Effect of replacing alfalfa with panicked-tick clover or sericea lespedeza in corn-alfalfa-based substrates on in vitro ruminal methane production

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ABSTRACT

Methane emissions from ruminant livestock contribute to total anthropogenic greenhouse gas emissions and reduce metabolizable energy intake by the animal. Condensed tannins (CT) are polyphenolic plant secondary compounds commonly produced by some perennial forage legumes that characteristically bind to protein, carbohydrates, and minerals. The degree to which CT may affect ruminant nutrition depends upon the concentration, structural composition, and biological activity of the CT. The objective of our experiment was to determine the effect of replacing alfalfa in a corn-alfalfa-based substrate with a legume containing CT on in vitro CH₄ production and the dynamics of fermentation using an in vitro gas production technique. All fermented substrates contained 50% ground corn as the energy concentrate portion, whereas the forage portion (50%) of each diet was comprised of alfalfa (control) or some combination of alfalfa and sericea lespedeza (SL) or panicked-tick clover (PTC). Our treatments consisted of PTC or SL 15, 30, and 45, which corresponded with 15, 30, or 45% replacement of the diet (alfalfa component) with either PTC or SL. Substrates containing 45% PTC or SL reduced in vitro CH₄ production. Treatments did not affect total gas production as compared with that of the control. Replacement of alfalfa with SL or PTC increased fermentable organic matter (FOM). The PTC treatment increased FOM by as much as 1.8% at the 45% replacement level, whereas FOM of SL 45 was increased by less than 1%. The replacement of alfalfa with PTC increased substrate nutritive value greater than replacement with SL. There were no correlations between any physicochemi-

cal constituent of the substrates and CH₄ production. A combination of factors associated with the inclusion of PTC and SL contributed to the in vitro CH₄ production, and CT in these forages was likely a major contributing factor. Further confirmation of these results on in situ or in vivo animal systems is required. If proven effective in an in vivo production scenario, replacement of commonly fed non-CT-containing legumes, such as alfalfa, with legumes containing CT might be a viable method to decrease the effect of animal agriculture on greenhouse gas production.

Key words: condensed tannin, greenhouse gas, legume, ruminant

INTRODUCTION

Methane emissions from ruminant livestock contribute to total anthropogenic greenhouse gas emissions (Patra, 2012). Conventional ruminant feeding methods that incorporate large amounts of carbohydrates are often identified as factors contributing to further increases in greenhouse gas emissions. Enteric CH₄ production is also a source of energy loss that can be attributed to inefficient digestion, primarily in the rumen of the ruminant animal (Johnson and Johnson, 1995), which results in a reduction of ME intake by the animal (Goel and Makkar, 2012). In intensive production systems, lactating dairy cattle are typically fed a diet consisting of approximately 50% concentrates and 50% roughage. Haylage produced from alfalfa (*Medicago sativa* L.), a forage legume containing no condensed tannin (CT), frequently comprises a substantial portion of the roughage present in these diets, whereas corn and corn by-products are often used as energy concentrates.

Condensed tannins are polyphenolic plant secondary metabolites that demonstrate multiple biological activities in ruminants, including protein binding (Jones and Mangan, 1977), anthelmintic activity (Athanasiadou et al., 2001), and reduction in enteric CH₄ emission

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(Grainger et al., 2009). Accurately determining CT concentration and biological activity in forages is difficult due to great diversity in their structural characteristics and reactivity (Schofield et al., 2001). A variety of methods can be used to estimate CT concentration and bioactivity, each having advantages and disadvantages. For example, the commonly used chemical assay, acid butanol method, which relies on the degradation of interflavan bonds to form anthocyanidin monomers and a subsequent color formation ranging from blue to red depending on the structural characteristics of the CT, may often underestimate CT due to the presence of intractable interflavan bonds (Yokota et al., 2013). This method provides no indication of CT-macromolecule binding activity, so other methods must be used to estimate the concentration of biologically active CT (Hagerman and Butler, 1978; Asquith and Butler, 1985). Condensed tannin concentrations in birdsfoot trefoil have been reported to range from 1 to 3% when determined by acid butanol (Coblentz and Grabber, 2013). However, when the well-established protein-precipitating assay of Hagerman and Butler (1978) was used in our laboratory to analyze the CT in birdsfoot trefoil, no protein binding ability was demonstrated (H. D. Naumann, unpublished data). Our objective was to determine the effects of replacing alfalfa in a simulated dairy-type diet containing 50% corn grain with forage legumes varying in CT concentration on in vitro methane emission and total gas production.

