ARTICLE

Do plant secondary metabolite-containing forages influence soil processes in pasture systems?

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Abstract

Grazed pastures are susceptible to N loss from urine/manure additions, which increases eutrophication, affecting the global N cycle. Plant secondary metabolites (PSM), such as condensed tannins (CT) and terpenes, influence silviculture soil dynamics by generally decreasing N mineralization. We investigated whether cattle-grazed pastures of non-traditional grass and legume forage monoculture strips including CT-containing sainfoin (*Onobrychis viciifolia* Scop.) and tall fescue (TF) [*Schedonorus arundinaceus* (Schreb.) Dumort.] influenced soil dynamics compared with traditional grass and legume forage monoculture strips of alfalfa (*Medicago sativa* L.), without tannins, and TF. Throughout the study, CT in sainfoin averaged 58.9 g kg^{-1} whereas alfalfa saponins averaged 5.7 g kg^{-1}. We observed greater soil microbial respiration ($p = .01$) in TF strips than legume strips, indicating greater microbial activity, and between legumes we found greater soil NO_3 ($p = .01$) in alfalfa than in sainfoin, although aboveground biomass and N differences were negligible. We also conducted a laboratory soil-feces incubation study to determine if feces from cattle foraging diets of legumes with or without CT influenced soil dynamics. Both feces treatments showed lower NO_3 ($p < .001$) than without feces, suggesting microbial inhibition. Dehydrogenase activity (DHEA) was lower ($p = .03$) in sainfoin than alfalfa feces, suggesting CT from sainfoin inhibit DHEA. To our knowledge this study is the first considering whether CT-containing sainfoin and saponin-containing alfalfa influence soil dynamics by assessing general differences in soil parameters. More research is needed to determine whether specific PSM mitigate N loss in pasture systems by slowing N mineralization.

1 | INTRODUCTION

Incorporating forages containing different PSM, such as CT and terpenes (i.e., saponins), may benefit animal

agricultural systems. When animals graze diverse forages, the different chemicals ingested in the process improves animal production while enhancing soil quality and nutrient cycling (Tracy et al., 2018). Diverse patchworks of vegetation containing PSM increase the rate of gain in foraging animals (Meuret & Provenza, 2015) while plant diversity, with inherently diverse biochemistries, increases the resilience of agroecosystems (Tracy et al., 2018). Potential mechanisms span from complementarities among resources to nutrient-cycling feedbacks, which increase nutrients in soils (Tilman & Snell-Rood, 2014; Tilman, Isbell, & Cowles, 2014). In addition, tannin-containing forages reduce methane emissions from grazing animals (Beauchemin, McGinn, Martinez, & McAllister, 2007; Boadi, Benchaar, Chiquette, & Massé, 2004; Pinares-Patiño et al., 2003; Woodward, Waghorn, & Laboyrie, 2004a, 2004b).

Nitrogen loss in agroecosystems is widespread, and the potential for NO_3 leaching under grazed pastures is greater than that of mowed pastures. This is because 60–90% of the ingested N is returned to the soil via manure and urine, creating hotspots of N in the soil that are prone to N loss (Di & Cameron, 2002; Haynes & Williams, 1993). The presence of CT or triterpenes in plants may ameliorate this problem as tannins and saponins bind to proteins in the gastrointestinal tract, increasing the ratio of fecal to urinary N (Barry & McNabb, 1999; Livingston et al., 1979; Waghorn, Shelton, McNabb, & McCutcheon, 1994), which slows release and leaching of N in pastures (Powell, Broderick, Grabber, & Hymes-Fecht, 2009). Condensed tannins are large polar molecules that remain in the gastrointestinal tract and are excreted in feces (Waghorn, 2008), which may then pose as recalcitrant substrate for soil microorganisms (Smolander, Kanerva, Adamczyk, & Kitunen, 2012).

The tannin content of detritus in forest systems is not easily decomposed, and heavily influences C and N mineralization (Kraus, Dahlgren, & Zasoski, 2003). In addition to CT, triterpenes affect both C and N cycling in soil (Adamczyk, Kiikkilä, Kitunen, & Smolander, 2013; Bradley, Titus, & Preston, 2000; Smolander et al., 2012). Carbon-based compounds such as tannins and terpenes generally inhibit N mineralization thereby increasing N immobilization (Adamczyk et al., 2013; Smolander et al., 2012; Winder, Lamarche, Constabel, & Hamelin, 2013). Research in silvicultural soil systems also shows these metabolites decrease decomposition rates and inhibit soil mesofauna and soil enzymatic activity (Adamczyk, Kitunen, & Smolander, 2009, 2011; Bradley et al., 2000; Joanisse, Bradley, Preston, & Munson, 2007; Lorenz, Preston, Raspe, Morrison, & Feger, 2000; Smolander et al., 2012; Adamczyk et al., 2013; Madritch & Lindroth, 2015). Research on how tannins and terpenes affect soil dynamics, conducted mainly in boreal forests, suggests these metabolites either bind to

> ### Core Ideas
> - Pasture soil systems may benefit from tanniferous or saponin-containing forages.
> - Soil microbial activity decreased under tanniferous and saponin-containing forages.
> - Pastures with tanniferous sainfoin contained lower soil nitrate levels than alfalfa pastures.

proteinaceous or other organic N compounds (offering an increased C resource for soil microbes), adsorb to soil minerals, transform, or become toxic to microbes - although their specific roles are not fully elucidated (Kelleher, Simpson, & Simpson, 2006; Smolander et al., 2012).

