Tannin-containing legumes and forage diversity influence foraging behavior, diet digestibility, and nitrogen excretion by lambs[1,2]

Sebastian Lagrange[†,‡,3] and Juan J. Villalba[†]

[†]Department of Wildland Resources, Quinney College of Natural Resources, Utah State University, Logan, UT 84322; and [‡]Estación Experimental Agropecuaria Bordenave, Instituto Nacional de Tecnología Agropecuaria. Bordenave, Buenos Aires, 8187, Argentina

ABSTRACT: Diverse combinations of forages with different nutrient profiles and plant secondary compounds may improve intake and nutrient utilization by ruminants. We tested the influence of diverse dietary combinations of tannin- (sainfoin-*Onobrichis viciifolia*; birdsfoot trefoil-*Lotus corniculatus*) and non-tannin- (alfalfa-*Medicago sativa* L.) containing legumes on intake and diet digestibility in lambs. Freshly cut birdsfoot trefoil, alfalfa, and sainfoin were offered in ad libitum amounts to 42 lambs in individual pens assigned to 7 treatments (6 animals/treatment): 1) single forage species (sainfoin [**SF**], birdsfoot trefoil [**BFT**], and alfalfa [**ALF**]), 2) all possible 2-way choices of the 3 forage species (alfalfa-sainfoin [**ALF-SF**], alfalfa-birdsfoot trefoil [**ALF-BFT**], and sainfoin-birdsfoot trefoil [**SF-BFT**]), or 3) a choice of all 3 forages (alfalfa-sainfoin-birdsfoot trefoil [**ALF-SF-BFT**]). Dry matter intake (DMI) was greater in ALF than in BFT ($P = 0.002$), and DMI in SF tended to be greater than in BFT ($P = 0.053$). However, when alfalfa was offered in a choice with either of the tannin-containing legumes (ALF-SF; ALF-BFT), DMI did not differ from ALF, whereas DMI in SF-BFT did not differ from SF ($P > 0.10$). When lambs were allowed to choose between 2 or 3 legume species, DMI was greater (36.6 vs. 33.2 g/kg BW; $P = 0.038$) or tended to be greater (37.4 vs. 33.2 g/kg BW; $P = 0.067$) than when lambs were fed single species, respectively. Intake did not differ between 2- or 3-way choice treatments ($P = 0.723$). Lambs preferred alfalfa over the tannin-containing legumes in a 70:30 ratio for 2-way choices, and alfalfa > sainfoin > birdsfoot trefoil in a 53:33:14 ratio for the 3-way choice. In vivo digestibility (**DMD**) was SF > BFT (72.0% vs. 67.7%; $P = 0.012$) and DMD in BFT tended to be greater than in ALF (64.6%; $P = 0.061$). Nevertheless, when alfalfa was offered in a choice with either sainfoin or birdsfoot trefoil (ALF-SF; ALF-BFT), DMD was greater than ALF ($P < 0.001$ and $P = 0.007$, respectively), suggesting positive associative effects. The SF treatment had lower blood urea nitrogen and greater fecal N/N intake ratios than the ALF, BFT, or ALF-BFT treatments ($P < 0.05$), implying a shift in the site of N excretion from urine to feces. In conclusion, offering diverse combinations of legumes to sheep enhanced intake and diet digestibility relative to feeding single species, while allowing for the incorporation of beneficial bioactive compounds like condensed tannins into the diet.

Key words: alfalfa, diverse forage diets, nitrogen excretion, preference, sainfoin, tannin-containing legumes

[1]This research was supported by grants from the National Institute of Food and Agriculture (NIFA), USDA (award no. 2016-67019-25086) and a fellowship to Sebastian Lagrange (INTA, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina). This paper is published with the approval of the Director, Utah Agricultural Experiment Station, and Utah State University, as journal paper number 9200.

[2]We acknowledge R. Lira and C. Spackman for technical support, and R. Stott for veterinary services.

[3]Corresponding author: sebastian.lagrange@aggiemail.usu.edu

Received April 19, 2019.

Accepted July 31, 2019.

J. Anim. Sci. 2019.97:3994–4009
doi: 10.1093/jas/skz246

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most high-yielding and nutritious forage available for feeding high-producing ruminants in North America (NAAIC, 2017). Nevertheless, its use in pure stands has been associated with increased risk of bloat (Wang et al., 2012) and large urinary nitrogen losses caused by the rapid degradation of alfalfa proteins in the rumen (Julier et al., 2003; Getachew et al., 2006; Dijkstra et al., 2013). In addition to ammonia volatilization to the atmosphere due to urinary N excretions (Whitehead, 2000), high levels of ammonia in urine "hot spots" are sources of nitrous oxide, a potent greenhouse gas (Forster et al., 2007) produced during microbial nitrification and denitrification processes (Oenema et al., 2005; Huang et al., 2014). Another problem with excesses of urinary N is the eutrophication of watersheds by nitrates, produced by ammonia oxidation, and then leached into ground water, streams, and lakes (Whitehead, 2000).