MATERIALS AND METHODS

Experimental Forages

We evaluated plant material consisting of leaves from herbaceous warm-season perennial legumes, *Desmodium paniculatum* (L.) DC. var. *paniculatum* (panicked-tick clover; **PTC**) and *Lespedeza cuneata* (Dum. Cours.) G. Don (sericea lespedeza; **SL**), both known to contain CT that inhibit methane emissions (Animut et al., 2008a; Puchala et al., 2012; Naumann et al., 2013). Forage legumes were collected from multiple 4- × 6-m plots at Stephenville, Texas (32°15'N, 98°12'W, altitude = 395 m) on a Windthorst sandy loam soil [Udic Paleustalf; 10 mg/kg of nitrate-N, 13 mg/kg of P, 206 mg/kg of K, 1416 mg/kg of Ca, 247 mg/kg of Mg, 15 mg/kg of S, 152 mg/kg of Na, 10.12 mg/kg of Fe, 0.59 mg/kg of Zn, 1.10 mg/kg of Mn, and 0.22 mg/kg of Cu using Mehlich III extraction (Mehlich, 1984)]. Leaves were dried at 55°C in a forced-air oven for 48 h, then ground to pass a 1-mm screen in a shear mill (Arthur H. Thomas Co., Philadelphia, PA) and stored for subsequent chemical analysis.

CT Purification, Quantification, and Characterization

Purification. We extracted CT from plant tissue (25 g) with 70:30 (vol/vol) acetone:water. Acetone was removed by evaporation under reduced pressure and replaced by methanol. Condensed tannins were purified from each plant species using Sephadex LH-20 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ; Strumeyer and Malin, 1975). The CT were released from the Sephadex by washing with 70:30 (vol/vol) acetone:water, and the purified CT were lyophilized. The purified CT from each individual species were used to develop species-specific standards for the acid butanol assays and for molecular weight determinations.

Quantification. We prepared plant extracts for each plant species by extracting 50 mg of plant tissue with 1 mL of 50:50 (vol/vol) methanol:water on a rotator for 15 min followed by centrifugation at 16,000 × *g* for 5 min at 4°C. We used the scaled-down protein precipitable phenolics method described by (Hagerman and Butler, 1978) to quantify biologically active CT (those that are readily extractable in the rumen environment and reactive with protein) in 3 replicate plant extracts. A 50-μL aliquot of plant extract supernatant was combined with 250 μL of Buffer A (0.20 *M* acetic acid, 0.17 *M* NaCl), 50 μL of BSA (5 mg/mL in Buffer A), and 50 μL of 50:50 (vol/vol) methanol:water and incubated at room temperature for 30 min before centrifuging at 16,000 × *g* for 5 min at 4°C. Supernatants were removed by vacuum aspiration and the protein-phenolic pellet was washed with 100 μL of Buffer A before recentrifuging and aspirating. Protein-phenolic precipitates (PPP) were dissolved in 800 μL of SDS (1% wt/vol)-triethanolamine (5% vol/vol) before adding 200 μL of FeCl₃ (0.01 *M* FeCl₃ in 0.01 *M* HCl). Absorbance at 510 nm was read after 15 min and PPP were quantified using standard curves prepared with stock solutions of CT extracts (1 mg/mL) in deionized water.

Characterization. We analyzed anthocyanidin monomer composition to determine the cyanidin-to-delphinidin ratio of CT from PTC and SL under conditions similar to those described by Li et al. (2010). A solution of purified CT in acid butanol (0.25 mg/mL) was prepared for each plant species (PTC and SL) and allowed to react in a boiling water bath for 45 min. Cyanidin and delphinidin analyses were conducted on the products of the acid butanol reaction using HPLC analysis (Waters Corporation, Milford, MA) controlled by Breeze Software (Waters Corporation). A 4.6- × 150-mm, 5-μm Waters Symmetry C₁₈ column (Waters Corporation) was used. A 50-μL subsample of the acid butanol-reacted sample was injected prior to separa-

Table 1. Substrate formulations¹ (% DM basis)

Substrate ²	Corn	Alfalfa	SL	PTC
CRN:ALF	50	50	0	0
PTC 15	50	35	0	15
PTC 30	50	20	0	30
PTC 45	50	5	0	45
SL 15	50	35	15	0
SL 30	50	20	30	0
SL 45	50	5	45	0

¹SL = sericea lespedeza; PTC = panicled-tick clover.

