley State University (FVSU) Agricultural Research Station, Fort Valley, GA (32°33′ N, 83°53′ W). Two types of leguminous forages were used for this experiment, SL and alfalfa. The SL was harvested from 10 randomly-selected locations within a well-established pasture (10 years old) at the FVSU Research Station, while the alfalfa was harvested in a similar manner from well-established plots (> 5 years) at the University of Georgia Agricultural Research Station in Blairsville, GA. Both forages were cut during July, 2015, placed in large black plastic garbage bags, and frozen until processed for silage making.

2.2 Ensiling Properties of SL, Alfalfa and Mixtures of SL and Alfalfa

In May, 2016, the frozen plant material of each type was chopped to 1-2 cm using a flail-type forage chopper. The chopped alfalfa was spread out in aluminum baking pans in the laboratory and wilted for 2 h to attain a final moisture of 60% to 65% [14], while the chopped SL had a similar moisture level initially and required no wilting. The chopped SL and alfalfa were then hand mixed into ratios of 100:0, 75:25, 50:50, 25:75 and 0:100, respectively, based upon wet weight of forage material. Each of the five treatment forage combinations was packed into laboratory scale (5.08 cm × 20.32 cm) PVC mini-silos (n = 12/treatment; Fig. 1). Each silo tube was filled to the top, with the material packed down with a wooden stick topped by a metal washer slightly smaller than the diameter of the silo tube (Fig. 2a). The silos were sealed with a plastic pipe plug with rubber ring (Fig. 2b) to make them air-tight. The sealed mini-silos were allowed to remain at room temperature for up to 84 d to permit ensiling to occur. Three mini-silos per treatment were opened after 1, 7, 21 and 84 d of ensiling, with the contents of each tube emptied into ziplock plastic bags and placed in a -20 °C freezer for later analysis (n = 3/treatment). Once all the samples were frozen, they were removed from the freezer and

each split into two sections, with one refrozen for later analysis, and the other freeze-dried. Freeze-drying of frozen samples was completed using a FreeZone 1 liter Benchtop Freeze Dry Systems, Model 77400 Series (LabConco Corp., Kansas City, MO, USA).

The fresh-frozen silage samples were thawed and used for determining water activity (a_w), moisture content and pH. Analysis of pH was completed using an Oakton™ pH 2700/PC 2700/lon 2700/CON 2700 Benchtop Multiparameter Meter (Cole-Parmer, Vernon Hills, IL, USA). Analysis of moisture content was completed using a Heratherm Oven (Thermo Electron LED GmbH, Langenselbold, Germany); similarly, analysis of water activity was completed using an AquaLab instrument (Decagon Devices. Inc., Pullman, WA, USA). In addition to the ensiled samples, unensiled samples of pure SL and alfalfa were analyzed for pH and water activity (not included in statistical analyses). Water activity of the unensiled forages was 0.97 and 0.96 a_w and pH values were 5.48 and 6.18, respectively, for 100% SL and 100% alfalfa samples.

All freeze-dried silage samples were ground to 1 mm particle size using a Wiley Mill grinder (Thomas

Fig. 1 Mini-silo used for ensiling sericea lespedeza (SL) alone and in mixtures with alfalfa.

(a)

(b)

Fig. 2 Silo forage packer (a) and plastic pipe plug with rubber ring sealer (b).

Scientific, Swedesboro, NJ, USA) and then analyzed in duplicate for DM, ash, unbound CT, bound CT, N, NDF, ADF and ADL. Analysis of N was completed using an Elementar Vario Macro Cube Carbon/Nitrogen analyzer (Vario Max, Elementar, Elementar Americas, Inc., Mt. Laurel, NJ, USA), with CP calculated as N × 6.25. Fiber analyses (NDF and ADF) were completed using the method of Van Soest *et al*. [15] using an ANKOM$^{200/220}$ Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Amylase and sodium sulfite were added for the NDF extraction, and NDF and ADF procedures were completed by sequential analysis. Analysis of ADL was completed using an ANKOM Daisy Incubator (Method 9, ANKOM Technology, Macedon, NY, USA). Ash was determined using AOAC (1984) protocols (Thermolyne, Type 48000 Furnace, Radnor, PA, USA). All forage quality data are presented on a DM basis. Unensiled samples of pure SL and alfalfa contained 38.5% and 39.3% NDF, 2.3% and 3.1% N, and 64.3 mg/g and 1.7 mg/g unbound CT, 49.3 mg/g bound CT, and 113.6 mg/g and 7.4 mg/g total CT, respectively.