A strategy to reduce the aforementioned environmental impacts while maintaining high levels of animal productivity entails the provision of alfalfa in a diverse diet with bioactive-containing forages that increase N retention and/or reduce the proportion of urinary N losses. For instance, polyphenols like condensed tannins (**CT**) in legumes like sainfoin (*Onobrichis vicifolia*) or birdsfoot trefoil (*Lotus corniculatus*) bind to proteins and protect them from degradation in the rumen (Scharenberg et al., 2007b; Theodoridou et al., 2010; Theodoridou et al., 2012), altering the fate of the excreted N to greater fecal to urinary ratios (Mueller-Harvey, 2006). A shift in the route of N excretion from urine to feces means more stable N fractions in manure since N is mainly bound to organic compounds like neutral detergent and acid detergent insoluble N, which potentially lessens N losses to the environment as ammonia (Whitehead, 2000; Grosse Brinkhaus et al., 2016; Stewart, 2018).

In addition to the benefits of tannin-containing legumes, a diversity of forages and biochemicals available in pasturelands may enhance the benefits described above because complementary relationships among multiple food resources in nature improve the fitness of herbivores (Tilman, 1982). Biodiversity in pasturelands may lead to positive associative effects among forages which improve the nutrition (i.e., N retention and diet digestibility) and welfare of livestock (i.e., reductions in stress caused by single forages with unbalanced nutrient profiles), while reducing environmental impacts. Sheep and goats eating mixed diets on rangeland display daily intakes 2 or more times greater than reference intake values obtained with animals fed single forages of similar nutritive value (Agreil and Meuret, 2004). On the other hand, differences in the chemical structures of CT in sainfoin and birdsfoot trefoil (McAllister et al., 2005) influence their capacities to bind proteins and microbial enzymes in the rumen (Mueller-Harvey et al., 2019), which may also lead to positive associative effects in diverse diets that influence protein degradability and the fate of nitrogen excretion.

Ruminants offered a diversity of forages (alfalfa, sainfoin, and birdsfoot trefoil) may be able to build a diet that enhances nutrient retention and diminishes ammonia formation in the rumen, and consequently urinary N loses, relative to animals fed single forages. This response may occur because herbivores develop preferences based on the postingestive consequences of the foods experienced during the foraging process (Provenza, 1995; Provenza and Villalba, 2006).

We hypothesized that a diversity of tannin- and nontannin-containing legumes in ruminant feeding systems would lead to complementary relationships among nutrients and CT that 1) increase the ratio of fecal to urinary N excretions, 2) reduce blood urea N (**BUN**), and 3) maintain or increase food intake and digestibility relative to single forages. Thus, the aim of this study was to test the synergistic effects of increasingly diverse combinations of tannin-containing legumes (sainfoin or birdsfoot trefoil) and alfalfa offered as single, binary, or trinary choices.

MATERIAL AND METHODS

The study was conducted at the Green Canyon Ecology Center, located at Utah State University in Logan (41°45′59″N, 111°47′14″W), according to procedures approved by the Utah State University Institutional Animal Care and Use Committee (approval 2470). The experiment took place from May 20 to June 13, 2015.

Animals and Treatments

Forty-two commercial Columbia-Polypay-Suffolk crossbred lambs (4 mo of age) with an average initial body weight (BW) of 24 ± 6 kg were housed outdoors under a protective roof in individual, adjacent pens measuring 1.5 by 2.5 m. Lambs were fed ad libitum amounts of alfalfa pellets for 7 d to determine dry matter intake (DMI) for each lamb. After this 7-d period, a 7-d adaptation period was carried out to familiarize lambs to their respective legume diets, which were also fed during an ensuing 10-d experimental period. Throughout the study, lambs had free access to culinary water and trace mineral salt blocks (mineral composition: minimum 96% NaCl, 320 mg/kg Zn, 380 mg/kg Cu, 2,400 mg/kg Mn, 2,400 mg/kg Fe, 70 mg/kg I, and 40 mg/kg Co).

Freshly cut forage from 2 tannin-containing legume species—sainfoin (*Onobrichis vicifolia*) and birdsfoot trefoil (*Lotus corniculatus*) and from the nontannin containing legume alfalfa (*Medicago sativa*) were offered in ad libitum amounts in 7 diet treatments as 1) single forage species (sainfoin [**SF**], birdsfoot trefoil [**BFT**], and alfalfa [**ALF**]), 2) all possible 2-way choices of the 3 forage species (alfalfa-sainfoin [**ALF-SF**], alfalfa-birdsfoot trefoil [**ALF-BFT**], and sainfoin-birdsfoot trefoil [**SF-BFT**]), or 3) a choice of all 3 forages (alfalfa-sainfoin-birdsfoot trefoil [**ALF-SF-BFT**]). Lambs were sorted by their average intake of alfalfa pellets during the previous 7-d period and then randomly assigned to the 7 treatment groups (6 lambs/group) such that treatments were balanced with regard to their intake capacity. Treatments were randomly distributed among pens.