²CRN:ALF = corn-alfalfa control; PTC 15 = 15% panicled-tick clover; PTC 30 = 30% panicled-tick clover; PTC 45 = 45% panicled-tick clover; SL 15 = 15% sericea lespedeza; SL 30 = 30% sericea lespedeza; SL 45 = 45% sericea lespedeza.

tion. Separation was achieved using a gradient of 0.13% trifluoroacetic acid in H₂O (mobile phase A) and 0.1% trifluoroacetic acid in acetonitrile (MeCN; mobile phase B) at 0.5 mL/min in a 45-min program as follows: 0 to 10 min, 5% B; 10 to 15 min, increase to 10% B; 15 to 20 min, increase to 20% B; 20 to 25 min, 20% B; 25 to 35 min, increase to 55% B; 35 to 40 min, 55% B; 40 to 45 min, decrease to 5% B. The eluate was analyzed at a wavelength of 550 nm. Peaks were identified by comparing the retention time and spectra with those of commercially prepared cyanidin and delphinidin standards (Sigma-Aldrich Co., St. Louis, MO), and peak areas were calculated with Breeze Software (Waters Corporation).

Experimental Substrates

The formulation and nutritional qualities of the experimental substrates are shown in Tables 1 and 2, respectively. We chose a corn and alfalfa-based control substrate (**CRN:ALF**) because of the prevalence of those 2 ingredients in commonly fed dairy and beef cattle diets in high-intensity production systems. All substrates contained 50% ground corn as the energy concentrate portion. The forage portion (50%) of each

substrate was comprised of alfalfa (control) or some combination of alfalfa and SL or PTC (Table 1).

Cumberland Valley Analytical Services Inc. (Maugansville, MD) analyzed each component for concentrations of NDF, ADF, CP, ash, ether extract, lignin, ADIN, and NPN and reported results on a percent DM basis. We calculated fermentable organic matter (**FOM**) by subtracting ash, lignin, ether extract, ADIN, and NPN from 100.

In Vitro Fermentation

Each treatment was fermented in duplicate in each of 2 fermentation chambers in 2 separate fermentation events. Methane and total gas production were determined using an in vitro gas production technique, as described by Tedeschi et al. (2009). The animals used as rumen fluid donors were housed and cared for according to the guidelines of the Animal Care and Use Committee for the USDA-ARS Southern Plains Agricultural Research Center (FASS, 2010). For each replicate, 2 ruminally cannulated steers, unadapted to forage containing CT and fed bermudagrass (*Cynodon dactylon* L. Pers.) hay, were used for rumen fluid collection. Rumen fluid was collected concurrently from both steers and mixed at the time of collection. The same animals, consuming the same bermudagrass diet, were used for both fermentation events. With the exception of nonsubstrate-containing blanks, a subsample (200 mg) of each forage species was transferred to a 150-mL Wheaton bottle, which served as the fermentation flask. Rumen fluid was filtered through cheesecloth to contain large particulate matter followed by glass wool to filter out small particulate matter while continuously mixing with CO₂ to maintain an anaerobic environment. A total of 14 mL of pH-stabilized (6.8–6.9), CO₂-ventilated, phosphate-bicarbonate medium (Goering and Van Soest, 1970) and 2 mL of boiled distilled water were added to the incubation flask containing the plant

Table 2. Substrate quality constituents¹ (% DM basis)

Substrate ²	CT	NDF	ADF	Lignin	Crude fat	FOM	N
CRN:ALF	0	23.6	24.4	5.3	2.8	78.1	1.94
PTC 15	3.0	22.0	19.3	4.9	3.2	78.5	1.93
PTC 30	6.0	20.5	14.2	4.6	3.6	79.0	1.93
PTC 45	9.0	18.9	9.2	4.2	4.0	79.5	1.92
SL 15	0.9	23.3	20.5	5.3	2.9	78.3	1.95
SL 30	1.8	23.0	16.6	5.4	3.1	78.5	1.97
SL 45	2.6	22.7	12.8	5.5	3.3	78.8	1.99

¹CT = bioactive condensed tannins as protein precipitable polyphenols; FOM = fermentable organic matter.

