Ruminal fermentation and enteric methane production of legumes containing condensed tannins fed in continuous culture

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ABSTRACT

A continuous-culture fermentor study was conducted to assess nutrient digestibilities, volatile fatty acid (VFA) concentrations, microbial protein synthesis, bacterial nitrogen (N) efficiency, and enteric methane (CH₄) production of four 50:50 grass-legume diets, randomly assigned in a 4 × 4 Latin square design. Four legumes with different concentrations of condensed tannins (CT) were tested: alfalfa [ALF; *Medicago sativa* L., non-CT legume]; birdsfoot trefoil [BFT; *Lotus corniculatus* L., low-CT legume]; crown vetch [CV; *Securigera varia* (L.) Lassen, moderate-CT legume]; and sericea lespedeza [SL; *Lepedeza cuneata* (Dum. Cours.) G. Don, high-CT legume]. Orchardgrass (*Dactylis glomerata* L.) was the common forage used in all diets. Four fermentors were evaluated over four 10-d periods by feeding 82 g of dry matter (DM)/d in 4 equal feedings. Methane output was recorded every 10 min. Effluent samples were collected during the last 3 d of the experiment, composited by fermentor and period, and analyzed for pH and VFA, as well as DM, organic matter, crude protein, neutral detergent fiber, and acid detergent fiber for determination of apparent and true nutrient digestibilities. Microbial protein synthesis and bacterial efficiency were estimated by analysis of N flows and purines. The CT concentrations were 3, 21, 38 and 76 g/kg of DM for ALF, BFT, CV, and SL diets, respectively. The SL diet had decreased fiber digestibilities and total VFA concentrations compared with the other diets. This resulted in the least total CH₄ production in the SL diet. Bacterial N efficiency per kilogram of

organic matter truly digested was lower in the SL diet than in the BFT and CV diets. The lowest CH₄ production per unit of digestible nutrients was also found in the SL diet. Further work should be conducted to find optimal diets (by testing other legumes, rations, and sources of CT) for reducing CH₄ emissions without negatively affecting ruminal digestion to maintain or improve productivity.

Key words: condensed tannin, digestibility, legume, methane

INTRODUCTION

Plant secondary metabolites (PSM) such as tannins, saponins, and essential oils are recognized as bioactive modifiers and methanogenesis inhibitors in the rumen (Patra and Saxena, 2010). These PSM are mainly characterized as modulators of microbial populations (Bodas et al., 2012) by changing ruminal fermentation process, modifying nutrient digestion, and altering biohydrogenation of fatty acids (Theodoridou et al., 2010). Incorporating PSM in animal diets have been shown to reduce methane (CH₄) production and decrease risk of metabolic diseases (such as bloat or acidosis), thus improving ruminant productivity and health (Moore et al., 2008; Rochfort et al., 2008).

Tannins are a group of polyphenolic compounds found in forages and other feeds commonly used for feeding ruminants, including trees, shrubs, legumes, cereals, and grains (Patra and Saxena, 2011). Condensed tannins (CT) comprise 1 of 2 large groups of tannins, designated as proanthocyanidins, and are one of the most ubiquitous compounds in the plant kingdom (Yoshida et al., 2005). Condensed tannins are found in the leaves, stems, flowers, and seeds of several legumes (Li et al., 2014). Chemical structure and concentration of CT vary among plant species, growth stages, and growing conditions, such as temperature, light intensity, nutrient stress, and even plant exposure to herbivory (Li et al., 2014). Legumes containing CT are currently gaining interest in ruminant nutrition studies (Broderick et al., 2017) due to interactions with rumen

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microbiota (Díaz Carrasco et al., 2017), which then affect carbohydrate fermentation, protein degradation, and lipid metabolism (Vasta et al., 2019).

Condensed tannins have been shown to prevent bloat (Min et al., 2006), decrease internal parasite loads (Athanasiadou et al., 2001), and reduce enteric CH₄ emissions (Puchala et al., 2012). Overall, the inhibitory effects of CT on ruminal methanogenesis have been ascribed to directly reducing CH₄ production associated with methanogenic archaea or protozoa while indirectly depressing ruminal fiber digestion (Patra and Saxena, 2010). It is generally agreed that adding CT to diets may be predominantly beneficial for reducing degradation of proteins and improving RUP, by decreasing ammonia (NH₃-N) concentrations in the rumen (Patra and Saxena, 2010). The binding ability of specific tannin-proteins determines the effects of CT legumes on protein degradability in the digestive tract by inhibiting the availability of nutrients to be used by rumen microorganisms (Kamra et al., 2008). Although legumes hold promise in terms of CH₄ abatement (Animut et al., 2008a,b), the forage sources and optimum CT concentrations to ensure that CH₄ emissions are lowered without negatively affecting nutrient digestion and animal performance are variable (Williams et al., 2011). Legumes containing low to moderate concentrations of CT (<50 g/kg of DM), such as birdsfoot trefoil (*Lotus corniculatus* L.) and crown vetch [*Securigera varia* (L.) Lassen], may benefit ruminants by improving protein utilization (Aerts et al., 1999) without negatively affecting intake and nutrient digestion (Patra and Saxena, 2011). However, legumes containing high CT concentrations (60–120 g/kg of DM), such as sericea lespedeza [*Lespedeza cuneata* (Dum. Cours.) G. Don], may depress intake, digestive efficiency, and animal productivity (Aerts et al., 1999). Combining forages containing CT with orchardgrass (non-CT) may be an alternative feeding strategy for farmers, particularly producers with grass-fed herds who cannot use supplements to decrease CH₄ emissions in forage-based systems.