Forages

Well-established and irrigated stands of sainfoin (cv. Shoshone), birdsfoot trefoil (cv. Langille), and alfalfa (cv. DK) seeded in August 2014 at the Utah State University Irrigated Pasture research facility in Lewiston, UT (41 56′N 111 52′W) provided the forages for this study. Pastures were irrigated using hand-line sprinkler sets running in 12-h cycles, which applied approximately 10.5 cm of water every 2 wk.

Legumes were harvested from three monoculture plots of 0.17-ha each morning between 0700 and 0900 h in June 2015 at around 10 cm from ground level using a flail harvester (Rem Manufacturing Ltd., Swift Current, SK, Canada) with particle sizes varying between 2 and 4 cm, and immediately transported to the Green Canyon Ecology Center for daily feeding. Birdsfoot trefoil and alfalfa were cut at late bud stage and sainfoin in late flowering stage.

Adaptation Period (May 27 to June 2)

During this period, lambs were familiarized with the treatment diets and the experimental protocol. Each morning at 1100 h, all lambs received freshly cut forage of each legume according to their assigned treatments, starting with 100 g (DM basis) on May 27. Different legume species in the 2- and 3-way choice treatments were offered in separate buckets that were simultaneously presented on a daily basis at random locations within each pen. Forage amounts were increased by 100 g daily until ad libitum amounts were fed to each lamb by the last day of the period (June 2). During adaptation, lambs offered SF and ALF were, in general, willing to consume greater amounts of forage than lambs offered BFT. Lambs eating ALF were monitored daily for symptoms of bloat (e.g., reduced intake, reluctance to move, distended rumen, and difficulty in breathing), which were not observed during the study.

Experimental Period (June 3 to June 13)

Each morning at 1100 h, all lambs received legumes according to their assigned treatments and no other food was offered until the following day. Different legume species were presented as described for the adaptation period. The amounts of each legume offered per lamb during the experimental period ranged between 400 and 2200 g/d (DM basis) and they were adjusted on a daily basis depending on individual lamb intake such that refused amounts were always greater than 15% of the initial amounts of forage offered (DM basis). Refusals from each animal and for each legume were removed and weighed daily at 0900 h before fresh forage was offered to all animals according to their respective treatment.

Measurements

Intake and preference. Dry matter intake of each legume was calculated on a daily basis for each lamb as the difference between the amount of forage offered and the amount of forage refused. Intake was expressed as g DM/kg BW. For multiple forage treatments, preference by lamb was estimated as the daily proportion of the DMI calculated for each legume species relative to the total amount of DMI.

Fecal DM output and in vivo digestibility calculations. Fecal DM output (**FO**) was determined using the concentration of an internal marker, acid detergent lignin (**ADL**), in the forage consumed and in feces (Van Soest, 2018). Fecal samples of at least 10 g (wet basis) were manually taken daily from the rectum of each lamb at 1300 h during the last 8 d of the experimental period (June 6 to June 13). Representative samples of forage offered and refused were collected daily during the same period. Forage and fecal samples were placed in plastic seal top bags, labeled, and immediately stored in a freezer at −20 °C until analyses. Samples were subsequently freeze dried (Free Zone 18 Liters, Labconco Corporation, Kansas City, MO) at −60 °C until 2 consecutive weights did not differ in a 24-h period, and subsequently ground to pass the 1-mm screen of a Wiley mill (model 4; Thomas Scientific Swedesboro, NJ). Fecal samples were then composited by lamb over the 8-d sampling period, combining approximately 2.5 g DM from each day. Samples of forages offered and refused were also composited over the 8-d period (0.75 g/d, DM basis) by species and analyzed in duplicates for ADL (see below). Fecal output was then determined using the following formula (Cochran and Galyean, 1994):

$$\mathrm{FO}\,(\mathrm{g/d}) = [\mathrm{DMI}\,(\mathrm{g/d}) \times \mathrm{ADL\ in\ feed}\ (\mathrm{g/g})] / \mathrm{ADL\ in\ feces}\,(\mathrm{g/g})\,.$$

The ADL concentration in feed was calculated by the ratio of the difference between the amounts of ADL offered and refused for each legume and DMI as follows:

$$[\mathrm{offered}\,(\mathrm{ADL_{ALF}} + \mathrm{ADL_{SF}} + \mathrm{ADL_{BFT}})\,\mathrm{g} - \mathrm{refused}\,(\mathrm{ADL_{ALF}} + \mathrm{ADL_{SF}} + \mathrm{ADL_{BFT}})\,\mathrm{g}] / \mathrm{DMI}\,(\mathrm{g})\,.$$

Once FO was determined, dry matter digestibility (**DMD**) was calculated for each lamb as DMD (%) = {[DMI (g/d) – FO (g/d)]/DMI (g/d)} × 100 (Cochran and Galyean, 1994).

