Effects of incorporation of sainfoin (*Onobrychis viciafolia* Scop.) with cool season grasses on *in vitro* digestibility and CH_4 emission

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Abstract
Sainfoin (*Onobrychis viciafolia* Scop.) is an important non-bloating perennial leguminous forage. The tannins in sainfoin alter protein metabolism in the rumen and have been implicated in altering both nitrous oxide and methane emissions. However, the effect of sainfoin when mixed with cool-season forages is unknown. Therefore, in this study, we evaluated the *in-vitro* fermentation of sainfoin hay mixed with two other perennial cool-season forages, meadow bromegrass and orchardgrass at five ratios (0:100, 25:75, 50:50, 75:50, and 100:0). Data on dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), gas production (GP) methane (CH_4) emissions, and ammonia production were collected 48 h post incubation. Ruminal fluid was sourced from three heifers fed with forage hay. Incubations were conducted with and without PEG (polyethylene glycol) as PEG negates the biological activity of tannins. Sainfoin had a higher nutritive value than the other two grass species as evidenced by the higher proportion of total nitrogen and lower proportion of ADF and NDF. The change in DMD, ammonia-N, NDFD, GP, and CH_4 emissions between sainfoin and grass only hay were 3.1, 9.2, -36.8, -1.76, and -1.2% respectively with the intermediate results for the mixture. The inclusion of sainfoin with cool-season grasses has positive effects on ruminal fermentation and lowered *in vitro* methane emissions as compared to grass alone.

Introduction
The fear of pasture bloat prevents Canadian cattle producers from using pure stands of 'Queen of forages' (alfalfa) pastures, and it is estimated that Canadian cattle producers forego a revenue stream of $30 to $50 million per year as a result. Sainfoin is a bloat-free (Berg et al., 2000) condensed tannin (CT) containing perennial forage legume (Wang et al., 2006) that has the capacity to produce high DM yield (Acharya et al., 2013; Goplen et al., 1991) and high-quality forage, (Frame et al., 1998; Sottie et al., 2014) for grazing or preservation as hay or silage. In addition, when sainfoin is consumed, the condensed tannins in sainfoin reduce the proteolysis of proteins by forming tannin/protein complexes in the rumen. These complexes disassociate in the abomasum, enabling the plant protein to be digested to amino acids that can be absorbed in the small intestine (McMahon et al., 1999). Other studies have also suggested that the tannins in sainfoin also reduce ruminal CH_4 emissions.

The advantages of incorporating legumes into perennial grass stands for improving productivity and nutritive value have been well documented (Lüscher et al., 2014). Earlier grass/sainfoin mixtures indicated an unfavorable grass-sainfoin interaction as sainfoin lacked the ability to persist in mixed grass stands (Cooper, 1979; Hanna et al., 1977; McGinnies & Townsend, 1983; Poudel & Acharya, 2022). However, newer sainfoin populations developed by Lethbridge Research and Development Center are more compatible with mixed grass pastures such as those that contain orchardgrass (Poudel & Acharya, 2022). The percentage DMY of new sainfoin populations in sainfoin-orchardgrass mixtures increased linearly over successive harvests under irrigation and remained constant under dryland conditions. However, the effects of sainfoin on the nutritive values of the sainfoin-grass mixtures has not been studied. Therefore, the objective of this study was to assess the impact of mixing different levels of sainfoin with orchardgrass or meadow bromegrass on the *in-vitro* ruminal fermentation.

Methods
Whole plant sainfoin (*Onobrychis viciafolia* Scop. cv. AC Glenview) mixed with orchardgrass (*Dactylis glomerata* cv. Greenview; OG) or meadow bromegrass (*Bromus commutatus* cv. AC Admiral; MB) were collected from two replicated plots that were seeded in 2019 at Lethbridge, AB. Samples were hand-harvested at ~10% bloom of sainfoin on the first spring regrowth using a pair of scissors in 2021 and 2022. The samples were then hand-separated by species and dried in a forced-air oven at 55°C for 72 h. The dried samples were ground using a Wiley mill to pass through a 1.0 mm screen. Sainfoin and grass samples