Unbound and bound CT, total CP, NO_3-N, NH_{4+}N,

and protein-precipitable phenolics were completed at a forage quality laboratory at the University of Missouri. Unbound and bound CT were analyzed using the method of Porter *et al*. [16] with samples and standards read using a Beckman Coulter DU730 UV/Vis spectrophotometer (Beckman Coulter Inc, Atlanta, GA, USA). The CT standards utilized in this procedure were purified from ensiled, freeze-dried SL samples using Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) as described by Terrill *et al*. [17].

Plant samples were analyzed for NO_3-N by the cadmium reduction method [18]. Ten grams (10 g) of dried and ground plant material was added to a 50 mL Erlenmeyer flask. Then, 25 mL of 2 M KCl solution was added to the flask using an extracting solution dispenser. The mixture was shaken for 5 min at 180-200 oscillations/min. The suspension was filtered into 30 mL receiving beakers using nitrate-free filter paper to provide a clean filtrate without contributing measurable amounts of NO_3-N to the filtrate. A portion of the filtrate was transferred to 10 mL test tubes for analysis. The NO_3-N content of the filtrated plant extracts was determined by using the nitrate reduction method (Quikchem No. 12-107-04-1-B) through the Lachat Flow Injection Analyzer [19]. NO_3-N was quantified using the following standard curve: a 1,000 ppm NO_3-N in 2 M KCl stock solution was prepared by weighing 1.444 g of potassium nitrate (KNO_3) into a 200 mL volumetric flask with 2 M KCl extracting solution, mixed and then stored in a refrigerator. A 100 ppm NO_3-N working stock solution was then prepared by adding 20 mL of the 1,000 ppm standard stock solution to a 200 mL volumetric flask, brought to volume with 2 M KCl, and mixed by inverting multiple times. The standard curve was then prepared by pipetting specific volumes of 100 ppm working stock solution into the corresponding volumetric flasks and diluting to volume with extracting solution (Table 1).

Plant samples were analyzed for NH_{4+}N by a modification of the phenolate method [18]. Samples were weighed and mixed with 2 M KCl solution, shaken, and filtered as described for the NO_3-N procedure. Ammonium-N content of the filtered extracts was determined using the ammonia phenolate method (Quikchem No. 12-107-06-1-B) through the Lachat Flow Injection Analyzer [19]. Ammonium-N was quantified using the following standard curve: a 1,000 ppm ammonium-N standard stock solution prepared in 2 M KCl was prepared by weighing 3.819 g of ammonium chloride (NH_4Cl) into a 200 mL volumetric flask with 2 M KCl extracting solution, brought to volume and inverted three times to mix. A working stock solution of 100 ppm NH_{4+}N in 2 M KCl and the standard curve for NH_{4+}N was then prepared as described for the NO_3-N procedure.

Protein precipitable phenolics were determined using the method of Hagerman and Butler [20] as modified by Cooper *et al.* [21] using crude 50:50 *v*/*v* methanol:water extracts of plant samples as opposed to purified tannins.

2.3 Statistical Analyses

All silage sample data were analyzed for effects of SL: alfalfa forage ratio, time and the interaction

Table 1 Stock solutions for analysis of NO_3-N in ensiled combinations of sericea lespedeza (SL, 0%, 25%, 50%, 75%, 100%) and alfalfa (100%, 75%, 50%, 25%, 0%).