Soil research involving PSM has increased over the past decade, particularly in silviculture, though the only research we are aware of regarding the influence of PSM-containing forages on pasture soils is with ergot alkaloids, with inconclusive results suggesting reduced soil microbial activity (Franzluebbers, 2006; Franzluebbers & Hill, 2005; Franzluebbers & Stuedemann, 2005; Franzluebbers et al., 1999; Omacini, Chaneton, Ghersa, & Otero, 2004). Leshem and Levin (1978) found decreased NO_3 in peat soils with alfalfa under laboratory conditions, suggesting this was due to "substances originating in the plant and released by the roots". To our knowledge no research has been done determining the effects of tanniferous or saponin-containing forages on pasture agricultural soil processes. Reduced N mineralization would benefit pasture systems in reducing N loss. We explored whether the same phenomena that appear in silvicultural soil occur in pasture soils with forages containing CT and saponins.

The objective of this study was to determine if extractable condensed tannins from sainfoin and/or saponins from alfalfa influence soil nutrient cycling in pasture systems. Although both sainfoin and alfalfa contain additional types of secondary metabolites (Barrau, Fabre, Fouraste, & Hoste, 2005; Rafińska, Pomastowski, Wrona, Górecki, & Buszewski, 2017), the focus of our study was limited to CT and saponins. We measured the concentration of extractable CT in sainfoin plant samples, saponins in alfalfa samples, and soil parameters such as inorganic N, soil respiration, and enzyme activity. We hypothesized that cattle-grazed pastures of non-traditional grass and legume monoculture strips including CT-containing sainfoin and TF would influence soil microbial activity to a greater extent than "traditional" grass and legume monoculture strips of saponin-containing alfalfa, without tannins, and TF. To illuminate the effects of manure on soil nutrient cycling, we performed a soil incubation study with cattle

feces from two different diets consisting of TF with either CT-containing sainfoin, or saponin-containing alfalfa.

2 | MATERIALS AND METHODS

2.1 | Field experiment

2.1.1 | Plot establishment

Plots were established in spring 2009 at Utah State University's Agriculture Research Field Station in Lewiston, UT at 41°57′4″ N, 111°52′26″ W. The study site consisted of (i) Kidman fine sandy loam (coarse-loamy, mixed, mesic Calcic Haploxeroll) and (ii) Lewiston fine sandy loam (coarse-loamy, mixed, mesic Aeric Calciaquoll). Part of one block was in the Kidman fine sandy loam, while the remaining blocks were in Lewiston fine sandy loam soil. Block effect was tested using SAS PROC SGPANEL (SAS Institute, Cary, NC), and we conclude that our measured responses were not affected by the difference in soil type. Average annual precipitation is 52.6 cm, with an average annual high and low temperatures of 16.2 and 1.14 °C, respectively (Utah Climate Center, 2019). Total annual precipitation during the four sample collection years (2009, 2010, 2011, and 2012) was 36.8, 42.6, 35.2, and 45.7 cm, respectively, while the average maximum and minimum temperatures, respectively, for each season (May through October) for each year were 23.8 and 6.56 °C, 23.8 and 6.39 °C, 23.3 and 6.61 °C, and 27.0 and 9.26 °C (Utah Climate Center, 2019).

The field experiment was designed around a grazing choice experiment (Maughan et al., 2014) where three 3.6-ha blocks were each divided into two 1.8-ha plots, one seeded with novel-endophyte TF variety 'Max Q' at 28 kg ha^{-1} and inoculated alfalfa variety 'Vernal' at 11 kg ha^{-1}, and the other seeded with TF and inoculated sainfoin variety 'Shoshone' at 33 kg ha^{-1}. Within each 1.8-ha plot there were three planting strips, each approximately 30.5 m wide and 132.6 m long, seeded with TF, legume, and TF/legume mixture (30:70 grass/legume rate), all of which were grazed as one unit. The mixture treatment did not persist and therefore was removed from the study, leaving monoculture strips of TF and legume in each plot. Thus, the design employed a blocked split-plot design with three blocks. The whole plot factor was legume type (tannin-containing sainfoin/saponin-containing alfalfa), with forage type as the subplot factor (tall fescue/legume). Pastures were grazed as described by Maughan et al. (2014). Briefly, Angus fall-born calves strip-grazed either TF-sainfoin or TF-alfalfa plots from May–September 2010, with 12 calves per plot, and June–September 2012, with 8 calves per plot. Strip-grazing was managed using temporary electric fencing which was moved daily, allowing

access to new forage every day. The pastures were irrigated using hand-line sprinkler sets running in 12-h cycles which applied approximately 10.5 cm of water every 2 wk. Tall fescue variety Kentucky 31 was the intended variety, yet after failed attempts to detect ergovaline, the seed was tested revealing the identity of cultivar 'Max Q'.

Due to over-winter crop failure in 2010–2011 (i.e., *Microtus pennsylvanicus* infestation) of alfalfa and sainfoin strips, the two legumes over all 3 blocks were reseeded in spring 2011 using a no-till drill at the aforementioned rates. Tall fescue strips were not affected by the rodent infestation. From June–September 2011 plots were swathed and baled. The project resumed after healthy regrowth in 2012.

2.1.2 | Soil sample collection and analysis

Previous to seeding and before irrigation commenced, in summer (July 6–7) 2009, ten baseline soil subsamples were composited after being collected in a zigzag pattern along each strip using a 5-cm diameter Giddings probe at depths of 0 to 30, 30 to 60, and 60 to 90 cm. Soil samples were sieved to pass a 2-mm screen and stored at 4 °C until analysis. Samples were analyzed within 2 wk according to recommendations for the western region (Gavlak et al., 2003) for nitrate-nitrogen (NO_3-N; using the Cadmium Reduction Method S-3.10) and ammonium (NH_4-N; Method S-3.50). A subset of these samples (0–30 cm depth) was analyzed for DHEA (Tabatabai, 1994). Soil samples were then air-dried and analyzed (Gavlak et al., 2003) for soil pH (Method S-2.20), electrical conductivity (EC; Method S-2.30), Olsen extractable P and K (Method S-4.10), and DTPA-extractable Fe, Zn, Cu, and Mn (Method S-6.10). Total and organic C was analyzed using a Skalar Primacs SLC model CS22 (Breda, Netherlands), using the two temperature (575–1035 °C) method (Chichester & Chaison, 1992). Total N was analyzed using a Skalar Primacs Solid Sample TN Analyzer (Breda, Netherlands). Baseline soil samples included 36 composite samples for analysis (2 legume types × 2 forage types × 3 depths × 3 blocks).