²CRN:ALF = corn-alfalfa control; PTC 15 = 15% panicled-tick clover; PTC 30 = 30% panicled-tick clover; PTC 45 = 45% panicled-tick clover; SL 15 = 15% sericea lespedeza; SL 30 = 30% sericea lespedeza; SL 45 = 45% sericea lespedeza.

material. Flasks were sealed with greased butyl rubber stoppers and crimp sealed. A 4-mL subsample of mixed rumen fluid was injected into each sealed flask and incubated in each of 2 fermentation chambers once they reached a temperature of 39°C. Pressure sensors were attached to each flask and the pressure within each flask was returned to zero by puncturing with a needle. Pressure readings were made automatically each 5 min for a 48-h period. Pressure data were recorded using Pico Technology software (Eaton Socon, UK). After 48 h of fermentation, incubation flasks were removed from the fermentation chamber and placed in an ice bath to halt any further gas production. Upon completion of the 48-h fermentation, individual gas samples, a mixture of the gases produced during the 48-h fermentation, were taken from the headspace of individual fermentation flasks using a 10-mL gas-tight syringe. Methane gas measurements were made by gas chromatography (Gow Mac Instrument Co., Bethlehem, PA) using a 5% CH₄ standard.

Statistical Analyses

We analyzed the data using PROC GLIMMIX of SAS (ver. 9.3; SAS Institute Inc., Cary, NC). The general linear model for the dependent variables CH₄ and total gas production was a randomized complete block design with 7 treatments. There were 2 separate fermentation chambers which were run 2 different times, resulting in 4 blocks (replications). Each block contained 7 treatments in which 1 of the treatments was a control and the other 6 treatments were arranged as a 2 by 3 factorial (2 legumes and 3 concentrations; 15, 30, and 45% of PTC or SL, **PTC-15**, **PTC-30**, **PTC-45**, **SL-15**, **SL-30**, and **SL-45**, respectively). Each treatment within each block was assigned to 2 fermentation bottles (sampling unit), which were averaged (experimental unit). The statistical model was

$$Y_{ij} = \mu + T_i + b_j + e_{ij},$$

where μ is the overall mean, T_i is the i th treatment fixed effect ($n = 7$), b_j is the j th blocking random effect ($n = 4$), and e_{ij} is the independent and identically, normally distributed random error. The error term is the interaction between treatment and block factors.

One degree of freedom orthogonal polynomial contrast was performed to test for linear, quadratic, and equability of linear and quadratic effects between legumes. Least squares means were estimated and were tested using Fisher's least significant difference ($P \leq 0.05$).

RESULTS AND DISCUSSION

Using SL in agricultural systems may require special management practices due to the possibility of invasiveness (USDA NRCS, 2006). *Sericea lespedeza* is an introduced plant species that occurs in the humid and subhumid regions of North America. It is listed as a noxious weed in Kansas and Colorado, which by law requires annual control to the extent that plant stands are prevented from producing seed. If promoted as forage, however, plant material is likely to be harvested before seed production due to assumed effects of maturity on nutritive value. Panicked-tick clover is a native species that occurs naturally in the humid and subhumid regions of North America. The overall goal of this experiment was to determine the effects of replacing alfalfa in a corn-alfalfa-based diet with forage legumes containing CT (SL or PTC). Substrates were isonitrogenous (19.5 g of N/kg of DM) and contained various amounts of CT depending on the amount of CT-containing forage replacing alfalfa. Physicochemical factors of dairy cattle diets have a direct effect on diet quality, DMI, and, subsequently, ME intake (Allen, 2000). The plant secondary metabolite component of the physicochemical composition has been shown to positively affect ME intake through mitigation of CH₄ production (Bodas et al., 2012).