100 ppm working solution (mL)	Volumetric flask (mL)	Working standard concentration NO_3-N (ppm)
5	500	1
25	500	5
25	250	10
50	250	20

(treatment by time) using mixed model repeated measures analysis for a completely randomized design [22]. The silage sample data were tested for normality and log-transformed prior to statistical analysis if not normally distributed. Data were reported as least squares means, with statistical inferences made using log-transformed data. The lsmeans were considered different at a level of $p \leq 0.05$.

3. Results

3.1 Silage Water Activity and Moisture Content

Water activity (a_w) was significantly affected by silage treatment, with increased ($p < 0.05$) water activity as the percentage SL in the diet increased (Table 2). The values ranged between 0.95 and 0.98 a_w. Overall moisture concentration had similar results, with a significant treatment effect ($p < 0.01$) and increasing moisture in the silages as percentage SL increased (Table 2). Moreover, there were no time or treatment × time interaction effects on water activity or moisture data.

3.2 Silage pH

There was a treatment × time interaction ($p < 0.01$) for pH (Fig. 3). The pH decreased significantly for all treatments over time compared to Day 1 ($p < 0.001$), however, the 100% SL silage was higher ($p < 0.05$) than all other treatments on Day 1. Treatment difference virtually disappeared after Day 1, with only one other on Day 84 in which 75% SL had a slightly higher ($p < 0.05$) pH than 0% SL.

3.3 NDF

There was a treatment by time interaction ($p = 0.0524$) on silage NDF values (Table 3). There was no effect of time for NDF of the 0%, 25% and 50% SL silages, while NDF concentrations on Days 7 and 84 were different ($p < 0.05$) for the 75% SL samples, and on Days 21 and 84 ($p < 0.05$) for the 100% SL samples compared to Day 1 (Table 3). On Day 1, the 100% SL samples had higher ($p < 0.01$) NDF than the 0% and 25% SL silages, while on Day 7, the 75% SL was higher ($p < 0.05$) than the 0% and 25% SL samples. By Day 84, the 75% and 100% SL silages had significantly higher ($p < 0.001$) NDF values than the two lowest SL treatment samples (0% and 25%).

3.4 ADF

There was a significant treatment by time ($p < 0.05$) interaction on ADF values of the silages (Table 4). ADF values did not change over time compared to Day 1 for the 0%, 25% and 50% SL samples, while the 75% and 100% SL silages had higher ($p < 0.01$) ADF concentrations on Day 84 only compared to Day 1 (Table 4). On Day 1, ADF was significantly higher ($p < 0.05$) for the 100% SL silage than that of 0% and 25% SL silages, while the 75% SL silage had greater ($p < 0.05$) ADF than the 25% SL silage on Day 21. By Day 84, both the 75% SL and 100% SL silages had higher ($p < 0.001$) ADF values than the 0%, 25% and 50% SL silages.

3.5 ADL

The treatment by time interaction was significant ($p < 05$) for ADL concentrations of the silages (Table 5). ADL values did not change over time for the 25% and 50% SL samples, while the 0%, 75% and 100% SL silages had higher ($p < 0.05$) ADL concentrations on

Table 2 Moisture content and water activity (a_w) of combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Constituent	Ensiled forage combination (%SL and A)				
	0:100	25:75	50:50	75:25	100:0
Moisture (%)	60.84 ± 0.56^a	60.78 ± 0.59^a	61.50 ± 0.60^a	63.34 ± 0.50^b	64.24 ± 0.50^b
Water activity (a_w)	0.953 ± 0.002^a	0.962 ± 0.002^b	0.969 ± 0.002^c	0.973 ± 0.002^c	0.982 ± 0.002^d

[a, b, c, d]Row means with unlike superscripts differ significantly at $p < 0.05$.