In spring (May 2–5) 2011 before irrigation commenced, soil samples were collected to determine treatment effects two years after plot establishment. Ten subsamples were composited after being collected in a zigzag pattern in each strip using a 2.5-cm diameter soil probe at depths of 0 to 10, 10 to 20, and 20 to 30 cm. Soil samples were sieved to pass a 2-mm screen and stored at 4 °C until analysis within 2 wk for NO_3-N, NH_4-N, and the top 10-cm increments analyzed for DHEA, as previously described.

In fall (October 9–19) 2012, end-of-study soil samples were collected. Five soil subsamples were collected in a zigzag pattern in each strip using a 5-cm diameter Giddings probe to a depth of 90 cm, and split into five

increments (0 to 10, 10 to 20, 20 to 30, 30 to 60, and 60 to 90 cm), each subsample composited for all five increments. Soil samples were sieved to pass a 2-mm screen and stored at 4 °C until analysis for NO_3-N and NH_4-N at increments of 0 to 30, 30 to 60, and 60 to 90 cm, and a subset (0–30 cm) was analyzed for pH, EC, total N, total and organic C, P, K, Zn, Fe, Cu, and Mn as described above. Dehydrogenase was analyzed at 0 to 10, 10 to 20, and 20 to 30 cm increments. A subset (0–10 cm) was analyzed for phenol oxidase (Prosser, Speir, & Stott, 2011) and soil respiration (Anderson & Domsch, 1978; Davidson, Galloway, & Strand, 1987; Smith, McNeal, & Cheng, 1985; Sparling, 1992), with ratios between microbial biomass to organic C calculated to determine metabolic efficiencies. Soil samples included 60 composite samples for analysis (2 legume types × 2 forage types × 5 depths × 3 blocks).

In November 2012, soil samples were collected, using 260-ml tins, from each strip (n = 12), weighed, then soil bulk density was measured, and soil porosity calculated, using methods developed by Blake (1965) and described by USDA ARS NRCS Soil Quality Institute (2001).

2.1.3 | Plant sample collection and analysis

In spring (May 14–19) 2010, before grazing commenced and 7 d between the 2-wk irrigation cycle, baseline samples from each plant species (alfalfa, sainfoin, and TF) were collected randomly within each monoculture strip when developmental morphology was similar for each species, at late vegetative growth (Flick and Mueller, 1989; Moore & Moser, 1995; Moore et al., 1991). Briefly, ten subsamples of each species (sainfoin, alfalfa, and TF), clipped at 5-cm above ground, were collected in each strip in a simple random approach. Composite samples were collected from each strip, in each plot, in each block, comprising six TF samples (2 legumes × 3 blocks), three alfalfa samples, and three sainfoin samples (3 blocks). Given that CT may be labile molecules (Mehansho, Butler, & Carlson, 1987), sainfoin samples were placed on dry ice in the field, then stored at −20 °C until freeze-dried. Alfalfa and TF samples were placed in drying ovens at 30 °C. Dried plant samples were ground to pass a 1-mm screen with a Wiley mill grinder (Thomas Scientific, Swedesboro, NJ), then stored in sealed plastic bags at −20 °C until chemical analyses.

In 2012 plant samples were collected 3 times (26 June, 14 August, and 18 September) from each plant species as described above. After sampling periods, plots were grazed, then regrowth was allowed between collections. Plant samples included 18 composite TF samples (2 legumes × 3 collection times × 3 blocks), nine composite alfalfa samples, and nine composite sainfoin samples (3 collection times × 3 blocks).

Each composite plant sample was analyzed for total N using a Skalar Primacs Solid Sample TN Analyzer (Breda, Netherlands). Sainfoin samples were analyzed for extractable CT as described by Mantz, Villalba, and Provenza (2008) and Clemensen et al. (2017), using butanol-HCl methods developed by Reed (1986). Alfalfa samples were analyzed for saponins using a modification of Lee, Vogel, Gardner, and Stegelmeier (2001) as described by Clemensen et al. (2017) using a foam test procedure, following methods developed by Patamalai, Hill, Camp, Hejtmancik, and Bridges (1990) and Wall, Eddy, McClennan, and Klumpp (1952).

Our field experiment had an overlapping grazing component discussed in Maughan et al. (2014) where plant samples were collected and analyzed for plant biomass and crude protein (CP). We present plant biomass and CP data from Maughan et al. (2014) after statistically analyzing the data using the experimental design and model described for the present study.

2.1.4 | Statistical analyses

Soil data from 2009, 2011, and 2012 were analyzed using ANOVA with a blocked split-plot design. The log transformation was used for organic C where normality and homogeneity of variance was not met, the untransformed data is presented. The whole plot factor was legume type (sainfoin and alfalfa), with forage type as the subplot factor (tall fescue and legume) with all treatments as fixed and blocks as random effects. Soil data for each year (2009, 2011, and 2012) were analyzed separately. Using a three-way factorial treatment structure (forage × legume × depth), main effects for soil depth were assessed for NO_3-N and NH_4-N at 0 to 30, 30 to 60, and 60 to 90 cm (from 2009 and 2012, year analyzed separately), and for DHEA at 0 to 10, 10 to 20, and 20 to 30 cm (from 2012). To assess differences in soil response variables over time, we compared means from baseline 2009 (0–30 cm depth) data to 2012 (0–30 cm) data for each measured variable.

For plant analyses, extractable CT and total N data from sainfoin, and saponins and total N data from alfalfa were analyzed using ANOVA of a randomized complete block design (RCBD) with one factor, time (June, July, and August 2012). Assumptions for normality and homogeneity of variance were met. Total N from tall fescue data were analyzed using ANOVA of a blocked split-plot design, with repeated measures (June, July, and August 2012), where all treatments as fixed and blocks as random effects. Assumptions for normality and homogeneity of variance were met. Total N and CP in legumes (alfalfa and sainfoin) were compared using ANOVA of a RCBD.