prepared from each plot of each year were then combined into ratios (sainfoin:grass; DM basis) of 0:100, 25:75, 50:50, 75:25, and 100:0 to be used for *in vitro* assessment. The experiment was conducted using the procedure described by Peng et al (2020). Briefly, 0.5 g of each forage mixture in F57 filter bags was anaerobically incubated in a serum vial at 39 °C with mixed rumen microbial inoculum with or without addition of polyethylene glycol (PEG; MW 3,500) for 48 h. Polyethylene glycol specifically binds with CT H-bonds to form PEG-CT complexes without affecting other compounds in the plant and therefore specifically inactivates CT activity. For each sample, two runs of incubation were conducted with two replicate vials in each run. The gas production (GP) was determined at 6, 9, 12, 24 and 48 h post-inoculation by measuring gas pressure of the headspace using a pressure transducer (model 15078–193; Fisher Scientific, Pittsburgh, PA, USA) that was then converted to a volume (mL) estimate using the equation of Mauricio et al. (1999). GP = 0.18 + 3.697Pt + 0.0824Pt2, where GP is gas production, mL; Pt is pressure transducer reading value, psi. Headspace gas samples were taken and analyzed for CH_4 concentration for calculation of CH_4 production. At the end of 48-h of incubation, residues were processed and analyzed for DM (and neutral detergent fibre (NDF) for determinations of *in vitro* DM (DMD) and NDF disappearance (NDFD) by subtracting the losses of DM and NDF from the bags from the initial DM and NDF incubated. Fermentation fluid was sampled and processed to analyze ammonia-N as described by Wang et al (2000).

Dry matter and organic matter (OM) were determined following AOAC, # 930.15 and AOAC, # 943.01 respectively (AOAC, 1999). Total N (TN) was estimated by flash combustion analysis using a NA1500 nitrogen analyzer (Carlo Erba Instruments, MI, Italy). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an Ankom 200 system (Ankom Technology Corp., Fairport, NY, USA) as described by McGinn et al. (2004). Data were initially analyzed using the MIXED procedure of SAS 9.4 by including grass type, harvest year, ratio of sainfoin and grass and PEG treatment in the model. No difference was observed between the two years and the effects of different ratios of forages and PEG were similar between the two grasses. Therefore, a final statistical analysis was conducted for each grass with different forage ratio and PEG treatment as a fixed effect and plot, harvest year, replicate vials and run of the incubation as a random factor. Differences between treatments mean were determined by the PDIFF option of LSMEANS in SAS and considered significant at $P < 0.05$. Simple regressions between N content in incubated substrate and ammonia-N concentration in fermentation fluid and between ADF content and DMD and NDFD were performed.

Results and Discussion

Chemical characteristics of sainfoin vs cool season grasses

In general, sainfoin was numerically lower in NDF and ADF, but higher in TN than meadow brome or orchardgrass (Table 1). The crude protein of sainfoin averaged across two years was 14.6% DM, which is comparable to that of alfalfa hay, but interestingly NDF concentration was lower than alfalfa hay suggesting sainfoin may be a more energy-rich forage (Andrzejewska et al., 2020; McDonald et al., 2021). However, there were no differences in the OM content between either of the grass species and sainfoin.

Table 1. Chemical compositions of sainfoin, meadow bromegrass and orchardgrass (mean±SE) harvested at 10% bloom of sainfoin.

Year	Forages	OM %[†]	NDF %	ADF %	TN %
2021	Sainfoin	92.8±0.47	40.1±3.12	29.6±2.59	2.3±0.22
	Meadow bromegrass	92.6±0.40	57.8±0.74	31.1±0.45	2.1±0.23
	Orchardgrass	92.5±0.22	60.8±0.54	33.0±0.18	1.7±0.10
2022	Sainfoin	92.3±0.19	35.3±1.03	26.0±0.63	2.5±0.07
	Meadow bromegrass (MB)	92.6±0.49	57.9±0.37	30.6±0.76	2.1±0.08
	Orchardgrass (OG)	92.1±0.13	56.2±0.14	31.3±0.22	2.1±0.07

[†]OM (organic matter), NDF (Neutral detergent fiber), ADF, (acid detergent fiber), TN (total Nitrogen)

Overall, forage mixtures containing MB had higher DMD (64.5 vs. 57.1; $P < 0.001$) and NDFD (38.8 vs. 32.2; $P < 0.001$) than that containing OG, likely due to the fact that OG contained higher ADF, a factor that can limit DM digestion. With the increase in the proportion of sainfoin in OG, the DMD increased but the increase was not evident when sainfoin was added to MG (Table 2). In contrast, the NDFD decreased in a similar pattern with increasing sainfoin for both grass species despite the fact that as sainfoin increased, the ADF concentration of the mixture decreased. Among the three forage species, only sainfoin contained CT which have been shown to decrease ruminal fibrolytic activity. The addition of PEG increased NDFD of both forage mixtures (MB; 40.1 vs. 37.2; $P < 0.001$); (OG; 33.6 vs. 31.1; $P < 0.01$), indicating CT in the forage mixtures decreased NDFD. Further analysis showed that the effects of CT on NDFD mainly occurred when proportions of sainfoin were high (e.g. higher than 50% for sainfoin:MB mixture). Interestingly, regardless of grass species, as the proportions of sainfoin in the forage mixtures decreased, NDFD initially increase and then decreased. This suggests that the NDFD of the forage mixture is affected by both dietary CT and ADF content, impacting the positive associate effect of combining sainfoin with grass (Figure 1).