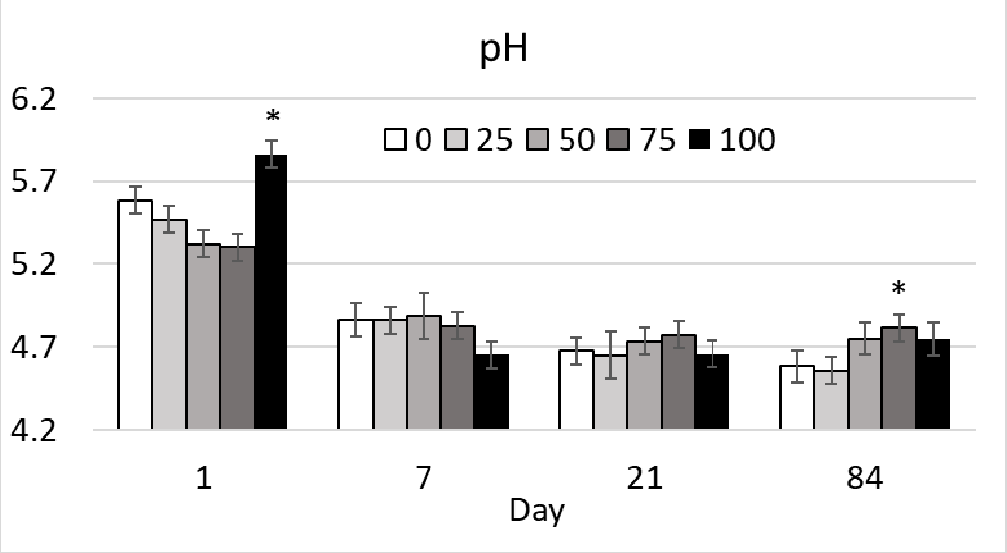

Fig. 3 The pH of mixtures of ensiled SL and alfalfa of 0% to 100% SL (0%, 25%, 50%, 75%, 100%) tested on 1, 7, 21 and 84 d of ensiling (*n* = 3/treatment/day).

There was a treatment × day interaction ($p < 0.003$). Bars with * differ within day among treatments except for Day 84 in which 75% SL was higher than 0% SL only.

Table 3 NDF (%DM) content of combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	39.1 ± 1.2[a]	38.5 ± 1.2[a]	40.3 ± 1.2[ab]	40.9 ± 1.2[ab]	43.9 ± 1.2[b]
7	39.6 ± 1.4[a]	37.8 ± 1.2[a]	41.6 ± 2.0[ab]	44.2 ± 1.2[b]*	41.7 ± 1.2[ab]
21	38.5 ± 1.2[ab]	35.3 ± 2.0[a]	41.4 ± 1.2[ab]	41.9 ± 1.2[b]	40.9 ± 1.2[ab]*
84	40.1 ± 1.4[a]	38.3 ± 1.2[a]	40.9 ± 1.4[a]	47.2 ± 1.2[b]*	48.0 ± 1.4[b]*

[a, b]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p = 0.0524$.

Table 4 ADF (%DM) content of combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	29.9 ± 0.9[a]	30.1 ± 0.9[b]	31.6 ± 0.9[b]	31.1 ± 0.9[b]	33.8 ± 0.9[b]
7	30.9 ± 1.1[a]	29.4 ± 0.9[a]	32.4 ± 1.6[a]	33.4 ± 0.9[a]	32.1 ± 0.9[a]
21	29.8 ± 0.9[a]	28.9 ± 1.6[a]	32.3 ± 0.9[b]	32.9 ± 0.9[b]	31.5 ± 0.9[ab]
84	31.4 ± 1.1[a]	30.7 ± 0.9[a]	32.0 ± 1.1[a]	37.8 ± 0.9[b]*	38.0 ± 1.1[b]*

[a, b]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p < 0.05$.

Day 84 than on Day 1. The 100% SL silage had a lower ($p < 0.05$) ADL value on Day 21 than it did on Day 1 (Table 5). On Day 1, ADL was higher ($p < 0.05$) for each of the SL silages compared with the 0% SL (pure alfalfa) silage. There were no differences due to silage treatment on Day 7, while the 0% and 25% SL silages had lower ($p < 0.05$) ADL values than the 50% and 75% SL samples on Day 21. By Day 84, the 75% and 100% SL silages had significantly higher ($p < 0.01$) ADL than the 0%, 25%, or 50% SL silage samples.