To our knowledge, no previous studies have investigated how incorporating legumes of varying CT concentrations into a 50% orchardgrass (*Dactylis glomerata* L.) diet will affect in vitro ruminal fermentation and enteric CH₄ production. Therefore, the objective of the current experiment was to determine the effect of 4 perennial legumes containing increasing CT concentrations on nutrient digestibilities, VFA production, microbial protein synthesis, bacterial N efficiency, and enteric CH₄ production of an orchardgrass-based diet. We hypothesized that the incorporation of a high CT-concentration legume (sericea lespedeza) to a non-

CT orchardgrass diet would reduce CH₄ production; however, there would be a negative effect on ruminal fermentation, specifically nutrient digestibilities, which may have potential to reduce animal productivity.

MATERIALS AND METHODS

Site, Diets, and Experimental Design

This study was conducted at the USDA-Agricultural Research Service Pasture Systems and Watershed Management Research Unit (University Park, PA) from February to April 2016. Orchardgrass herbage was harvested on May 15 and June 27, 2015, from a 2-yr-old monoculture established at the Pennsylvania State University Russell Larson Agricultural Research Farm (Rock Springs, PA). Orchardgrass was harvested at a vegetative stage of growth (36 cm tall; 21% DM), typical of high-quality pastures found in temperate regions of the United States (Soder et al., 2013). Alfalfa [*Medicago sativa* L. ‘Pioneer 55H94’ and ‘WL353LH’] was harvested on August 13, 2015, from 2 separate plots of a multiple-year monoculture established at the Pennsylvania State University Haller Farm (State College, PA) at flowering stage (53 cm tall; 27% DM). ‘Ober’ birdsfoot trefoil was harvested on July 17 and August 27, 2015, from a 1-yr-old monoculture planted at the Russell Larson Agricultural Research Farm (Rock Springs, PA), at a vegetative stage of growth (20 cm tall; 25% DM). The site consisted of a Hagerstown silt loam soil (Fine, mixed, semiactive, mesic Typic Hapludalfs) with 3 to 8% slopes. Potassium and P were applied based on soil test recommendations at the time of planting, and N was applied early- and mid-season using the Pennsylvania State University Extension recommendation for each forage species. Crown vetch was harvested on July 24, 2015 from a multiple-year monoculture established on a commercial farm in Warriors Mark, Pennsylvania, at flowering stage (48 cm tall; 57% DM). Sericea lespedeza was harvested on July 10 and July 30, 2015 from a multiple-year monoculture planted at the University of Kentucky Spindletop Research Farm (Lexington, KY) at flowering stage (90 cm tall; 41% DM). The site consisted of a Bluegrass-Maury silt loam soil (Fine, mixed, active, mesic Typic Paleudalfs) with 2 to 6% slope. Potassium and P were applied based on soil test recommendation at the time of planting, and N was applied early- and mid-season using the University of Kentucky recommendation for sericea lespedeza. Soil tests were routinely conducted in each of the plots under study. A plot harvester (HEGE 212; Wintersteiger AG, Waldenburg, Germany; 1.5-m-wide swath), set to a 10-cm stubble height, was used to harvest all plots

in the morning (at maximum 25–30°C). Within 30 min of harvest, all forages were placed in cloth bags and frozen (–4°C) until being freeze-dried (Ultra 35 Super ES; Virtis Co. Inc., Gardiner, NY). Freeze-dried forage samples were ground to pass through a 2-mm sieve (Wiley mill; Thomson Scientific Inc., Philadelphia, PA).

Continuous-Culture System and Fermentor Operation

The continuous-culture fermentor study was conducted at the USDA-Agricultural Research Service, Pasture Systems and Watershed Management Research Unit (University Park, PA) from May to July 2016. Total DM fed to all fermentors was fixed at 82 g/d for the duration of the experiment. Diets were randomly assigned to fermentors in a 4 × 4 Latin square design during 4 consecutive 10-d periods. Four legumes, containing increasing CT concentrations, were tested: alfalfa (**ALF**; no-CT legume), birdsfoot trefoil (**BFT**; low-CT legume), crown vetch (**CV**; moderate-CT legume), and sericea lespedeza (**SL**; high-CT legume). Orchardgrass was the common ingredient used in all diets. Representative samples of freeze-dried forages were collected from each diet at the beginning of this study and analyzed for nutrient concentrations at a commercial laboratory (Dairy One Laboratories, Ithaca, NY; results are presented in Table 1).