All statistical models were fitted using the GLIMMIX procedure in SAS/STAT Version 13.2 in the SAS System for Windows Version 9.4 (SAS Institute, Cary, NC). Tukey's test was used to adjust pairwise mean comparisons for family-wise Type I error. P < .05 was considered to be statistically significant.

2.2 | Laboratory incubation study

2.2.1 | Soil and feces collection and analysis

Soil for the incubation study was collected from the top 30 cm of an ungrazed grass pasture from the previously described field experiment site, air dried, then sieved through a 2-mm screen (Baitilwake, Salomez, Mrema, & De Neve, 2012). Fresh cattle feces was collected from the aforementioned grazing experiments (Maughan et al., 2014) of calves eating monoculture strips of either saponin-containing alfalfa or CT-containing sainfoin, both with monoculture strips of TF. Feces was collected after calves consumed monoculture strips of either sainfoin and TF, or alfalfa and TF, for two weeks. Fresh fecal samples were put on ice in the field, then triplicate subsamples of each feces type (alfalfa-TF and sainfoin-TF) were air-dried and analyzed for pH as described above, and total N and C using Leco FP-528 total combustion (St. Joseph, MI). The remaining composite fresh fecal samples were then stored at −20 °C until freeze dried to best preserve potential tannins (Terrill, Windham, Evans, & Hoveland, 1990) in the feces (Waghorn, 2008). To obtain uniformity fecal samples were then ground and sieved through a 2-mm screen to be used in the soil incubation experiment.

2.2.2 | Experimental design and analysis

The incubation study employed a RCBD with repeated measures, where feces from two different diets (alfalfa-TF or sainfoin-TF) were mixed with soil and incubated at 24 °C for 56 d. Each treatment had four replicates containing 500 g of air-dried soil, in addition to control treatments without addition of feces to baseline potential N flushing effects, as we did not pre-incubate soil used in this study.

Determining the amount of feces to apply was based on N content in the feces and applied at a rate of 350 kg total N ha^{-1} with 1.3 g cm^{-3} soil bulk density (the average soil bulk density of the field site) (Honeycutt et al., 2005). Feces from sainfoin diets contained slightly greater N than feces from alfalfa diets. Therefore, we averaged the amount of feces to be added from each diet, so each feces treatment

had the same amount of feces applied, totaling 4.19 g per subsample of soil (Equation 1).

$$\frac{350 \text{ kg N}}{\text{Hectare} - \text{furrow slice}} \times \frac{\text{cm}^3}{1.3 \text{ g soil}} \times \frac{100 \text{ kg DM}}{2.14 \text{ kg N}}$$
$$\times \frac{500 \text{ g soil}}{\text{subsample}} \quad (1)$$

On day 0, distilled water was added to each treatment to reach 18% moisture by slowly misting while mixing. Percent moisture was dependent on our soil type and the observable maximum moisture content before oversaturation-induced soil structure collapse. Samples were placed in sealed quart-size Ziploc bags with protruding straws to allow exchange of gases and minimize water loss, then compacted to reach a bulk density of roughly 1.3 g cm^{-3}, and incubated for 56 d at 24 °C. The initial weight of all samples was recorded and moisture content monitored, adding distilled water when evaporation loss was > 5% (relative) of 18% moisture (Baitilwake et al., 2012; Honeycutt et al., 2005).

Sampling for analyses occurred at days 0, 3, 7, 14, 21, 28, 42, and 56. Samples were analyzed for NO_3, NH_4, DHEA, organic C, and total N as described above. Total N and organic C was measured at the beginning and end of the incubation period (days 0 and 56). Two feces types with a control treatment totaled 96 samples for analysis (3 treatments × 4 replications × 8 sample days).

2.2.3 | Statistical analysis

Soil data were analyzed using an ANOVA by SAS 9.4 PROC GLIMMIX (SAS Institute, Cary, NC) of a RCBD with a single factor (alfalfa-TF or sainfoin-TF diet) using repeated measures (days 0, 3, 7, 14, 21, 28, 42, and 56). Tukey's test was used for mean comparisons. P < .05 was considered to be statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Field experiment

Baseline soil samples taken in 2009 before experimental plots were seeded showed no differences among response variables between impending treatment plots (Table 1). In spring 2011, after two growing seasons, there were no differences in soil parameters between alfalfa and sainfoin plots, nor between TF or legume plots.

Our end-of-study October 2012 data showed legume forage strips having 4.2-fold greater ($p = .05$; Table 2) soil NO_3 at 0 to 30 cm than TF strips. These results support

TABLE 1 Soil results presented from baseline 2009 field experiment, prior to planting. Main effect means and standard errors (SE) for forage type (grass vs legume) and legume type (tannin-containing sainfoin vs saponin-containing alfalfa, without tannins) are shown. There were no interaction effects for any soil variables, nor depth interaction effects