Quality constituents of experimental substrates are shown in Table 2. We observed an increase in substrate quality with increasing levels of alfalfa replacement by warm-season perennial legumes. The replacement of alfalfa with PTC resulted in a decrease in NDF as compared with CRN:ALF, ranging from a 6.7% decrease for PTC-15 to a 19.9% decrease for PTC-45. The addition of SL resulted in a decrease in NDF ranging from 1.3 to 3.8%. We observed a similar trend relative to ADF. The replacement of alfalfa with PTC reduced ADF by 20.9% for PTC-15 and 62.3% for PTC-45. Alfalfa replacement with SL reduced ADF by 16% and 47.5% for SL-15 and SL-45, respectively. Replacement of alfalfa with warm-season perennial legumes increased FOM of all diets. However, FOM was not affected to the same degree as NDF and ADF. When we replaced alfalfa with PTC, we observed an increase in FOM by as much as 1.8% at the 45% replacement level, whereas FOM of SL-45 was increased by less than 1% as compared with CRN:ALF. Overall, the replacement of alfalfa with PTC increased substrate nutritive value to a greater extent than replacement with SL. Condensed tannin concentration in the substrate increased with increasing replacement of alfalfa with each of the warm-season perennial legumes.

The physicochemical constituents of diets affect the dynamics of ruminal fermentation (Tedeschi et

Figure 1. Effect of replacing alfalfa with paniced-tick clover (PTC) or sericea lespedeza (SL) in a corn-alfalfa (CRN:ALF)-based dairy substrate on ruminal CH₄ production. PTC or SL 15, 30, 45 = percent replacement (15, 30, or 45%) of the alfalfa component of the substrate with either PTC or SL; FOM = fermentable organic matter. Least squares means with the same letter (a–c) are not different ($P \leq 0.05$); SEM = 10.1.

al., 2009). There were no correlations between any physicochemical constituent of the substrates and CH₄ production. It is not likely that any one constituent is solely responsible for the decrease in CH₄. Rather, a combination of factors associated with the inclusion of PTC and SL contributed to the CH₄ produced during *in vitro* fermentation. We speculate that the CT in these forages was a major contributing factor.

The *in vitro* CH₄ production resulting from fermentation of experimental substrates is shown in Figure 1. The test of orthogonal polynomial contrast indicated that CH₄ production decreased linearly ($P < 0.0001$) as alfalfa was replaced with PTC and SL. We observed no difference ($P > 0.10$) in CH₄ production among PTC-15, SL-15, and CRN:ALF (130.5, 132.2, and 110.8 g/kg of FOM, respectively), despite ADF values that differed by as much as 5.1%. Fermentation of PTC-30 and SL-30 produced 85.5 and 89.7 g of CH₄/kg of FOM, respectively, which also did not differ ($P > 0.06$) from the CRN:ALF control despite differences in ADF values ranging from 7.8 to 10.2. However, fermentation of PTC-45 resulted in the least amount of CH₄ produced (38.1 g/kg of FOM), which was 54% less than that of SL-45 (84.0 g/kg of FOM; $P = 0.002$) and 65% less than that of CRN:ALF ($P < 0.0001$). The ADF for PTC-45 and SL-45 treatments differed from the control by 62 and 48%, respectively, which could have contributed to the reduction in CH₄ production. We

should also mention that despite the lack of correlation between crude fat and CH₄, the PTC-45 treatment had the greatest concentration of crude fat, which could have also contributed to a reduction in CH₄. Inclusion of dietary fat in cattle diets has been reported to be a viable method for mitigation of enteric CH₄ (Grainger and Beauchemin, 2011). Total gas production (L/kg of FOM) by fermentation of experimental substrates containing CT did not differ ($P = 0.24$) from that of the CRN:ALF control (Figure 2).