For this study, a 4-unit single-flow continuous-culture fermentor system (Applikon Biotechnology, B.V., Schiedam, the Netherlands; Dillard et al., 2019) was used. Fermentors were fed 4 times daily (0730, 1030,

1400, and 1900 h) in equal proportions to simulate diurnal patterns in grazing. Solid mean retention time and liquid dilution rate of the fermentors were adjusted daily to 24 h and 10%/h, respectively, which was achieved by regulation of buffer input and effluent volume removal. A full description of continuous-culture operation to determine total effluent volume removed per day has been described by Dillard et al. (2019).

Ruminal fluid and digesta samples were collected from a ruminally fistulated, nonlactating, nonpregnant 4-yr-old Holstein cow (794 kg of BW) cared for in accordance with the Pennsylvania State University Animal Care and Use (IACUC no. 46212) guidelines. The donor animal was housed and maintained on a diet of silage, hay, and grain (75:25 forage-to-concentrate ratio, ad libitum) in a feed bunk for a total of 13.8 kg of DM of available feed per cow per day at the Pennsylvania State University Dairy Research Farm (University Park, PA). The total mixed ration contained a vitamin and mineral premix at 1.8% of total DMI, which was included to meet NRC (2001) nutrient recommendations for a nonlactating dairy cow. Approximately 3 h after feeding, 7 L of ruminal fluid was collected with a hand pump into a prewarmed insulated container and maintained at 39°C. Solid digesta was collected by hand from the ventral, central, and dorsal areas of the rumen. Liquid and whole digesta samples were transported to the USDA-Agricultural Research Service laboratory in separate containers. Within 15 min of collection, fluid was strained through 4 layers of cheesecloth and 1.50 L was poured into each of the prewarmed fermentation jars until it cleared the overflow spout. Solid digesta

Table 1. Chemical composition of forages and diets fed during continuous-culture fermentation

Item ¹	Forage					Diet ²			
	Orchardgrass	Alfalfa	Birdsfoot trefoil	Crown vetch	Sericea lespedeza	ALF	BFT	CV	SL
OM (%)	92.6	90.7	93.7	92.5	94.7	91.7	93.2	92.6	93.7
CP (%)	23.0	26.6	17.5	16.1	11.9	24.8	20.3	19.6	17.5
RDP (% of CP)	79.0	82.0	69.0	65.0	32.0	80.5	74.0	72.0	55.5
SP (% of CP)	38.0	51.0	31.0	47.0	30.0	44.5	34.5	42.5	34.0
NDF (%)	55.4	34.7	17.8	54.5	50.8	45.1	36.6	55.0	53.1
ADF (%)	32.4	26.7	12.1	44.4	46.5	29.6	22.3	38.4	39.5
Lignin (%)	6.3	10.0	3.6	12.5	6.3	8.2	5.0	9.4	6.3
NFC ³ (%)	10.8	27.2	55.3	18.6	29.1	19.0	33.0	14.7	19.9
Ether extract (%)	3.40	2.20	3.20	3.20	2.90	2.80	3.30	3.30	3.15
TDN (%)	60.0	59.0	75.0	52.0	63.0	59.5	67.5	56.0	61.5
RFV	107	183	416	93	96	145	262	100	102
CT (g/kg of DM)	3.5	2.3	38.3	72.4	147.7	2.9	20.9	38.0	75.6

¹SP = soluble protein; RFV = relative feed value; CT = condensed tannins.

²Calculated using actual nutrient composition and proportion of individual ingredients (DM basis); ALF = 50% orchardgrass + 50% alfalfa; BFT = 50% orchardgrass + 50% birdsfoot trefoil; CV = 50% orchardgrass + 50% crown vetch; SL = 50% orchardgrass + 50% sericea lespedeza.

³Calculated as NFC (%) = 100 – [CP (%) + NDF (%) + ether extract (%) + ash (%)].

was mixed by hand and 32 g was added to each vessel following the protocol of Dillard et al. (2019).

Each of the 4 periods consisted of a 7-d diet adaptation followed by a 3-d sampling collection. During the 10 d of each period, effluent was collected in 4-L plastic containers located inside of a freezer to maintain an effluent temperature of 4°C and inhibit microbial fermentation (Dillard et al., 2019). During the 7-d adaptation period, the overflow containers were weighed daily and then the contents discarded. During the 3-d collection period, daily effluent collections were mixed according to the methods of Soder et al. (2016).