Soil property, g^{-1} soil	Forage			Legume		
	Grass	Legume	SE	Alfalfa	Sainfoin	SE
Ammonium, µg (0-30 cm depth)	ND[b]	ND		ND	ND	
Ammonium, µg (30-60 cm depth)	1.05	1.41	0.28	1.36	1.09	0.63
Ammonium, µg (60-90 cm depth)	2.86	3.31	0.40	3.26	2.91	0.59
Nitrate, µg (0-30 cm depth)	2.11	2.29	0.12	2.28	2.12	0.11
Nitrate, µg (30-60 cm depth)	0.67	0.50	0.11	0.64	0.54	0.17
Nitrate, µg (60-90 cm depth)	0.51	0.50	0.08	0.49	0.52	0.17
Dehydrogenase, µg TPF[a]	2.75	2.82	0.16	2.88	2.69	0.06
Total N, mg	0.74	0.70	0.04	0.75	0.69	0.04
Organic C, mg	7.25	7.83	0.50	7.60	7.48	0.41
Olsen P, µg	13.6	13.7	1.09	14.8	12.5	2.35
Olsen K, µg	182	187	12.3	194	175	11.5
Zinc, µg	1.74	1.34	0.48	1.36	1.72	0.51
Iron, µg	8.03	8.06	0.04	8.38	7.71	0.37
Copper, µg	0.77	0.77	0.03	0.81	0.74	0.06
Manganese, µg	9.35	9.58	0.36	9.79	9.14	0.85
Soil property						
C to N ratio	9.87	11.4	0.66	10.4	10.8	0.32
pH	8.19	8.21	0.03	8.21	8.18	0.02
EC, dS m^{-1}	0.28	0.29	0.01	0.29	0.28	0.03

[a] TPF = triphenyl formazan

[b] ND = no detection

other studies showing greater N-availability in leguminous systems (Cadisch, Schunke, & Giller, 1994), yet contradict results discussed by Hooper and Vitousek (1998) where they expected greater N immobilization in perennial bunchgrasses but observed varying results, which were largely dependent on season, and in some cases (i.e., November) N-fixing legumes showed greater immobilization than bunchgrasses. Supporting other grazing preference observations (Rutter, 2006), the grazing portion of this study (Maughan et al., 2014) showed preference for legumes, which could explain greater soil NO$_3$ in the legume strips. However, separate from scanning observations of forage preference, overall time calves spent in plots was quite evenly distributed as observed in the field, and by indication of fecal spots.

Interestingly, we observed 3.4-fold greater soil NO$_3$ ($p = .01$) in saponin-containing alfalfa plots than in CT-containing sainfoin plots at 0 to 30 cm depth (Table 2). Both sainfoin and alfalfa fix N efficiently, yet Krall and Delaney (1982) found sainfoin was more proficient at N-fixation, which implies there would subsequently be more available soil N in sainfoin plots. Plant biomass over the 2012 season was greater in sainfoin than in alfalfa ($p < .01$), with no differences in September 2012 ($p = .93$), which
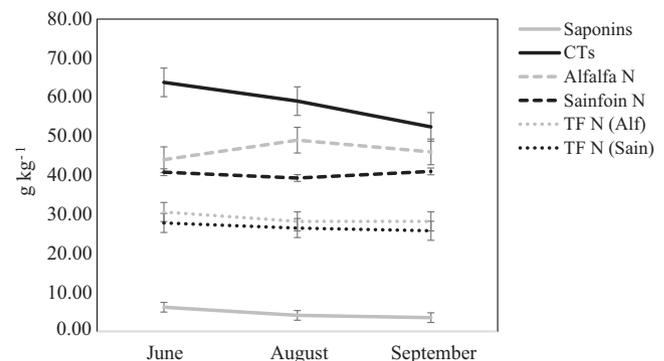
FIGURE 1 Main effect means and standard error bars from plant tissue responses shown over the 2012 growing season (June, August, and September) including condensed tannins (CT) and total N in sainfoin, saponins and total N in alfalfa, and total N in tall fescue (TF) monoculture strips growing in the alfalfa (Alf) or sainfoin (Sain) plots

was nearer to the time of our soil collection (data not shown; see Maughan et al., 2014). Moreover, differences in soil total N between legume plots was negligible ($p = .75$; Table 2), as were differences in plant tissue total N between alfalfa and sainfoin ($p = .07$; Figure 1) over the 2012 season.

TABLE 2 Soil results presented from the end-of-study October 2012 field experiment. Main effect means and standard errors (SE) for forage type (grass vs legume) and legume type (tannin-containing sainfoin vs saponin-containing alfalfa, without tannins) are shown. There were no interaction effects for any soil variables, nor depth interaction effects

Soil property, g^{-1} soil	Forage			Legume		
	Grass	Legume	SE	Alfalfa	Sainfoin	SE
Ammonium, µg (0-30 cm depth)	0.33	0.00	0.22	0.00	0.30	0.33
Ammonium, µg (30-60 cm depth)	0.17	0.04	0.16	0.00	0.31	0.24
Ammonium, µg (60-90 cm depth)	0.00	0.00	0.13	0.00	0.02	0.03
Nitrate, µg (0-30 cm depth)	1.54 [*]	8.06 [*]	2.36	7.82 [**]	1.78 [**]	0.74
Nitrate, µg (30-60 cm depth)	0.81	1.43	0.52	1.48	0.77	0.78
Nitrate, µg (60-90 cm depth)	0.00	0.87	0.40	0.59	0.24	0.40
Dehydrogenase, µg TPF (0-10 cm depth)[a]	10.3	9.86	0.85	9.44	10.7	0.89
Dehydrogenase, µg TPF (10-20 cm depth)	5.43	4.34	0.48	4.79	4.97	0.26
Dehydrogenase, µg TPF (20-30 cm depth)	3.70	3.24	0.35	3.44	3.50	0.15
Total N, mg	1.23	1.25	0.05	1.24	1.25	0.04
Organic C, mg	7.82	7.12	0.51	7.68	7.25	0.49
Olsen P, µg	7.77 [*]	10.20 [*]	1.55	9.03	8.92	1.60
Olsen K, µg	199 [*]	140 [*]	17.8	177	162	19.2
Zinc, µg	1.40	1.43	0.26	1.41	1.42	0.24
Iron, µg	10.7	9.86	0.87	10.4	10.2	0.86
Copper, µg	1.32	1.49	0.45	1.57	1.23	0.45
Manganese, µg	10.1	9.74	0.64	9.85	10.0	0.64
Phenol Oxidase, µg Dopachrome	1.28 [**]	1.44 [**]	0.05	1.33	1.39	0.06
Microbial respiration, µg	4.41 [**]	2.96 [**]	0.33	3.56	3.81	0.36
Microbial biomass, µg	649	668	46.8	653	664	54.3
Readily Mineralizable C, µg	28.0 [**]	21.6 [**]	2.07	25.3	24.3	2.09
Microbial biomass C/Organic C	836	951	69.4	864	922	79.8
qCO$_2$ (Mic. respiration Mic. Biomass^{-1})[b]	0.007 [*]	0.005 [*]	0.001	0.005	0.006	0.001
Porosity, mg	5.18	4.97	0.14	5.12	5.03	0.13
Soil property						
C to N ratio	6.38	5.69	0.42	6.21 [*]	5.85 [*]	0.40
pH	8.42	8.40	0.02	8.41	8.41	0.02
EC, dS m^{-1}	0.22	0.21	0.03	0.23	0.20	0.03
Bulk Density, g cm^{-3}	1.28	1.33	0.04	1.29	1.32	0.03