Productions of total fermentation gas and CH_4 were not affected by PEG but were affected by the sainfoin/grass ratio and grass species. Regardless of PEG, both GP and CH_4 production decreased as the proportion of sainfoin increased for MB, but it decreased for OG. These results might reflect the difference in their ruminal fermentability as well as interactions among forage components within the mixtures. However, the exact reason for this response is unknown and requires further investigation.

Regardless of grass species and PEG treatment, ammonia-N concentration in fermentation fluid increased with increasing sainfoin in the mixture. The inclusion of PEG significantly increased ($P < 0.001$) ammonia-N concentration with higher proportions of sainfoin in the mixture (Table 2, Figure 2). This result is consistent with the common observation that dietary CT decreases ruminal protein degradation and thereby ruminal ammonia production.

Table 2. Effects of incorporation of sainfoin into grass on dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), gas production (GP), CH_4 production and ammonia-N concentration after 48-h ruminal incubation *in vitro*.

Traits	Grass	PEG	Grass: sainfoin ratio						*P values*	
			0:100	25:75	50:50	75:25	100:0	SEM	Ratio	PEG
			% DM							
DMD†	Meadow	PEG	62.3^{abA*}	59.5^{b}	62.6^{aA}	61.3^{ab}	60.8^{ab}			
		NO-PEG	59.1^{abB}	57.5^{b}	60.4^{aB}	60.9^{a}	59.7^{a}	1.12	<0.001	<0.001
	Orchard	PEG	59.8^{a}	59.2^{a}	56.7^{b}	56.8^{b}	55.9^{b}			
		NO-PEG	58.0^{a}	57.6^{ab}	55.8^{b}	56.2^{bc}	55.6^{c}	1.43	<0.001	0.017
NDFD	Meadow	PEG	31.1^{dA}	35.5^{cA}	42.3^{bA}	44.2^{b}	47.3^{a}			
		NO-PEG	25.4^{dB}	31.4^{cB}	39.0^{bB}	44.3^{a}	45.9^{a}	1.92	<0.001	<0.001
	Orchard	PEG	27.7^{cA}	32.4^{bc}	33.5^{b}	36.3^{ab}	38.1^{a}			
		NO-PEG	22.8^{dB}	28.6^{c}	30.7^{c}	35.4^{ab}	37.9^{a}	1.9	<0.001	0.01
GP	Meadow	PEG	91.2	88.9	93	96.2	97.9			
		NO-PEG	83.8^{b}	85.1^{b}	94.7^{a}	97.6^{a}	98.0^{a}	8.2	<0.001	0.222
	Orchard	PEG	91.6^{ab}	93.4^{a}	85.0^{b}	86.4^{ab}	84.1^{b}			
		NO-PEG	92.1	88.7	87.3	85.8	85.1	6.71	<0.001	0.762
CH_4	Meadow	PEG	11.3^{bA}	11.0^{b}	12.0^{a}	12.0^{a}	12.0^{a}			
		NO-PEG	10.4^{bB}	10.9^{b}	11.9^{a}	12.2^{a}	12.1^{a}	0.65	<0.001	0.291
	Orchard	PEG	11.3^{a}	11.2^{a}	10.0^{b}	10.1^{b}	10.2^{b}			
		NO-PEG	11.1^{a}	10.6^{ab}	10.0^{b}	10.5^{b}	10.3^{b}	0.44	<0.001	0.833
Ammonia-N	Meadow	PEG	21.7^{aA}	21.4^{abA}	20.4^{bA}	19.9^{bA}	18.2^{c}			
		NO-PEG	19.8^{aB}	20.0^{aB}	19.2^{abB}	18.0^{bcB}	17.5^{c}	2.04	<0.001	<0.001
	Orchard	PEG	20.4^{abA}	21.0^{aA}	19.8^{bc}	19.3^{c}	19.2^{c}			
		NO-PEG	18.9^{B}	19.1^{B}	19.1	18.9	19.1	2.09	0.058	<0.001

†DMD (Dry matter disapperance), NDFD (neutral detergent fiber disappearance), GP (gas production)
*different capital letters within a column indicate significant effects of tannin ($P<0.05$); different small letters within a row indicate effects grass:sainfoin ratio ($P<0.05$).