Neutral detergent fiber digestibility (**NDFD**) and acid detergent fiber digestibility (**ADFD**) were calculated by determining the concentration of neutral detergent fiber (NDF) or acid detergent fiber (ADF) in forages, refusals, and feces (see below), and then applying the formula (Cochran and Galyean, 1994):

$$\mathrm{NDFD\ or\ ADFD}\,(\%) = \{[\mathrm{NDF\ or\ ADF\ in\ feed}\,(\mathrm{g/d}) - \mathrm{NDF\ or\ ADF\ in\ feces}\,(\mathrm{g/d})] / \mathrm{NDF\ or\ ADF\ in\ feed}\,(\mathrm{g/d})\} \times 100.$$

The NDF or ADF concentration in feed was calculated by the ratio of the difference between the amounts of NDF or ADF offered and refused for each legume and DMI as follows:

$$\mathrm{NDF\ concentrationin\ feed}\,(\mathrm{g/g}) = [\mathrm{offered}(\mathrm{NDF_{ALF}} + \mathrm{NDF_{SF}} + \mathrm{NDF_{BFT}})\ \mathrm{g} - \mathrm{refused}(\mathrm{NDF_{ALF}} + \mathrm{NDF_{SF}} + \mathrm{NDF_{BFT}})\mathrm{g}] / \mathrm{DMI}\,(\mathrm{g})\,,$$

then NDF in feed (g/d) = DMI (g/d) × NDF concentration in feed (g/g).

NDF in feces (g/d) = FO (g/d) × NDF concentration in feces (g/g).

ADF in feed (g/d) and ADF in feces (g/d) were calculated as described for NDF in feed and feces.

Digestible dry matter intake (**DDMI**) was calculated as the product of DMI (g/d) and DMD.

The ratio of nitrogen excreted through the feces to consumed nitrogen (**Fecal N:Intake N**) was calculated by analyzing N concentration in the forage (offered and refusals) and fecal samples. The N excreted through the feces (g per lamb) was calculated by multiplying FO by the N concentration in feces. Intake of N was estimated for each lamb by difference between the total amount of the N offered with the legumes and the total amount refused every day as follows:

$$\mathrm{Intake\ N}\,(\mathrm{g/d}) = \mathrm{Offered}\,(\mathrm{N_{ALF}} + \mathrm{N_{SF}} + \mathrm{N_{BFT}}) - \mathrm{Refused}\,(\mathrm{N_{ALF}} + \mathrm{N_{SF}} + \mathrm{N_{BFT}})\,.$$

Blood Analyses

Blood samples (without EDTA added; Becton Dickinson Vacutainer System; Becton Dickinson and Company, Franklin Lakes, NJ; 10 mL serum vacutainer tubes) were collected via jugular venous puncture at 1000 h from each lamb prior to the beginning of the experimental period on May 29 and at the end of the experimental period on June 12. Samples were allowed to clot for 45 min before being centrifuged (1500 rpm for 15 min). The serum was extracted, placed in 1.5-mL microcentrifuge tubes and immediately submitted to the Utah Veterinary Diagnostic Laboratory (Logan, UT) for BUN analyses. The assay was performed with a Siemens Dimension Xpand Plus analyzer (Siemens Healthcare Diagnostics, Newar, DE) using Siemens urea N flex reagent, in an enzymatic method which uses urease enzyme in a bichromatic rate technique.

Chemical Analyses

One representative sample of each legume offered (alfalfa, sainfoin, and birdsfoot trefoil) was taken daily before feeding, as well as one representative sample of refusal per legume. Legume and refusal samples were placed in paper bags and dried in a forced-air oven (VWR Scientific Inc., Radnor, PA) at 60 °C for 48 h to determine moisture content and report voluntary intake on a DM basis.

One additional sample of each legume offered was collected at the same time, along with 1 additional sample of each legume refusal, and frozen in plastic seal top bags. Samples were subsequently freeze-dried at −60 °C and ground to pass a 1-mm screen of a Wiley mill (model 4; Thomas Scientific Swedesboro, NJ). Both legume and refusal samples were composited by species over the 10-d experimental period, taking approximately 2.0 g DM from each sample (samples from 06/03 to 06/13) and used for chemical analyses.

Composited forage, refusal, and fecal samples were analyzed in duplicates for DM, N, ADF, and aNDF concentrations. Dry matter was determined by drying the samples at 105 °C for 3 h in a forced-air drying oven as recommended by the National Forage Testing Association (Shreve et al., 2006). Crude protein was calculated by analyzing the N concentration of the samples using a Leco FP-528 N combustion analyzer (AOAC, 2000; method 990.03) and applying the 6.25 conversion factor (Jones, 1931). aNDF (Mertens, 2002) and ADF (AOAC, 2000; method 973.18) determinations were modified by using Whatman 934-AH glass microfiber filters with 1.5-µm particle retention and a California Buchner funnel in place of fritted glass crucible. Determinations of ADL were modified from (Robertson and Van Soest, 1981) as follows: fiber residue and filter from the ADF step was transferred to a capped tube and 45 mL of 72% sulfuric acid was added. Tubes were gently agitated for 2 h and filtered onto a second filter (same type as above) which was then rinsed, dried, weighed, and finally ashed for 2 h in a furnace to remove lignin organic matter.

Analyses of total CT in legume samples were conducted in triplicate (assaying the samples 3 times in the same day), according to the butanol-HCl-acetone spectrophotometric assay of Grabber et al. (2013), using purified CT from sainfoin and birdsfoot trefoil as the reference standard.