Plant secondary metabolites, namely CT, have been shown to reduce enteric CH₄ production by ruminants (Patra and Saxena, 2010; Puchala et al., 2012). However, apparent differences in CT from different plant sources have an effect on the degree with which ruminal CH₄ production is affected. In our study, PTC-45 and SL-45 effectively reduced ruminal CH₄ production and had CT concentrations of 8.7 and 2.3%, respectively. The PTC-45 treatment resulted in a 65.6% reduction in CH₄, whereas that of the SL-45 treatment was 24.2%. Neither treatment resulted in a decrease in total gas production compared with CRN:ALF, suggesting that overall digestibility was not negatively affected by the presence of CT. The PTC-15 treatment had 3.0% CT, similar to that of SL-45. However, the PTC-15 treatment was not effective at reducing ruminal CH₄ production. We speculate that CT produced by SL are more biologically active (antimethanogenic) than CT

Figure 2. Effect of replacing alfalfa with paniced-tick clover (PTC) or sericea lespedeza (SL) in a corn-alfalfa (CRN:ALF)-based dairy substrate on ruminal total gas production. PTC or SL 15, 30, 45 = percent replacement (15, 30, or 45%) of the alfalfa component of the substrate with either PTC or SL; FOM = fermentable organic matter. Least squares means with the same letter (a,b) are not different ($P \leq 0.05$). SEM = 22.6.

produced by PTC, based on results reported by Naumann et al. (2014) in which CT from SL demonstrated greater than 4 times more biological activity (protein-binding ability) than CT from PTC. In their study, CT from SL had a protein bound-to-protein precipitable phenolic ratio of 1.36 as compared with 0.32 for that of PTC.

Condensed tannin concentration, rather than molecular characteristics of CT, is a common measure in CT-CH₄ mitigation studies. However, when comparing across plant species, it is apparent that CT concentration has little, if any value in predicting the effects of CT on ruminal CH₄ production. Greater birdsfoot trefoil (*Lotus pedunculatus*) with 9.9% CT decreased ruminal CH₄ production by as much as 42% compared with alfalfa, a non-CT-producing forage legume (Tavendale et al., 2005). Tan et al. (2011) measured the effects of leadtree (*Leucaena leucocephala*) CT at dietary concentrations ranging from 2 to 6%. At 6% CT, ruminal CH₄ and total gas were reduced by 63 and 42%, respectively, almost 4% less CT and a 20% greater reduction in CH₄ than that reported by Tavendale et al. (2005). Naumann et al. (2013) evaluated the effects of several warm-season perennial legumes containing CT on CH₄ and total gas production in vitro. In their study, the forage species were fermented alone without inclusion of any other dietary substrates in an effort to screen plants for their ability to reduce CH₄ production. The CH₄ reductions observed were unrealistic, as animals would not likely consume these forages alone, without inclusion of any other dietary substrates. Of the species surveyed, Illinois bundleflower (*Desmanthus illinoensis*), tall bushclover (*Lespedeza stuevei*), powderpuff (*Mimosa strigillosa*), and yellow puff (*Neptunia lutea*) had CT concentrations of 8, 12, 12, and 8%, respectively. These 4 species reduced ruminal CH₄ by 34.8, 87.2, 80.1, and 48.4%, respectively, with no reduction in total gas production compared with a legume containing no CT, rhizoma perennial peanut (*Arachis glabrata*). The PTC and SL evaluated by Naumann et al. (2013) had CT concentrations of 12 and 8%, respectively, and both reduced CH₄ and total gas production. Relative to total gas production, the PTC-45 (9% CT) and SL-45 (2.6% CT) treatments evaluated in our study had no effect on total gas, which may be the result of fermentation of these forages with corn and alfalfa as opposed to just forage alone. The PTC-45 treatment in our study had a CT concentration similar to that of the Illinois bundleflower and yellow puff reported by Naumann et al. (2013). However, we observed a reduction in ruminal CH₄ production by the PTC-45 treatment of 1.8 and 1.4 times more than Illinois bundleflower and yellow puff, respectively. We observed a decrease in CH₄ mitigation

by PTC-45 relative to the PTC fermented alone by Naumann et al. (2013). In that study, CH₄ was reduced by 79% and total gas was reduced by 28% as compared with rhizoma perennial peanut, a forage containing no CT. For CH₄ reduction, the efficacy of PTC-45 was 16% less than that of PTC alone. Despite a reduction in efficacy, the addition of PTC to a simulated dairy diet containing substrates of corn grain and alfalfa reduced CH₄ production by 66% and did not negatively affect total gas production in vitro.