Daily effluent samples (100 mL/sample) were taken inside of each vessel for protozoal count during the 3-d collection period immediately before the 1030 h feeding. Vessel protozoal cell counts were completed using the Sedgewick-Rafter chamber by Dehority (2005). A 50-mL sample of effluent was also collected on the 3 collection days and strained through 8 layers of cheesecloth. Then, 3 mL of 25% (vol/vol) *m*-phosphoric acid was added to 15 mL of filtrated sample for determination of NH₃-N (Chaney and Marbach, 1962), and for VFA (Erwin et al., 1961) via gas chromatography [Varian 330 Gas Chromatograph (FID detector), Varian 4290 Integrator; Supelco, 1975, modified to use a 80/120 Carbowax B-DA/4% Carbowax 20M column] at the Rumen Fermentation Profiling Laboratory (West Virginia University, Morgantown).

An additional 1 L of effluent was collected each day of the collection period and composited at the end of each period. Composited effluent samples were freeze-dried, ground to pass through a 1-mm sieve, and stored in a sealed plastic bag for later analyses of DM, OM, NDF with α -amylase, ADF, CP (AOAC International, 2006), and total purines (Makkar and Becker, 1999). During the last day of each period, the contents of each fermentor vessel were processed for bacterial isolation according to the methods of Griswold et al. (1996) with the modification used by Soder et al. (2016) of replacing initial centrifugation for blending particulate-phase bacteria for 30 s and strained through 2 layers of 53- μ m Nitex fabric (Wildco, Buffalo, NY) into a 2-L plastic container with 5 mL of 50% sulfuric acid added to retard microbial growth. Concentrations of total purines in effluent and bacterial isolates were then used to partition effluent N flow into bacterial and nonbacterial fractions, and to calculate true DM and OM digestibilities and flows by using the equations of Soder et al. (2016).

Methane Collection and Measurements

Methane measurements were taken every 10 min using a photoacoustic gas monitor (LumaSense Technolo-

gies Inc., Santa Clara, CA) connected to a multiport sampler (CAI, Inc., Orange, CA) that directed the flow of gas from each vessel. Each cycle required 140 cm³ of the approximately 1,500-mL of headspace gas available. Total daily CH₄ production was calculated by determining the difference in CH₄ volume (measured CH₄ concentration multiplied by the headspace volume) between each of the 10-min samples, and then summing these values for every 24-h period during the collection days following the equations of Dillard et al. (2019). Thus, total daily CH₄ production = Σ [CH₄ volume_a – CH₄ volume_b], where CH₄ volume_a equals CH₄ volume determined by multiplying the headspace volume by the measured CH₄ concentration, and CH₄ volume_b equals CH₄ volume 10 min before CH₄ volume_a, over each 24-h period.

Nutrient Analyses

Forage samples of orchardgrass, alfalfa, birdsfoot trefoil, crown vetch, and sericea lespedeza were analyzed by wet chemistry (Dairy One Laboratories, Ithaca, NY) according to the following procedures: DM (method 930.15; AOAC International, 2006), CP (method 990.03; AOAC International, 2006), RDP (Cornell *Streptomyces griseus* enzymatic digestion; Coblenz et al., 1999), soluble protein (SP; Licitra et al., 1996), ether extract (method 2003.05; AOAC International, 2006), and NDF [Ankom Technology method 6 (NDF in Feeds – Filter Bag Technique for A200; Fairport, NY); with heat-stable α -amylase and sodium sulfite used in the NDF procedure (inclusive of ash); Mertens (2002)]. The NFC were calculated using the equation: NFC (%) = 100 – [CP (%) + NDF (%) + ether extract (%) + ash (%)]. Condensed tannin concentrations were determined by following the methods of Grabber et al. (2013), using butanol-HCl-iron assay, at the Department of Plants, Soils, and Climate (Utah State University, Logan, Utah).

Effluent samples and microbial pellets were analyzed for DM, OM (methods 930.15 and 942.05, respectively; AOAC International, 2006), and CP concentrations (micro-Kjeldahl digestion using 75-mL calibrated tubes with CuSO₄/K₂SO₄ catalyst; method 976.06; AOAC International, 2006). The NDF concentration of the effluent was determined by the methods of Van Soest et al. (1991) with α -amylase and sodium sulfite used in the NDF procedure (inclusive of ash). Concentrations of total purines (Makkar and Becker, 1999) in effluent and microbial isolates were used to partition effluent N flow into bacterial and nonbacterial fractions, and to calculate true DM and OM digestibilities and flows. Apparent (DM, OM, CP, NDF, and ADF) and true (DM and OM) digestibilities of nutrients, N fractions

flow, and bacterial efficiency were calculated using the equations previously reported by Soder et al. (2016).

Statistical Analyses

All data were analyzed as a 4×4 Latin square design using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC), fitted to the following model:

$$Y_{ijk} = \mu + P_i + F_j + D_k + e_{ijk},$$

where Y_{ijk} = observations for dependent variables, μ = population mean, P_i = mean effect of i th period, F_j = mean effect of j th fermentor, D_k = mean effect of k th diet, and e_{ijk} = residual error. Period and diet were fixed effects, while fermentor and residual error were random effects.

For all statistical analyses, significance was declared at $P \leq 0.05$, and tendencies at $0.05 < P \leq 0.10$ after application of Fisher's least significance difference protected test. There were no period $\times$ fermentor or period $\times$ diet interactions; therefore, only main effects are reported below.