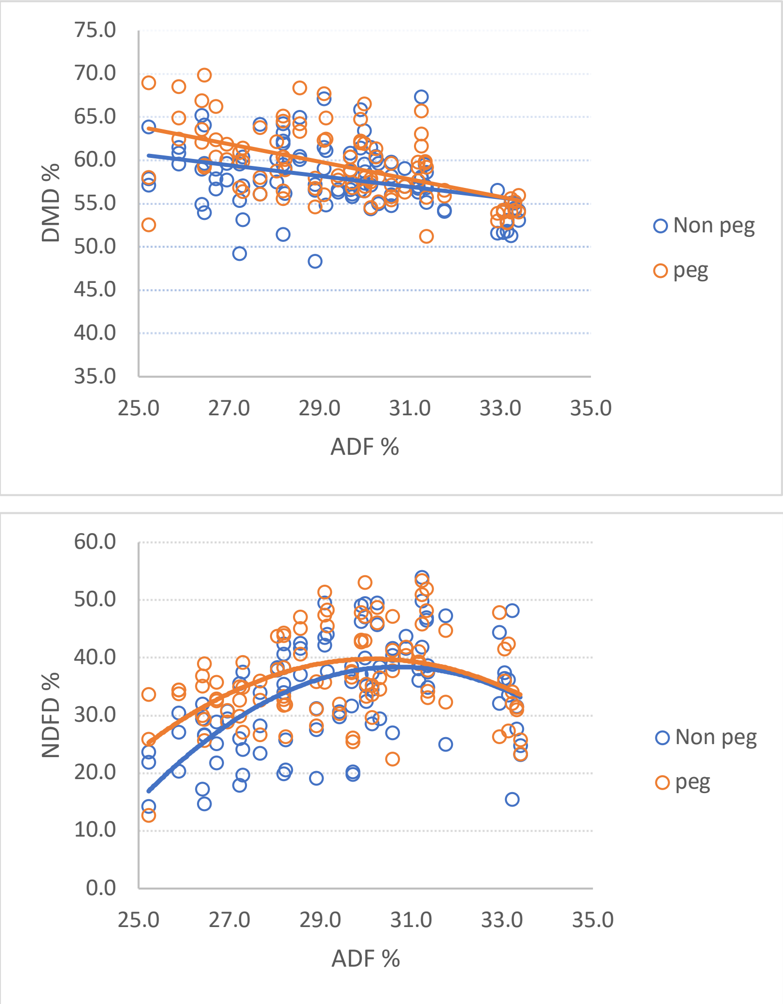

Figure 1. Relationships between dietary concentration of acid detergent fiber (ADF) with dry matter disappearance (DMD) (upper) and neutral detergent fiber disappearance (NDFD) (lower) after 48-h ruminal fermentation *in vitro*.

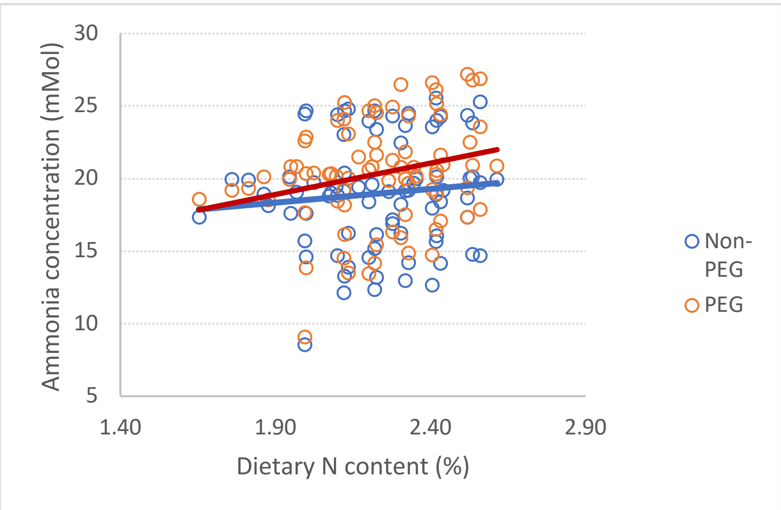

Figure 2. Relationship between dietary-N and ammonia-N concentration in the fermentation fluid after 48-h ruminal fermentation *in vitro*.

Conclusions and/or Implications

This study suggests that incorporation of > 50% sainfoin into either MB or OG hay will improve the ruminal digestibility and reduce GHG emissions *in vitro*. However, further *in vivo* studies with confined and grazing cattle are needed to confirm these responses

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