Statistical Analyses

Dry matter intake, DDMI, and FO were analyzed using a repeated measure design with day as the repeated measure. Diet (single forage species, 2-way and 3-way choices), day, and the interaction diet x day were the fixed factors. Lambs (nested within diet) were included in the model as the random factor. The variance–covariance structure used was the one that yielded the lowest Akaike information criterion (compound symmetric). Nutritional composition of diets and feces, DMD, NDFD, ADFD, Fecal N excretion, Intake N, Fecal N:Intake N ratio, and BUN, were analyzed as a completely randomized design, with diet as the fixed factor and lamb nested within diet as the residual component. BUN values were analyzed with initial BUN as a covariate. All analyses were computed using PROC GLIMMIX in SAS/STAT (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows). Least squares means (LSMeans) were compared pairwise using the Least Significant Difference test (LSD) when F-ratios were significant ($P < 0.05$) and reported along with their standard errors (SEM). A tendency was considered when $0.05 < P < 0.10$.

To explore the potential associative effects in the 2- and 3-way choice treatments, the difference between the values observed for each response variable in a choice treatment and a linearly predicted value for the same variable was calculated as

$$\text{Associative effect}\,(\%) = 100 \times [(\text{Observed value} - \text{Estimated value}) / \text{Estimated value}].$$

The estimated value was calculated as the weighted average of the values measured for each one of the legumes in the choice when they were fed as a single treatment (i.e., ALF, BFT, or SF). As an example, the estimated values for DMI in the ALF-SF choice was calculated as (DMI_{ALF} × proportion of alfalfa selected in the choice) + (DMI_{SF} × proportion of sainfoin selected in the choice).

Preplanned contrasts were performed to compare observed vs. estimated values using the LSMESTIMATE statement in PROC GLIMMIX. Contrasts were specified as the arithmetic difference between the observed value for the specific binary or trinary diet and the estimated value from the average of their components. Preplanned contrasts were also performed to compare the average of the 3 singles diets vs. binary (2-way choices) or singles vs. trinary treatments (3-species diets). A difference between the singles and binary or trinary diet groups or between observed and estimated values for a specific choice was considered significant when P values were <0.05.

Proportion of each legume consumed within binary and trinary treatments (preference) was analyzed with day (fixed factor) as the repeated measure and lamb as the random factor. The confidence interval of the intercept was used to determine the range in which the true average proportion selected can vary. A legume species was considered “preferred” or “not preferred” in a specific 2- or 3-way choice treatment, when the average proportion selected (intercept) for the legume was higher or lower than 0.50 or 0.33, respectively, and the confidence interval for the intercept did not include 0.50 or 0.33, respectively.

Assumptions of homoscedasticity of variance and normality were tested using studentized residuals and no apparent deviations from such assumptions were found. Normality of the random effect (lambs within diet) was tested using probability plots in PROC UNIVARIATE.

RESULTS

Chemical Composition of the Forages and Feces

The chemical composition of the legumes offered in the study, as well as the composition of refusals is reported in Table 1. On average across legumes, the refused forage was of lower nutritional quality than the forage on offer (i.e., lower CP, and greater ADF, aNDF, and ADL concentrations). Nevertheless, this difference was less evident for birdsfoot trefoil, which showed similar CP values between offered and refused forage.

An estimation of the nutritional composition of the diets consumed by the lambs is reported in Table 2. The CP concentration was similar between BFT and ALF treatments (P = 0.469), and both diets had greater CP concentration than SF (P < 0.001). In contrast, the SF treatment presented the greatest concentrations of NDF, ADF, and ADL, followed by BFT and then by ALF with the lowest values (P < 0.05). Thus, when alfalfa was consumed with birdsfoot trefoil in 2-way choices (ALF-BFT), the CP concentration of the diet was greater (P < 0.001) and the concentration of ADL tended to be lower (P = 0.052) than in the ALF-SF treatment, due to the presence of sainfoin. The nutritional quality of the ALF-SF-BFT and ALF-SF treatments was similar.

Condensed tannin concentrations were greater (~2X) in SF than in BFT (P < 0.001). Alfalfa is a nontannin-containing legume, confirmed by the low values of CT (Table 2).

Fecal CP concentration was lower than the concentration observed in the ingested forages, with the exception of SF and SF-BFT treatments (Table 2), which presented greater values in the feces. SF also revealed greater protein concentration in feces than the ALF (P < 0.001) and ALF-BFT (P = 0.004) treatments, and this parameter also tended to be greater in SF than in BFT (P = 0.096). Fecal CP concentration was also greater in BFT than in ALF (P = 0.008). Fecal NDF, ADF, and ADL concentrations were on average ~1.5X, 1.5X, and ~3.5X the concentration observed in the forages, respectively. Lambs fed SF showed the greatest fecal concentrations of NDF, ADF, and ADL among the single diets (P < 0.05; Table 2).