RESULTS AND DISCUSSION

Diet Composition and Donor Rumen Fluid

Chemical composition of forages and diets are reported in Table 1. The use of composite sampling for nutrient and CT analyses precluded statistical comparisons among diets. The SL diet had the lowest numerical CP, RDP, and SP concentrations, whereas the ALF diet showed the greatest values. The CV and SL diets showed the greatest numerical NDF and ADF concentrations, whereas the BFT diet showed the low-

est values. The BFT diet had the greatest NFC and relative feed value. Concentrations of CT were numerically the lowest in ALF diet and the greatest in the SL diet, with the BFT and CV diets showing intermediate levels.

Concentrations of CT for the birdsfoot trefoil and sericea lespedeza were within the range of those previously found by Jonker and Yu (2017; 21–47 g/kg of DM) and Müir et al. (2018; 121–142 g/kg of DM). However, alfalfa and crown vetch showed numerically higher CT concentrations than those reported by Jonker and Yu (2017; 0.5 g/kg of DM) and Terrill et al. (1992; 16 g/kg of DM), respectively. Those differences might be due to variations in soil fertility (Barry and Forss, 1983), growth conditions (Li et al., 2014), harvesting stages (Schreurs et al., 2007), or methodology used for analyses (Terrill et al., 1992).

While the use of a single donor cow for rumen fluid has been accepted for in vitro research (e.g., Foskolos et al., 2015; Lascano et al., 2016; Dillard et al., 2017), it has become more commonplace to use multiple donor animals to reduce variation in source of rumen inoculum. However, Yáñez-Ruiz et al. (2016) found no research assessing the minimum number of donor animals needed to provide a representative source of rumen inoculum from a statistical analysis perspective. Additionally, animal welfare and institutional animal care and use committee concerns also monitor and emphasize the use of the minimum number of animals in research. Given that the main goal of in vitro systems is to compare relative differences between treatments within a study, and not make absolute numerical comparisons to in vivo conditions, the use of a single donor cow provides such a means when animal numbers are limiting or animal welfare concerns are at the forefront.

Table 2. Nutrient digestibilities of alfalfa, birdsfoot trefoil, crown vetch, and sericea lespedeza fed with orchardgrass during continuous-culture fermentation

Item	Diet ¹				SEM	P-value
	ALF	BFT	CV	SL		
Apparent digestibility (%)						
DM	45.7 ^a	45.6 ^a	44.0 ^b	40.3 ^c	0.66	<0.001
OM	61.9 ^a	61.1 ^a	59.6 ^b	56.1 ^c	0.65	<0.001
NDF	52.9 ^a	53.2 ^a	51.6 ^a	39.4 ^b	1.42	<0.001
ADF	53.4 ^a	53.9 ^a	51.1 ^a	33.8 ^b	1.83	<0.001
True digestibility (%)						
DM	69.2 ^b	75.1 ^a	68.7 ^{bc}	64.1 ^c	1.57	0.006
OM	77.5 ^b	81.3 ^a	76.6 ^b	70.5 ^c	1.02	<0.001

^{a-c}Mean values in the same row with different superscripts differ ($P < 0.05$).

¹ALF = 50% orchardgrass + 50% alfalfa; BFT = 50% orchardgrass + 50% birdsfoot trefoil; CV = 50% orchardgrass + 50% crown vetch; SL = 50% orchardgrass + 50% sericea lespedeza.

Nutrient Digestibilities

Apparent DM and OM digestibilities were lowest ($P < 0.001$) for SL, intermediate for CV, and highest in ALF and BFT diets (Table 2). Apparent NDF and ADF digestibilities were decreased ($P < 0.001$) in the SL compared with the other diets. True DM digestibilities were greater ($P = 0.006$) in BFT. True OM digestibilities were greatest ($P < 0.001$) in the BFT diet and lowest in SL.

Decreased fiber digestibilities of the SL diet were likely a result of high indigestible fiber fractions in that legume compared with the others (Buxton and Redfearn, 1997). The reduced nutrient digestibilities in the SL diet agree with previous results, where tannin extracts or tannin-containing plants are used, at concentrations >5 g of CT/kg of DM, both in vitro (Bhatta et al., 2009) and in vivo (Animut et al., 2008a,b). It is generally suggested that high CT concentrations may have adverse effects on nutrient utilization and productivity of animals, but the response is dependent on the type of tannins (Waghorn, 2008). Moderate to high CT concentrations have been shown to exert antimicrobial actions in the rumen (Patra and Saxena, 2009), which provoke adverse effects on ruminal fermentation and

digestion of feeds, and may explain why nutrient digestibilities were lower for SL than for the other 3 diets. Additionally, depressed fiber digestibilities in SL might be due to reduced degradation of fiber by preventing the attachment of microorganisms to plant cell walls (McAllister et al., 1994), inhibiting microbial degrading enzymes, or altering the functionality of ruminal microorganisms (Jones et al., 1994).