3.6 Ash Concentration

Ash, or total concentration of minerals in the silages, was significantly affected by treatment ($p < 0.001$), but not day, with ash levels decreasing ($p < 0.001$) as percentage SL in the silage mix increased. Percentage

ash SL means ± standard error were 8.06 ± 0.03, 6.96 ± 0.04, 6.21 ± 0.05, 4.99 ± 0.02 and 3.63 ± 0.02 for ensiled forage mixtures of 0%, 25%, 50%, 75% and 100% SL, respectively.

3.7 N Concentration

There was a treatment × time interaction ($p < 0.001$) for N concentration of the silages, with decreasing values as the percentage SL in the silage mix increased (Table 6). As CP is N × 6.25, data are not shown for CP. However, for both N and CP, values differed from Day 1 within treatment ($p < 0.05$) for the 0% and 50% SL samples on Days 21 and 84, and for the 25% SL samples on Day 84 only. On Day 1, N and CP were higher ($p < 0.05$) in the 0% and 25% SL silages than the other combinations, while on Day 7, the 0% and 50% SL silages were not different, but were each higher ($p < 0.001$) than for the 25%, 75% and 100% SL silages. On Day 21, N and CP were the highest ($p < 0.001$) in the 0% SL silage, there were no differences between the 25% and 50% SL values, while the 75% and 100% SL silages had the lowest ($p < 0.01$) concentrations. On Day 84, there was a linear decrease ($p < 0.01$) in N and CP in the silages as percentage SL in the mixture decreased.

3.8 NO_3-N Concentration

There was a treatment × time interaction ($p < 0.001$) for NO_3-N data (Table 7). All the nitrate values decreased ($p < 0.001$) after Day 1, except for 100% SL, which did not differ from Day 1 to Day 84. On Day 1, NO_3-N concentration was less ($p < 0.05$, except for 0% versus 25%, $p = 0.09$) with increasing percentage SL in the silage. On Day 7, only 100% was lower ($p < 0.05$) than 0%. There were no treatment differences in NO_3-N for Days 21 and 84 (Table 7).

3.9 $NH_{4+}N$ Concentration

There was significant treatment × time interaction ($p < 0.001$) in $NH_{4+}N$ concentrations in the silages (Table 8). Ammonia levels increased ($p < 0.05$) over time for each treatment as compared to Day 1, but decreased ($p < 0.05$) with increasing percentage of SL in the silage at each time point, but between Day 21 and Day 84, the 0%, 25% and 75% SL samples slightly increased in their ammonia concentrations, while the other two treatment samples remained the same (50% SL) or slightly decreased in ammonia (100% SL) (Table 8).

Table 5 ADL (%DM) content of combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	8.57 ± 0.66[a]	10.59 ± 0.66[b]	10.57 ± 0.66[b]	11.01 ± 0.66[b]	12.11 ± 0.66[b]
7	9.44 ± 0.80[a]	9.14 ± 0.66[a]	11.34 ± 1.13[a]	10.82 ± 0.66[a]	11.14 ± 0.66[a]
21	8.98 ± 0.66[a]	8.59 ± 1.12[a]	11.71 ± 0.66[b]	11.39 ± 0.66[b]	9.65 ± 0.66a[b]*
84	10.73 ± 0.80[a]*	10.21 ± 0.66[a]	11.77 ± 0.80[a]	16.47 ± 0.66[b]*	15.92 ± 0.80[b]*

[a, b]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p < 0.05$.

Table 6 Percentage of N concentration in combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	2.51 ± 0.04[a]	2.14 ± 0.04[a]	2.00 ± 0.04[b]	1.90 ± 0.04[b]	1.88 ± 0.64[b]
7	2.58 ± 0.04[a]	2.16 ± 0.04[b]	2.56 ± 0.06[a]	1.94 ± 0.04[b]	1.81 ± 0.04[b]
21	2.65 ± 0.04[a]*	2.25 ± 0.06[b]	2.29 ± 0.04[b]*	1.93 ± 0.04[c]	1.74 ± 0.04[c]
84	2.68 ± 0.05[a]*	2.44 ± 0.04[b]*	2.23 ± 0.05[c]*	1.97 ± 0.04[d]	1.81 ± 0.04[e]

[a, b, c, d, e]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p < 0.001$.