[*]Significant at the .05 probability level

[**]Significant at the .01 probability level

[a]TPF = triphenyl formazan

[b]qCO$_2$ = microbial efficiency

We extracted CP data from the overlapping grazing study (Maughan et al., 2014), which showed greater CP concentration ($p = .001$) in alfalfa than in sainfoin over the 2012 season. This could explain the greater soil NO_3 in alfalfa plots. However, when each collection time was compared, only one time (15 August) showed significant differences between alfalfa and sainfoin (Table 3), and differences in CP were less pronounced in September, nearer the time of our soil collection.

Furthermore, total N content in TF was greater ($p = .04$) in TF monoculture strips growing in saponin-containing alfalfa plots than in CT-containing sainfoin plots in 2012 (Figure 1), indicating greater plant-available N in alfalfa plots. Prior research conducted by Ta and Faris (1987) using the ^{15}N dilution method showed that alfalfa 'excretes' more N than red clover and birdsfoot trefoil legumes, yet there was no comparison to sainfoin in the study. Dubach and Russelle (1994) compare alfalfa and birdsfoot trefoil and conclude that these two legumes differ in their mechanisms of N contributions to soil, alfalfa transferring more through root decomposition while trefoil transfers more through nodule decomposition, but differences between

TABLE 3 Compiled data extracted from overlapping grazing study (Maughan et al., 2014) showing crude protein differences between alfalfa and sainfoin legumes in 2012

Collection date	Crude protein		
	Alfalfa	Sainfoin	*p*-value
	————mg g^{-1}————		
6 June	192	167	0.98
3 July	166	140	0.97
25 July	249	179	0.07
15 Aug.	227	151	0.04
5 Sept.	216	168	0.46
11 Sept.	203	189	1.00

the two legumes were negligible. Although we did not assess root:shoot, a study comparing alfalfa and sainfoin showed that root:shoot biomass was significantly greater in sainfoin than alfalfa (Bingcheng, Shan, Li, & Jiang, 2007).

In 2012, combined means of soil NO_3 in sainfoin legume strips (3.03 µg g^{-1}) were not significantly different to soil NO_3 in TF monoculture strips growing in sainfoin plots (0.53 µg g^{-1}; $p = .40$; SE = 1.73) nor TF monoculture strips growing in alfalfa plots (2.55 µg g^{-1}; $p = .96$; SE = 1.73). From baseline soil results in 2009 to end-of-study soil results in 2012, alfalfa plots increased in soil NO_3 ($p < .001$) but sainfoin plots showed no differences ($p = .99$). This may suggest CT-containing sainfoin forages inhibited nitrification, which would support studies in boreal forest soils (Adamczyk et al., 2013, 2019; Smolander et al., 2012). However, our measurements did not assess denitrification as it was outside the scope of our experimental capacity. Tannins may precipitate proteinaceous substances such as organic N compounds, typically at low to neutral pH (Adamczyk, Salminen, Smolander, & Kitunen, 2012; Salminen & Karonen, 2011), yet soil from our study plots was more alkaline (pH > 7). Therefore, if CT-containing sainfoin forages inhibited nitrification, it may be due to providing more recalcitrant C substrate for soil microbes, and/or being toxic to soil microbes (Smolander et al., 2012).

In a review of studies evaluating the influence of CT on C and N mineralization and soil microbial communities, Smolander et al. (2012) found CT have mostly inhibitory effects. Yet, apart from soil NO_3, our 2012 data revealed no differences in microbial activity between saponin-containing alfalfa and CT-containing sainfoin plots (Table 2). However, TF monoculture strips had greater soil microbial respiration ($p = .01$) and readily mineralizable C ($p = .01$; Table 2) than legume strips, which may indicate microbial inhibition from both the legumes. Alternatively, lower microbial respiration in legumes could

indicate C-limitation, as readily mineralized C was greater in TF monoculture strips than legume strips. Though lower microbial respiration in legumes could be due to the soil disturbance during the reseeding event in the legume strips in early summer 2011. Zak, Holmes, White, Peacock, and Tilman (2003) evaluated soil microbial activity with different plant species and found greater microbial respiration with increased plant diversity, which was largely due to increased plant production. The studies of Zak et al. (2003) included *Lespedeza capitata*, known to contain CT, among other legumes, grasses, forbs, and trees, yet the study did not distinguish between individual species or inherent PSM and the potential ensuing effects on soil microbial activities.

Both legumes showed greater ($p = .02$; Table 2) microbial efficiency (qCO_2) than TF monoculture strips, indicating greater substrate quality (e.g. nutrient availability) in legumes. Consistent with our data, in plant-biodiverse experimental plots, Eisenhauer et al. (2010) found overall greater microbial respiration and microbial biomass in plots containing grasses than plots containing legumes, while the plots with legumes had greater microbial efficiency. The lack of difference ($p = .68$) in qCO_2 between CT-containing sainfoin plots and saponin-containing alfalfa plots may suggest that CT-containing sainfoin did not affect the ability of microbes to process organic matter anymore than saponin-containing alfalfa forages. This could imply soil microbial inhibition by both CT and saponins, consistent with other studies showing terpenes and CT having similar effects on soil nutrient cycling (Adamczyk et al., 2013). Supporting this hypothesis, legumes showed greater phenol oxidase activity ($p = .001$; Table 2) than TF monoculture strips, indicating the presence of phenolic molecules (Sinsabaugh, 2010) in the legumes.