Fermentor pH, Protozoal Counts, and VFA Production

The SL diet had higher ($P < 0.05$) mean pH than the ALF and BFT diets, but was not different from the CV diet (Table 3). Greater pH in SL might be attributed to better buffering capacity in that legume due to higher CT concentrations and lower RDP. Fermentor minimum pH was lower ($P < 0.05$) in the BFT diet than in the CV and SL diets, with ALF diet being intermediate. Maximum fermentor pH was greater ($P < 0.05$) in the SL diet compared with the other 3 diets. According to Van Soest (1994), low-fiber diets (such as those found at the current trial in BFT) tend to have higher rates of digestion and acid production, thereby substantially lower ruminal pH. This agrees with other

Table 3. Fermentor pH, VFA concentrations and molar proportions, protozoal count, and total CH₄ production of alfalfa, birdsfoot trefoil, crown vetch, and sericea lespedeza fed with orchardgrass during continuous-culture fermentation

Item	Diet ¹				SEM	P-value
	ALF	BFT	CV	SL		
pH						
Mean	6.52 ^b	6.41 ^b	6.60 ^{ab}	6.80 ^a	0.075	0.026
Minimum	6.37 ^{ab}	6.19 ^b	6.46 ^a	6.57 ^a	0.076	0.038
Maximum	6.73 ^b	6.75 ^b	6.84 ^b	7.14 ^a	0.085	0.026
VFA						
Total (mmol/L)	66.7 ^a	73.2 ^a	57.0 ^b	36.8 ^c	2.25	<0.001
Individual VFA (mol/100 mol)						
Acetate (A)	64.48	62.95	65.35	64.71	1.363	0.627
Propionate (P)	24.60	27.61	25.14	25.07	1.179	0.247
Butyrate (B)	9.27 ^{ab}	8.84 ^a	9.0 ^a	10.22 ^b	0.367	0.049
Isobutyrate	0.61 ^a	0.00 ^b	0.14 ^b	0.00 ^b	0.046	<0.001
Valerate (V)	0.92	0.60	0.37	0.00	0.302	0.249
Isovalerate	0.12	0.00	0.00	0.00	0.062	0.436
A:P	2.62	2.38	2.60	2.58	0.135	0.202
A+B:P	3.00	2.60	2.96	2.99	0.151	0.170
A+B:P+V	2.89	2.54	2.91	2.99	0.158	0.218
Protozoa (cells $\times 10^5$ /mL)	2.13 ^b	2.23 ^b	2.14 ^b	2.88 ^a	0.081	0.002
Total CH ₄ production (mg)						
CH ₄ /d	276.7 ^a	244.1 ^{ab}	207.1 ^b	28.4 ^c	43.38	<0.001
CH ₄ /g of OM	3.6 ^a	3.2 ^{ab}	2.7 ^b	0.4 ^c	0.57	<0.001
CH ₄ /g of NDF	6.5 ^a	6.7 ^a	5.8 ^a	0.8 ^b	1.11	<0.001
CH ₄ /g of digestible OM	5.4 ^a	4.8 ^{ab}	4.2 ^b	0.6 ^c	0.84	<0.001
CH ₄ /g of digestible NDF	6.4 ^a	5.5 ^{ab}	4.9 ^b	0.9 ^c	1.04	<0.001

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹ALF = 50% orchardgrass + 50% alfalfa; BFT = 50% orchardgrass + 50% birdsfoot trefoil; CV = 50% orchardgrass + 50% crown vetch; SL = 50% orchardgrass + 50% sericea lespedeza.

continuous-culture fermentor studies (Soder et al., 2012, 2013) where fermentor pH was decreased by increasing nutrient digestibilities. Kolver and de Veth (2002) predicted that mean pH to optimize digestion of pasture was 6.35, lower than that found in the current study; however, they also mentioned that nutrient digestion and synthesis of microbial protein were largely insensitive to pH across a broad range of pH values (5.8–6.8).

Total VFA were lower ($P < 0.001$) in SL and CV diets compared with ALF and BFT diets (Table 3). In the current study, a reduction in VFA production in those diets can be linked to decreased nutrient digestibilities and changes on the composition of ruminal microbiota (Vasta et al., 2019). There were no differences ($P > 0.05$) among diets in molar proportions of acetate, propionate, valerate, isovalerate, or ratios of A:P, A+B:P, and A+B:P+V (when A = acetate, P = propionate, B = butyrate, and V = valerate). Molar proportions of butyrate were greater ($P < 0.05$) in the SL diet than in the BFT and CV diets. Contrarily, molar proportions of isobutyrate were higher ($P < 0.001$) in the ALF than in the other 3 diets. The relatively low isobutyrate and isovalerate values in this study may have been due to CT concentration of the diet. In a meta-analysis of the relationship between methane formation and dietary CT level, Jayanegara et al. (2012) showed an inverse relationship between branched-chain VFA (isobutyrate and isovalerate) and an increase in CT concentrations. Alternatively, Hristov et al. (2012) found that variability in VFA data was greater for continuous-culture fermentor systems compared with in vivo data.