Table 7 NO_3-N concentration (ppm) in combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	110.5 ± 1.6[a]	93.1 ± 1.6[a]	73.6 ± 1.6[b]	40.3 ± 1.6[c]	13.3 ± 1.6[d]
7	27.6 ± 5.1[a]*	13.7 ± 4.4[ab]*	12.5 ± 7.5[ab]*	13.2 ± 4.4[ab]*	10.0 ± 4.4[b]
21	13.0 ± 1.7[a]*	11.5 ± 2.0[a]*	11.7 ± 1.7[a]*	9.3 ± 1.7[a]*	12.4 ± 1.7[a]
84	13.3 ± 1.5[a]*	13.7 ± 1.5[a]*	12.0 ± 1.6[a]*	8.5 ± 1.5[a]*	11.3 ± 1.5[a]

[a, b, c, d]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p < 0.001$.

Table 8 NH_{4+}N concentration (ppm) in combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Time of ensiling (d)	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
1	2,339 ± 32[a]*	1,723 ± 32[b]*	1,275 ± 32[c]*	767 ± 32[d]*	93 ± 32[e]*
7	3,153 ± 36[a]	2,507 ± 35[b]	1,711 ± 47[c]	961 ± 35[d]	262 ± 35[e]
21	3,662 ± 51[a]	2,701 ± 58[b]	1,942 ± 51[c]	1,065 ± 51[d]	313 ± 51[e]
84	3,965 ± 12[a]	3,155 ± 11[ab]	1,979 ± 13[b]	1,288 ± 11[bc]	198 ± 11[d]

[a, b, c, d, e]Row means with unlike superscripts differ significantly at $p < 0.05$. Column means with * differ from Day 1, $p < 0.05$ in each ratio. There was a treatment by day interaction, $p < 0.001$.

Table 9 CT (mg/g DM) content of combinations of SL (0%, 25%, 50%, 75%, 100%) and alfalfa (A, 100%, 75%, 50%, 25%, 0%) ensiled for 84 d.

Constituent	Ensiled forage combination (SL:A)				
	0:100	25:75	50:50	75:25	100:0
	mg/g DM				
Unbound CT	4.8 ± 3.4[a]	8.5 ± 3.8[b]	27.4 ± 3.8[c]	38.4 ± 3.0[d]	49.8 ± 3.2[e]
Bound CT	7.0 ± 1.7[a]	14.8 ± 1.8[b]	23.9 ± 1.9[c]	33.0 ± 1.5[d]	36.5 ± 1.5[d]
Total CT	11.9 ± 3.9[a]	23.8 ± 4.3[a]	51.2 ± 4.7[b]	71.5 ± 3.3[c]	90.7 ± 3.3[d]

[a, b, c, d, e]Row means with unlike superscripts differ significantly at $p < 0.05$.

3.10 CT

There was a significant treatment effect ($p < 0.001$) on unbound (extractable) CT, with increasing concentrations as percentage SL in the silage mixtures increased (Table 9). There was also an effect of day, in which there was less ($p < 0.05$) unbound CT concentration (pooled by treatment) by Day 84 (17.3 ± 2.9 mg/g DM) compared with samples taken on Day 1 (26.2 ± 2.8 mg/g DM), Day 7 (28.9 ± 3.4 mg/g DM) and Day 21 (30.8 ± 3.2 mg/g DM).

Treatment effects on bound CT ($p < 0.001$) followed a similar pattern to extractable CT, with a linear increase ($p < 0.05$) in bound CT levels in ensiled material as percentage SL in the silage increased (Table 9). There was no effect of day or treatment × day.