Covariate analyses showed similar soil organic C from 2009 to 2012 between TF and legume strips, and between alfalfa and sainfoin legumes. Yet total soil N increased ($p < .0001$) from 2009 to 2012 in all treatments. Thus, C to N ratio decreased in all treatments from 2009 to 2012 ($p < .0001$). Between legumes, results from 2012 showed saponin-containing alfalfa plots having greater ratio of C to N than CT-containing sainfoin plots ($p = .04$; Table 2), yet total N differences were negligible ($p = .75$), as were organic C differences ($p = .17$; Table 2). Variation in soil K between TF and legume strips widened from 2009 ($p = .99$) to 2012 ($p = .01$). As legumes use more soil K, unsurprisingly soil K in TF strips was greater ($p = .02$) than legumes. Soil P decreased from 2009 to 2012 ($p < .001$), particularly in TF strips ($p = .001$) and alfalfa strips ($p = .01$). Results from 2012 showed greater soil P in legume than TF monoculture strips ($p = .05$; Table 2).
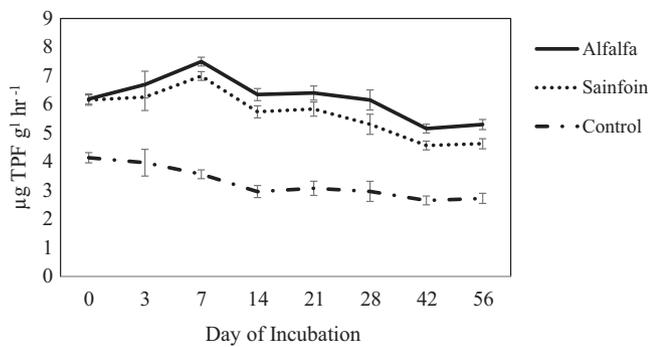
FIGURE 2 Incubation study – Main effect means and standard error bars from dehydrogenase activity (DHEA), measured in μg triphenyl formazan (TPF) g^{-1} hr^{-1}, from cattle feces from two different diets of either CT-containing sainfoin, saponin-containing alfalfa, and a control without the addition of feces, measured eight times over 56 d

FIGURE 3 Incubation study – Main effect means and standard error bars from NO$_3$ results from cattle feces additions from two different diets of either CT-containing sainfoin or saponin-containing alfalfa (without tannins), and a control without the addition of feces, measured eight times over 56 d

Plant secondary metabolites and total N in plants typically fluctuate over time (Cheeke, 1998; Clemensen et al., 2017; Tava, Odoardi, & Oleszek, 1999). However, throughout this study alfalfa, sainfoin, and TF, respectively, showed no differences over time in N (p = .47; Figure 1) or saponin concentration (p = .51); N (p = .56) or CT (p = .07); or N (p = .71), with no interaction effects for each variable within each species. The lack of differences among species could be due to a small sample size.

S. Adamczyk et al. (2013) found that larger terpenes such as saponins show similar patterns of decreased soil N mineralization and nitrification as seen with monoterpenes and with tannins. In our study, CT in sainfoin legumes averaged 58.9 g kg^{-1} whereas saponins in alfalfa averaged 5.7 g kg^{-1} during the 2012 grazing season (Figure 1). This substantial difference in PSM concentration between alfalfa and sainfoin could help explain the considerable difference in soil NO$_3$ concentration between plots of the two legumes, indicating greater saponin concentrations in alfalfa may influence soil nutrient cycling more. However, Lu and Jorgensen (1987) reported impacts of alfalfa saponins on rumen microbes at 2 and 4% concentration.

3.2 | Laboratory incubation study

Feces from alfalfa-TF diets had 7.28 pH, 3.0 mg N g^{-1}, and 47.8 mg C g^{-1}. Feces from sainfoin-TF diets had 7.26 pH, 3.6 mg N g^{-1}, and 44.8 mg C g^{-1}. We hypothesized that feces from CT-containing sainfoin diets would inhibit microbial activity more than feces from saponin-containing alfalfa diets, without tannins. Supporting our hypothesis, combined means of DHEA during our incubation study were greater (p = .03) in feces treatments from alfalfa diets than from sainfoin diets (Figure 2), indicating

reduced microbial activity in feces treatments from CT-containing sainfoin diets. However, this difference could be because of greater CT concentration in sainfoin than saponin concentration in alfalfa, as shown in the field data results. Dehydrogenase activity fluctuated throughout the incubation in both feces treatments (alfalfa-TF and sainfoin-TF) and the control (soil without feces) (p < .001), while both feces treatments showed greater (p < .001; Figure 2) DHEA than the control, supporting other studies showing increased DHEA with N inputs (Chu et al., 2007; Cooper & Warman, 1997). Dehydrogenase enzyme activity may indicate overall soil microbial activity (Wolinska & Stepniewsk, 2012), and represents a biological indicator of soil health (Chellemi & Porter, 2001). Although we did not measure CT in fecal samples, recent results suggest that the concentration of CT in cattle feces is proportional to the concentration of CT in the forage consumed (Stewart et al., 2019).