Protozoal counts were greatest ($P < 0.05$) in the SL diet compared with the other 3 diets. Chiquette et al. (1989) observed that protozoal counts tended to increase when feeding ewes a CT-rich diet (3% CT) compared with a low-CT diet (1% CT), but was only a significant increase 3 h after feeding. Animut et al. (2008a) found a linear increase in protozoal counts as the percentage of CT-containing Kobe lespedeza (*Lespedeza striata*) was added to a sorghum-sudangrass (*Sorghum bicolor*) diet.

Methane Production

Total CH₄ production (mg of CH₄/d) was lower ($P < 0.001$) in the SL diet than in the other 3 diets (Table 3). This decrease was mainly attributed to a reduction in apparent fiber digestion in the SL diet, but also to lowering apparent and true OM digestibilities. Furthermore, CH₄ per gram of OM, per gram of NDF, per gram of digestible OM, and per gram of digestible NDF fed were the lowest ($P < 0.001$) for the SL diet. No differences ($P > 0.31$) were observed among the other 3

diets for CH₄ per gram of NDF fed. However, ALF had greater CH₄ per gram of OM fed, digestible OM fed, and digestible NDF fed than CV or SL ($P = 0.03$, 0.04, and 0.04, respectively). The high CT concentration in SL diet may decrease CH₄ production by reducing fiber digestion (indirect effect), whereas hydrolysable tannins (such as those in sweet chestnut) act by inhibiting the growth or activity of methanogens or hydrogen-producing microbes (direct effect) (Goel and Makkar, 2012). Animut et al. (2008a) suggested that alterations in protozoal activity among diets differing in CT concentrations resulted in a change in supply of substrates to methanogens may be partly responsible for changes in CH₄ emissions. Decreases in both CH₄ production and in apparent total-tract DM and OM digestibilities found in goats fed lespedeza (Animut et al., 2008a,b) also support the idea that observed CH₄ mitigation in the SL diet may be due to decreased nutrient digestibilities. Toxicity of phenols and other tannin fractions in the SL diet to rumen microbes and methanogens may also have contributed to the observed effect. Animut et al. (2008b) reported that inhibition of methanogens by CT might be a primary result of suppressed fiber degradation that limits H₂ derived from synthesis of acetate. They suggest that depressed fiber degradation could be due to a reduced number of cellulolytic bacteria, formation of tannin–cellulose complexes, or impaired bacterial adhesion to substrate and fibrolytic activity of rumen microbes.

Decreased CH₄ emissions and pasture DMI were previously reported by Puchala et al. (2005) after feeding Angora goats sericea lespedeza (177 g of CT/kg of DM) versus crabgrass [*Digitaria ischaemum* (Schreb.) Schreb. ex Muhl.]/tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort., nom. cons.; without CT], respectively. Animut et al. (2008a) also observed that feeding different levels of Kobe lespedeza dosed at 1:2, 2:1, and 3:0 in a mixture with sorghum-sudangrass reduced CH₄ production linearly in goats, which was attributed to the presence of CT in that legume. Furthermore, Animut et al. (2008b) reported a reduction in CH₄ emissions (51%) produced by goats fed sericea lespedeza (140 g of CT/kg of DM) as sole feed compared with diets with polyethylene glycol (which deactivates CT).

Nitrogen Metabolism

Nitrogen intake was greater ($P < 0.001$) in ALF, BFT, and CV diets than in the SL diet (Table 4). Fermentor NH₃-N concentrations were greater ($P < 0.001$) in ALF than in BFT and SL diets, with the CV diet showing an intermediate level. True CP degradability was greater ($P = 0.02$) in ALF and CV diets than in

Table 4. Nitrogen metabolism of alfalfa, birdsfoot trefoil, crown vetch, and sericea lespedeza fed with orchardgrass during continuous-culture fermentation

Item	Diet ¹				SEM	P-value
	ALF	BFT	CV	SL		
N intake ² (g/d)	4.04 ^a	3.44 ^b	3.35 ^c	3.07 ^d	0.006	<0.001
NH ₃ -N (mg/dL)	22.5 ^a	9.3 ^c	17.6 ^b	11.2 ^c	0.74	<0.001
True CP degradability (%)	90.8 ^a	75.7 ^b	93.6 ^a	72.9 ^b	4.48	0.020
N flows (g/d)						
Total N	2.57 ^a	2.72 ^a	2.34 ^b	2.24 ^b	0.076	0.001
NH ₃ -N	0.96 ^a	0.40 ^c	0.75 ^b	0.47 ^c	0.032	<0.001
Non-NH ₃ -N	1.61 ^b	2.33 ^a	1.59 ^b	1.76 ^b	0.072	<0.001
Bacterial N	1.31 ^{bc}	1.68 ^a	1.43 ^b	1.14 ^c	0.071	0.003
Dietary N	0.30 ^{ab}	0.65 ^a	0.16 ^b	0.62 ^a	0.110	0.031
Bacterial efficiency						
OM truly digested (g of N/kg)	22.5 ^{bc}	27.0 ^a	24.5 ^{ab}	21.0 ^c	1.09	0.013

^{a-d}Means within a row with different superscript differ ($P < 0.05$).