There was a significant treatment effect ($p < 0.001$) on total (unbound + bound) CT, with increasing concentrations as percentage SL in the silage mixtures increased (Table 9). There was also a day effect ($p < 0.05$), with less total CT by Day 84 (38.8 ± 3.9 mg/g DM) compared with samples taken on Day 7 (55.5 ± 4.4 mg/g DM) and Day 21 (55.0 ± 4.2 mg/g DM), and a tendency ($p = 0.0559$) to be less than those sampled on Day 1 (50.0 ± 3.6 mg/g DM).

3.11 Protein-Precipitable Phenolics

The concentration of total protein-precipitable phenolics in the pure, unensiled SL sample was 20 g/kg, or 2%, while there were no detectable protein-precipitable phenolics in any of the ensiled samples regardless of SL level.

4. Discussion

Water activity represents a measure of unbound water that is available for microbial growth activity. The water activity values for each of the ensiled samples in the current investigation were above the threshold value of 0.95 a_w, so there was adequate moisture for the microbial fermentation required for proper ensiling. The initial moisture levels of the unensiled SL and alfalfa were similar, at 61.2% and 61.5%, respectively. This moisture level is considered adequate for proper ensiling of leguminous forages to preserve feeding quality [4, 23].

The silage in each of the mini-silos was visually observed and given an olfactory test as they were opened. All the treatment combinations appeared to have ensiled adequately in this study, as the silage in each tube smelled sweet and had a greenish-yellow color as appropriate for ensiled material. The terminal pH values for each of the treatment silages ranged between 4.6 and 4.8, which is also an indication that each of the mixtures properly ensiled. The majority of the pH decline for each of the treatment silages occurred by Day 7 in this investigation. In a similar study with ensiled mixtures of sainfoin and alfalfa, Wang *et al*. [24] reported final pH values of less than 4.5 for each of the mixtures, with a linear decrease in pH over time. After Day 7, pH continued to decline through Day 84 in only the 0% and 25% SL silages in the current study, although at a slower rate than initially (Table 3).

Ensiling appeared to have a greater effect on fiber (NDF, ADF, ADL) over time as the percentage SL in the mixture increased. For the 75% and 100% SL mixtures, the NDF, ADF and ADL levels all increased on Day 84 compared with Day 0. This may be related to challenges in use of the detergent analysis system with CT-containing samples [25, 26]. Terrill *et al*. [25] reported increased precipitation of CT and CP in NDF and ADF when oven-dried (55 °C) and freeze-dried SL samples were analyzed without sodium sulfite added (greater CT and CP precipitation in NDF than ADF). Similar results were reported by Pagan *et al*. [26] for a range of different CT-containing plants using the Ankom system (ANKOM Technology, Macedon, NY, USA) with NDF and ADF analyzed separately, with ADF values generally greater than NDF (sodium sulfite added) [26]. Sequential NDF-ADF analysis with sodium sulfite added during the NDF step reportedly removed the CT precipitants in both of these investigations [25, 26]. The samples in the current study were analyzed using appropriate protocol to reduce CT interference with detergent fiber analysis [26] but ensiling of SL may have altered CT behavior in the system. Analysis of NDF and ADF residues of ensiled SL may shed light on the fate of CT and CP in the detergent analysis system, but unfortunately, these residues were not analyzed in the current investigation.

Level of ash in the samples decreased as the percentage SL in the silage decreased, indicating a higher mineral concentration in alfalfa than in SL. The N concentration of the silages also increased as percentage alfalfa in the mixture increased (Table 7), which is to be expected, as alfalfa generally has greater CP values than SL [27]. The most important effect of ensiling on leguminous forages is likely on the form of N rather than on total N of these plants. In the current study, increasing the percentage SL in the ensiled mixtures reduced proteolysis by reducing the amount of both NO_3-N and NH_{4+}N produced during ensiling. Ensiling of forages has been recommended to reduce nitrate concentrations [28], and this was confirmed in the current investigation, as level of NO_3-N was similarly low for all of the forage treatment mixtures by the end of the study period (Table 8).