Intake and Preference

On average across diets, DMI differed throughout the experimental period (P < 0.001;

Table 1. Nutritional composition (g/kg DM [mean (SEM)]) of legumes offered in the study and refusals

	Legumes	CP[1]	aNDF[2]	ADF[3]	ADL[4]	CT[5]
Offered	Alfalfa	177.0 (2.8)	376.0 (10.0)	317.0 (9.9)	65.0 (1.3)	1.8 (0.1)
	Birdsfoot Trefoil	191.0 (3.5)	374.0 (11.6)	333.0 (11.9)	70.8 (2.9)	13.0 (0.4)
	Sainfoin	138.0 (5.6)	430.0 (13.7)	383.0 (12.1)	86.2 (4.3)	27.1 (1.1)
Refusals	Alfalfa	134.0	514.0	427.0	95.9	0.8
	Birdsfoot Trefoil	191.0	461.0	394.0	88.9	9.9
	Sainfoin	112.0	581.0	508.0	115.1	14.1

[1]CP= crude protein.

[2]aNDF= amylase-treated neutral-detergent fiber.

[3]ADF= acid-detergent fiber.

[4]ADL= acid-detergent lignin.

[5]CT= Condensed tannins.

Table 2. Nutrient concentration of diets and feces (lsmean; g/kg DM) when lambs were fed single forages, and 2- and 3-way choices of those forages: alfalfa (ALF), birdsfoot trefoil (BFT), and sainfoin (SF)

	Diet	CP[2]	aNDF[3]	ADF[4]	ADL[5]	CT[6]
Nutrient concentration[1]	ALF	188.7[a]	338.5[cd]	287.1[c]	56.6[c]	2.1[e]
	BFT	191.0[a]	353.9[bc]	318.9[b]	66.6[b]	13.7[c]
	SF	147.7[d]	376.4[a]	338.3[a]	75.8[a]	31.2[a]
	ALF-SF	180.2[b]	325.1[de]	282.8[c]	57.8[c]	13.7[c]
	ALF-BFT	195.0[a]	314.1[e]	274.3[c]	53.2[c]	5.9[d]
	SF-BFT	160.7[c]	363.5[ab]	328.1[ab]	72.0[a]	26.9[b]
	ALF-SF-BFT	181.7[b]	313.7[e]	277.4[c]	56.3[c]	15.5[c]
	S.E.M	2.2	7.6	6.0	1.6	1.1
	Diet effect	<0.001	<0.001	<0.001	<0.001	<0.001
Feces	ALF	142.2[d]	512.7[c]	411.2[e]	160.0[c]	
	BFT	157.0[bc]	574.7[b]	524.5[b]	206.8[b]	
	SF	166.0[ab]	614.8[a]	561.0[a]	270.9[a]	
	ALF-SF	159.2[abc]	540.2[c]	467.2[c]	201.6[b]	
	ALF-BFT	149.7[cd]	508.5[c]	436.7[de]	174.0[c]	
	SF-BFT	168.0[a]	598.7[ab]	549.3[ab]	258.9[a]	
	ALF-SF-BFT	160.0[abc]	534.2[c]	459.8[cd]	207.3[b]	
	S.E.M	3.7	11.7	9.2	5.5	
	Diet Effect	<0.001	<0.001	<0.001	<0.001	

[a–e]LSmeans in a column with different letters differ ($P < 0.05$).

[1]Nutrient Concentration: Concentration of nutrients in lambs' diets calculated as: (Amount of forage offered x concentration of the nutrient in the forage – Amount of forage refused x concentration of the nutrient in the refusal)/DMI.

[2]CP= Crude protein.

[3]aNDF= amylase-treated neutral-detergent fiber.

[4]ADF= acid-detergent fiber.

[5]ADL= acid-detergent lignin.

[6]CT= Condensed tannins.

Figure 1). Averaged across days, DMI in ALF was greater than intake displayed by lambs fed BFT ($P = 0.002$; Table 3) and DMI in the SF treatment tended to be greater than in the BFT treatment ($P = 0.053$). Nevertheless, when alfalfa was offered in a choice with either of the 2 tannin-containing legumes (ALF-SF or ALF-BFT), total DMI did not differ from ALF ($P = 0.503$ and $P = 0.377$, respectively). Similarly, DMI in the SF-BFT treatment did not differ from SF ($P = 0.584$).

Comparisons between observed and estimated values did not reveal any positive or negative associative effects regarding DMI for lambs offered binary or trinary choices ($P > 0.10$; Table 3). Nevertheless, DMI was on average 10% greater when lambs were allowed to choose between 2 legume species than when fed single species (36.6 vs. 33.2 g/kg BW, respectively, $P = 0.038$), and overall DMI tended to be greater for 3-way choices than for single species (37.4 vs. 33.2 g/kg BW, $P = 0.067$; Table 3). In contrast, DMI did not differ between treatments when lambs were offered choices between 2 or 3 legume species (37.4 vs. 36.6 g/kg BW, respectively; Table 3).