¹ALF = 50% orchardgrass + 50% alfalfa; BFT = 50% orchardgrass + 50% birdsfoot trefoil; CV = 50% orchardgrass + 50% crown vetch; SL = 50% orchardgrass + 50% sericea lespedeza.

²N intake (g/d) = dietary N (g/d) + urea-N from buffer (g/d).

BFT and SL diets. The decreased degradability of CP in the SL diet may be attributed to the formation of tannin-protein complexes that prevent degradation by rumen microbes, which is beneficial for post-ruminal utilization (Mueller-Harvey, 2006). It has been proposed that higher concentrations of CT in diets, such as those found in the SL diet, which remain free after binding with proteins, may depress fiber digestion by complexing with lignocellulose, thus preventing microbial digestion or by directly inhibiting cellulolytic microorganisms and activities of fibrolytic enzymes, or both (Patra and Saxena, 2010).

Effluent total N was greater ($P = 0.001$) in ALF and BFT diets than in CV and SL diets (Table 4). Effluent NH₃-N was highest ($P < 0.001$) in ALF diet and lowest in BFT and SL diets. Effluent non-NH₃-N was greater ($P < 0.001$) in the BFT diet than in the other 3 diets. Bacterial N flow was highest ($P = 0.003$) in the BFT diet. Dietary N flow was greater ($P = 0.031$) in BFT and SL diets than in the CV diet; however, ALF showed an intermediate level among them. Bacterial efficiency (g N/kg of OM truly digestible) was greater ($P = 0.013$) in the BFT diet than in the SL diet; however, ALF and CV diets showed intermediate levels between them.

While the amount of DM fed was kept constant in all fermentors, N intake differed across diets due to differences in CP concentration of diets. It is expected that the increase in dietary CP fed (related to diets tested with SL, CV, BFT, and ALF) would result in increased non-NH₃-N flow, as well as flows of NH₃-N and bacterial N due to greater N input into the system (Brandao and Faciola, 2019). Fermentor NH₃-N concentrations are in line with those reported in previous studies conducted by Williams et al. (2010), where a reduction in NH₃-N

concentrations (27.8%) and flow (26%) occurred with birdsfoot trefoil compared with alfalfa in continuous cultures fed with 55:45 forage to concentrate ratio. According to Brandao and Faciola (2019), fermentors fed with high CP diets (such as ALF in the current study) showed greater NH₃-N accumulation than those fed low CP diets (such as BFT, CV, and SL in the current study). However, the reduction in NH₃-N concentration and flow in the SL diet is notable because the SL diet contained the highest content of CT, which may have resulted in increased N binding, as shown by its lower RDP concentration. Devant et al. (2000) showed that degradability of CP in the rumen affects N digestibility and retention. Therefore, it is important to maintain a minimum concentration of ruminal NH₃-N, not only for adequate microbial fermentation, but also for adequate fiber digestion. Due to differences in true CP degradability among diets, it can be hypothesized that the rate of NH₃-N release from ALF diet (without CT) was greater than that of the 3 diets containing CT. Previous studies (Soder et al., 2012, 2013) also showed lower NH₃-N concentrations, N flows (total N, NH₃-N, non-NH₃-N, and bacterial N), and bacterial efficiency by using a 100% orchardgrass diet compared with a 50:50 orchardgrass:alfalfa mixture (similar to that tested in the current study). At high CT concentrations, lower bacterial efficiency and total VFA concentration were observed in the SL diet, suggesting that overall ruminal fermentation might be compromised due to low nutrient digestibilities.

CONCLUSIONS

Condensed tannin concentrations significantly influenced in vitro ruminal fermentation and enteric CH₄

production in diets containing 50:50 grass:legume. When compared with cool-season perennial legume diets (ALF, BFT, and CV), the warm-season legume SL diet, which also contained the greatest CT concentrations, had decreased apparent NDF and ADF digestibilities, total VFA concentration, N intake, and total CH₄ production. In addition, the lowest apparent and true DM and OM digestibilities and bacterial N efficiency per kg of OM truly digested were found in the SL diet. However, tradeoffs in ruminal fermentation (reduced nutrient digestibilities, total VFA concentration, and bacterial N efficiency) must be considered when SL (a high-CT legume) is used to lower CH₄ production. Legumes containing moderate concentrations of CT also decreased CH₄ production compared with the ALF diet, but with less negative effects on ruminal fermentation. Further work should be conducted in vitro to find optimal diets (e.g., testing other legumes, rations, and concentrations of CT) that reduce CH₄ emissions without negatively affecting ruminal digestion.

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