The primary effect of CT in this study appears to be on ammonia production of the silages. There was a linear decrease in NH_{4+}N as the percentage SL increased in the ensiled mixtures (Table 9). A similar effect on ammonia production has been reported in ensiled sulla (*H. coronarium*) [5] and sainfoin (*O.*

viciifolia) [9]. Several authors have suggested that CT may inhibit proteolysis in silage [29, 30]. Hymes-Fecht *et al*. [7] reported that CT in birdsfoot trefoil (*L. corniculatus*) hay or silage limited proteolysis during conservation and ruminal fermentation of this forage in animals. Niezen *et al*. [5] compared the effect of ensiling sulla in different combinations with harvested grass pasture (0/100, 25/75, 50/50, 25/75, and 100/0) on ammonia concentration and reported a reduction in ammonia from 5.9% to near zero as sulla increased from 0 to 100%.

Reduced proteolysis is considered a positive outcome for leguminous silages [4]. In a study comparing alfalfa or red clover ensiled alone or in combination with birdsfoot trefoil, Hymes-Fecht *et al*. [7] reported increased milk production and milk fat when dairy cows were fed silages containing *L. corniculatus.* The nutritional consequences of reduced proteolysis as the percentage of SL in the silage mixture increased could not be determined in the current study and will be the subject of future investigations.

Unbound (extractable), bound (unextractable) and total CT all increased in the samples as the percentage SL increased in the ensiled mixtures. This was to be expected, as SL has been reported as a high-CT forage for decades [31-33]. What was not expected and is more difficult to explain is why unbound and total CT both decreased over time (Day 84 samples). Possible explanations for this drop in CT may be interference of other substances in the ensiled samples with color development in the detection assay used or perhaps changes in the structure of CT that render it less sensitive to butanol-HCl. Variable CT recovery was reported by Terrill *et al*. [34] from purified *L. pedunculatus* CT added to digesta recovered from sections of gastrointestinal tract of sheep fed a non-CT forage, with lower recovery from ruminal and small intestinal compared with abomasal samples. These authors attributed lowered CT recoveries to conformational changes in the CT molecule so that it was no longer reactive with butanol-HCl, or possibly from interference of other digesta constituents.

In addition to CT concentration and structure, total protein precipitable phenolics is another indicator of CT bioactivity. In the current investigation, there were no detectable protein-precipitable phenolics in any of the ensiled samples regardless of SL level, and the concentration in the unensiled SL sample was 20 g/kg, or 2%, which is quite low. This would suggest little or no bioactivity in these SL mixtures after ensiling. However, CT extracted and purified from the ensiled treatment mixtures regained its ability to precipitate protein (H. Naumann, unpublished data), suggesting that unidentified factors may have reduced the bioactivity of SL during the ensiling process, or perhaps prior to ensiling. The SL and alfalfa forage utilized for this study were frozen for several months and then thawed prior to packing into the mini-silos for ensiling. The un-ensiled material was thawed and then refrozen prior to freeze-drying. Freezing and thawing SL plant material even for a short period was reported by Terrill *et al*. [33] to reduce extractable CT concentrations relative to freeze-drying the samples. To avoid this possibility in future research, SL and alfalfa should be chopped, packed and ensiled fresh, with no freezing and thawing of the plant material.

Concerning the bioactivity of ensiled SL, this was assessed in *in vivo* feeding trials with goats using the same plant material used in the current *in vitro* investigation [35]. Terrill *et al*. [35] reported significant reductions in GIN egg counts and coccidial oocyst counts in feces of young goats fed both sun-dried (hay) and ensiled SL diets compared with a Bermuda grass hay-based ration.

5. Conclusions

SL can be successfully ensiled in different combinations with alfalfa, while reducing proteolysis of the ensiled material compared with alfalfa alone, potentially increasing the feed value of alfalfa silage.

In particular, production of ammonia during ensiling was linearly reduced as percentage SL increased. These effects were likely due to the tannins in this plant, as unbound and total CT linearly increased as the percentage SL in the silage mixture increased. The nutritional consequences and anti-parasitic bioactivity of ensiling SL alone or in different combinations with alfalfa were not determined in this study and should be the focus of future research work with this nutraceutical forage.

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