When offered the 2-way choice diets (ALF-SF, ALF-BFT, or SF-BFT), alfalfa was preferred over sainfoin or birdsfoot trefoil (alfalfa > sainfoin and alfalfa > birdsfoot trefoil; Table 3), and sainfoin was preferred over birdsfoot trefoil (sainfoin > birdsfoot trefoil). Similarly, for the 3-way choice treatment, alfalfa was the most and birdsfoot trefoil the least preferred legume during the feeding period (alfalfa > sainfoin > birdsfoot trefoil; Table 3). Intake of each legume within each choice treatment expressed as g/kg BW is shown in Figure 2. A day effect was detected for treatments containing birdsfoot trefoil ($P < 0.01$; Table 3), driven by an increase in the proportion of birdsfoot trefoil selected by lambs towards the end of the experimental period and the concomitant decline in the proportions selected of the other components in the choice.

Digestibility and Fecal Output

Dry matter digestibility was SF > BFT ($P = 0.012$) and digestibility in BFT tended to be greater than in the ALF treatment ($P = 0.061$; Table 4). Nevertheless, when alfalfa was offered in a choice with sainfoin or birdsfoot trefoil, the inclusion of these tannin-containing legumes to the diet increased DMD relative to the single treatment

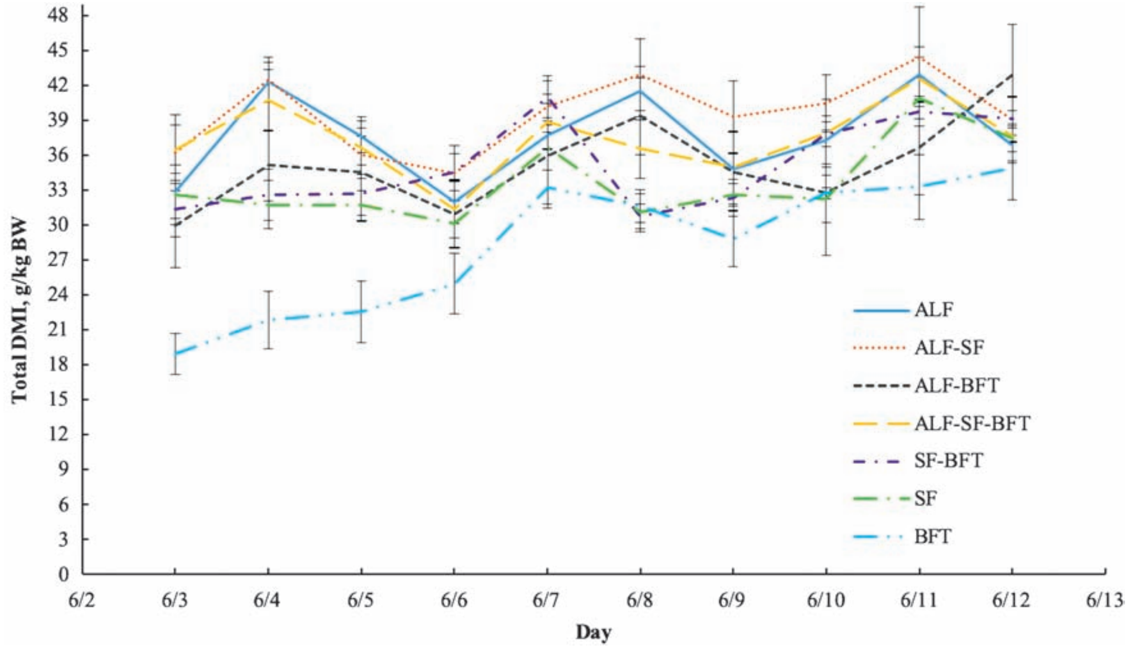

Figure 1. Daily total dry matter intake during the experimental period (g·kg BW^{-1}·d^{-1}; DM basis) of single forages and 2- and 3-way choices of those forages by lambs. Lambs were offered tannin-containing legumes (sainfoin; SF and birdsfoot trefoil; BFT) and the nontannin-containing legume alfalfa (ALF). Means are for 6 lambs per treatment. Bars represent SEM.

Table 3. Total dry matter intake (lsmeans) of legumes and proportions of these legumes selected by lambs when they were presented as a single forage or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT), and sainfoin (SF)

		Proportions[3]		
Diets	Total DMI, g·kg BW^{-1}·d^{-1}	ALF	SF	BFT
ALF	37.6^{ab}			
BFT	28.3^{c}			
SF	33.7^{bc}			
ALF-SF	39.4^{a}	**0.67** (0.52–0.81)	**0.33** (0.19–0.48)	
ALF-BFT	35.1^{ab}	**0.71** (0.60–0.81)***		**0.29** (0.19–0.40)***
SF-BFT	35.2^{ab}		**0.71** (0.63–0.80)***	**0.29** (0.20–0.37)***
ALF-SF-BFT	37.4^{ab}	**0.53** (0.32–0.74)**	**0.33** (0.11–0.55)**	**0.14** (0.08–0.20)***
SEM	1.9			
P				
Diet effect	0.008			
Date effect	<0.001			
Diet x Date effect	<0.001			
2-species choice vs. singles[1]	0.038			
3-species choice vs. singles	0.067			
3-species vs. 2-species choice	0.723			
Associative effects[2]	%—(*P*-value)			
ALF-SF-BFT	6.8 (0.303)			
ALF-SF	8.6 (0.201)			
ALF-BFT	0.8 (0.907)			
SF-BFT	9.5 (0.216)			

[a–c]Total DMI LSmeans with different letters differ ($P < 0.05$).