Sericea lespedeza also reduces CH₄ when fed to ruminants in in vivo studies. Puchala et al. (2005) reported that goats grazing on SL pasture produced 57% less CH₄ than goats grazing a mixture of crabgrass and tall fescue. This level of efficacy is similar to that of SL reported on by Naumann et al. (2013), which resulted in a 60% reduction in vitro. Those authors speculated that decreases in CH₄ emissions by goats were due to the greater concentration of CT from SL as compared with crabgrass-tall fescue. Animut et al. (2008b) also reported reductions in CH₄ emissions by goats consuming SL alone or SL mixed with annual lespedeza (*Lespedeza striata*) and quebracho (*Schinopsis* spp.), both CT-containing plant species. Polyethylene glycol was used in Animut et al. (2008b) to inhibit activity of CT and results suggested CT to be a major factor contributing to CH₄ reduction. Puchala et al. (2012) fed SL to goats as either fresh forage or hay and reported a decrease in CH₄ emissions of 37% for fresh forage and 40% for hay as compared with alfalfa. Polyethylene glycol treatment used in Puchala et al. (2012) indicated that CT from SL was primarily responsible for CH₄ mitigation. Reporting the ability of SL to inhibit ruminal CH₄ production is not novel. However, previous studies have focused on the use of SL as forage or feed for small ruminants (sheep and goats) either by itself or as part of forage-based diets. Others have neglected to address the question of whether or not SL will reduce CH₄ when incorporated into a dairy cow-type diet that contains a concentrate fraction such as corn. We observed a decrease in CH₄ mitigation by SL-45 as compared with the SL fermented alone by Naumann et al. (2013). In their study, CH₄ was reduced by 60% and total gas was reduced by 25% as compared with rhizoma perennial peanut. The ability of SL-45 to reduce CH₄ decreased by 60% compared with that of SL alone was reported in their previous study. The SL-45 was not as effective at reducing CH₄ as compared with the sericea lespedeza pasture reported on by Puchala et al. (2005) or sericea lespedeza fresh forage or hay reported on by Puchala et al. (2012). Although less effective as compared with previous reports, the addition of SL to a simulated dairy diet containing substrates of corn grain

and alfalfa reduced CH₄ production by 24% and had no negative effect on total gas production in vitro.

Our analysis indicated that ratio of procyanidin to prodelphinidin of PTC and SL were 60:40 and 0:100, respectively. The structure of CT relative to anthocyanidin composition may be a critical factor in the role of CT bioactivity (Hagerman et al., 1998; Soares et al., 2007; Hagerman, 2012). Anthocyanidins differ in the hydroxylation pattern of the B-ring constituent of the proanthocyanidin. For example, the anthocyanidins propelargonidin, procyanidin, and prodelphinidin possess 1, 2, and 3 B-ring hydroxyl groups, respectively. It is possible that the greater number of hydroxyls present, the greater the biological activity of the CT. This is because hydroxyl groups serve as substitution groups or binding sites for macromolecules, such as protein, carbohydrates, lipids, and minerals. If this characteristic is a determining factor in biological activity (CH₄ mitigation), then the smaller the procyanidin-to-prodelphinidin ratio in CT composition, the greater the biological activity of the CT. Given the reports by (Naumann et al., 2014) that biological activity of CT from SL was greater than biological activity of CT from PTC, we speculated that CT composed primarily of prodelphinidin has greater antimethanogenic properties. Considering the observed procyanidin-to-prodelphinidin ratios of SL and PTC in our study, the theory that hydroxylation pattern is a contributing factor to CH₄ mitigation seems plausible. However, more data from a wider range of CT containing species is needed to draw a conclusion relating procyanidin-to-prodelphinidin ratio to biological activity of CT.

CONCLUSIONS

Results from our study suggest that replacement of alfalfa with 45% of PTC or SL in corn-alfalfa-based substrates will suppress ruminal CH₄ with no compromise in total gas production (i.e., FOM) in vitro. We speculate that CT present in the PTC and SL are in large part responsible for the CH₄ reduction observed. However, the only way to know for sure would be to include purified CT from these forages in in vitro fermentations. Further evaluations of the experimental substrates using whole forages as opposed to only leaf material in in situ or in vivo animal systems would be required before adoption for commercial application. If proven effective in vivo, replacement of commonly fed non-CT-containing legumes, such as alfalfa, with CT-containing legumes might be a viable method to decrease the effect of animal agriculture on greenhouse gas production.

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