Cattle feces collected from both the alfalfa and sainfoin diets had relatively low C to N ratio (< 18), which generally results in greater mineralization (Robertson & Groffman, 2007). On day 0 there were no differences in C to N ratio (p ≥ .97), organic carbon (p ≥ .18), or total N (p ≥ .99) between both feces treatments and the no feces control. From day 0 to day 3 there were no differences in NO$_3$ between treatments (p = 1.0; Figure 3), yet from day 7 through 28 the control treatment showed greater NO$_3$ (p < .001) than both feces treatments. This may suggest nitrification inhibition from treatments with feces of both diets (CT-containing sainfoin and saponin-containing alfalfa). Our results support other soil manure incubation studies showing initial immobilization of N with feces additions (Abbasi, Hina, Khalique, & Khan, 2007; Baitilwake et al., 2012; Probert, Delve, Kimani, & Dimes, 2005). Lower NO$_3$ in both feces treatments could be explained simply by fecal manure having more recalcitrant
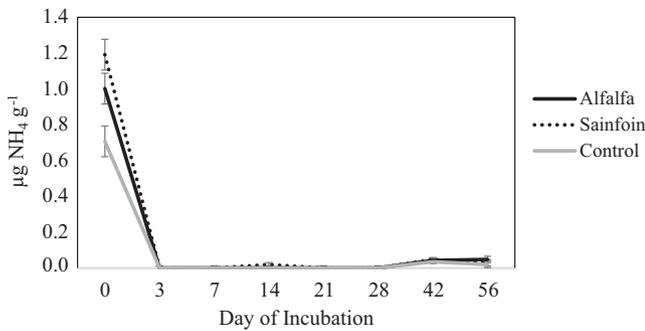
FIGURE 4 Incubation study – Main effect means and standard error bars from NH_4 results from cattle feces additions from two different diets of either CT-containing sainfoin or saponin-containing alfalfa (without tannins), and a control without the addition of feces, measured eight times over 56 d

substrate which is digested (mineralized) more slowly, releasing available N more gradually. Towards the end of the incubation study NO_3 appeared to increase in alfalfa feces treatments while appearing to decrease in sainfoin feces and control treatments. Unfortunately, the incubation study ended at day 56 when shifts in NO_3 levels may have been emerging, with NO_3 appearing to increase in the alfalfa diet treatment.

Decreased nitrification in both feces treatments suggests that feces from CT-containing sainfoin and saponin-containing alfalfa diets may inhibit nitrification, which may thereby increase N immobilization. Delve et al. (2001) emphasize how the quality of manure is greatly influenced by what animals consume, including how diets with CT render feces with greater and more recalcitrant N. This may suggest that incorporating CT- or saponin-containing forages into pasture systems may slow the release of plant-available N, reducing the potential loss of N in pasture agricultural systems.

Ammonium concentration on day 0 was greater ($p \leq .05$) in both the feces treatments than the control, with no difference between feces type ($p = .67$; Figure 4). Ammonium then decreased to undetectable levels from day 3 to day 42, except for the sainfoin diet treatment which showed traces (0.02 $\mu g\ NH_4\ g^{-1}$) on day 14. Proceeding day 0, NH_4 was most likely nitrified to NO_3, due to the observed increase in NO_3 concentration for all treatments from days 0 to 3, or immobilized by microorganisms, fixed to exchange sites, or volatilized. Unfortunately, our measurements did not assess denitrification as it was outside the scope of our experimental capacity. Traces of NH_4 (0.02–0.04 $\mu g\ g^{-1}$) were then observed in all treatments on days 42 and 56, suggesting NH_4 was released from exchange sites and/or the immobilized N was mineralized at this point during the incubation period. Interestingly, when the sainfoin feces

treatment showed traces of NH_4 on day 14, NO_3 in this treatment was at its lowest point (4.14 $\mu g\ NO_3\ g^{-1}$).

From the beginning (day 0) of the incubation study to the end (day 56), total N means in all treatments increased ($p = .0003$) from 1.10 to 4.26 mg g^{-1}, yet there were no differences between treatments ($p = 1.0$), nor interaction effects ($p = .92$). Our observed increase in N over the incubation study is consistent with other incubation studies (Abbasi et al., 2007; Baitilwake et al., 2012), showing an overall increase in total N.

There were no differences ($p = .90$) in organic C from day 0 to 56 (11.3 to 11.2 mg g^{-1}), with no interaction effects between treatment and day ($p = .81$). Organic C was greater ($p = .02$) in the alfalfa feces treatment (12.8 mg organic C g^{-1}) than the control treatment (9.59 mg organic C g^{-1}). The increased organic matter content from the alfalfa feces treatment enhancing DHEA supports observed correlations between DHEA and soil organic C (Burgos, Madejón, & Cabrera, 2002), although incubation studies are not representative of field studies. As total N increased and organic C remained constant in all treatments, C to N ratio decreased ($p < .0001$) from day 0 to 56 in all treatments (10.7 to 3.02), with no differences between treatments ($p = .53$) and no interaction effects between treatment and day ($p = .92$).

4 | CONCLUSIONS

We hypothesized that the presence of CT in grass-legume systems would inhibit N nitrification, which could then mitigate soil N loss in pasture systems. Our results show greater soil NO_3 in alfalfa plots than in tanniferous sainfoin plots, suggesting nitrification inhibition from tannins in the sainfoin pasture system. However, more rigorous experiments are required to support our hypotheses. This study had the challenges inherent of grazing experiments (i.e., reduced sample size) aimed at illuminating soil responses to shifts in plant species and/or management. An additional layer of complexity arises when delving into the potential effects PSM may have amid soil systems in field experiments. This research requires further testing which could include more specific soil incubation studies involving particular PSM. It may also be of interest to standardize the influence of PSM on soil processes, by evaluating a gradient of particular metabolites to determine quantifiable thresholds which prompt changes in soil dynamics. Field experiments might include comparisons between different cultivars of each legume, such as comparing alfalfa varieties Vernal and Lahontan (a low saponin variety) to distinguish the possible influence of saponins on pasture soil. The use of resin bags and/or Plant Root Simulator Probes (PRS probes) may also help elucidate these

processes. Additionally, responses may not be attributable to just one specific metabolite but to different mixtures of metabolites. We did not examine the effects of other PSM in this study, nor the influence of what the novel endophyte from TF may have on soil processes.

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