[1]Indicate that these are preplanned contrasts between 2-way, 3-way choices and single diets.

[2]Associative effects (%): 100 × [(observed value − Estimated value)/Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

[3]Proportions: numbers between parentheses represent lower and upper values for 95% confidence interval of the mean; a legume species was considered “preferred” or “not preferred” when the average proportion selected was higher or lower than 0.50 (2-way choice) or 0.33 (3-way choice) and the confidence interval for the intercept did not include 0.50 or 0.33, respectively. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ represent date effect for the proportion selected within each diet.

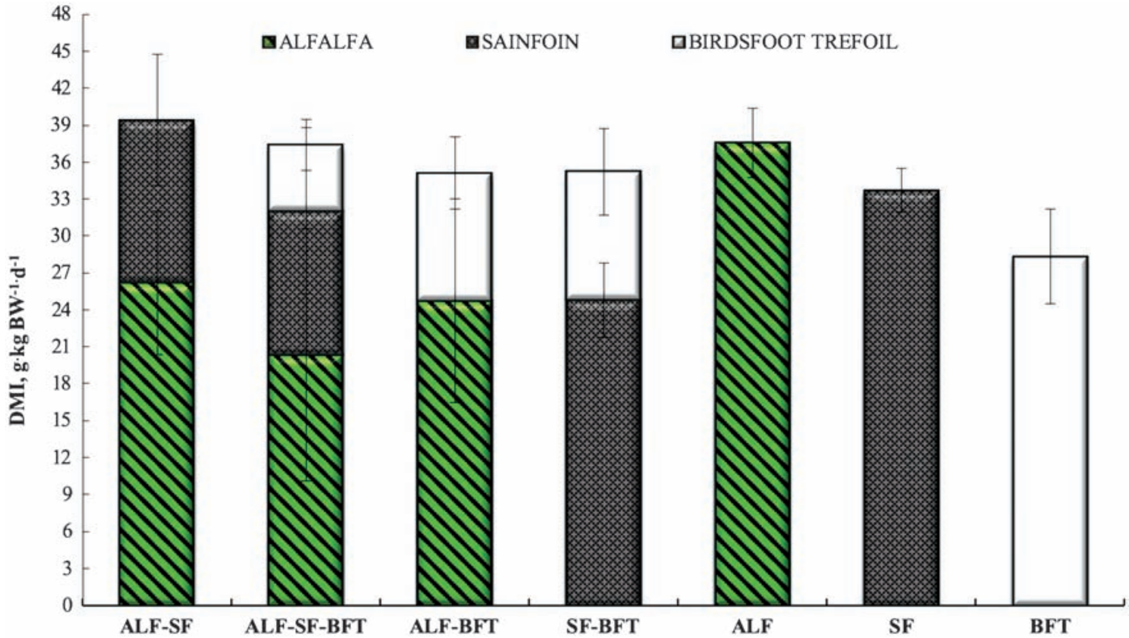

Figure 2. Dry matter intake (g·kg $BW^{-1}·d^{-1}$) of each legume consumed in the choice treatments. Bars represent 95% confidence intervals.

Table 4. Dry matter, NDF, and ADF digestibility (lsmeans), digestible dry matter intake, and fecal output (g·kg $BW^{-1}·d^{-1}$; DM basis) of legumes presented as single forages or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT), and sainfoin (SF)

Diets	DMD[1], %	NDFD[2], %	ADFD[3], %	DDMI[4], g·kg $BW^{-1}·d^{-1}$	FO[5], g·kg $BW^{-1}·d^{-1}$
ALF	64.6[d]	46.5[c]	49.3[cd]	24.2[b]	13.4[a]
BFT	67.7[cd]	47.6[c]	46.9[d]	19.1[c]	9.1[b]
SF	72.0[ab]	54.3[a]	53.6[ab]	24.3[b]	9.4[b]
ALF-SF	71.2[ab]	52.3[ab]	52.6[ab]	28.0[a]	11.3[ab]
ALF-BFT	69.3[bc]	50.7[b]	51.5[bc]	24.0[b]	11.1[ab]
SF-BFT	72.2[ab]	54.2[a]	53.4[ab]	25.4[ab]	9.8[b]
ALF-SF-BFT	72.6[a]	53.5[a]	54.8[a]	27.0[ab]	10.3[b]
SEM	1.1	0.9	0.9	1.1	0.9
P					
Diet effect	<0.001	<0.001	<0.001	0.001	0.033
Date effect				<0.001	<0.001
Diet x Date effect				<0.001	<0.001
2-species vs. singles[6]	0.005	0.001	0.001	0.001	0.874
3-species vs. singles	0.002	0.001	<0.001	0.002	0.765
3- vs. 2-species choice	0.209	0.327	0.033	0.359	0.681
Associative effects[7]			%–(*P*-value)		
ALF-SF-BFT	7.6 (0.001)	8.7 (0.001)	8.7 (0.001)	15.0 (0.014)	-9.9 (0.291)
ALF-SF	6.2 (0.006)	6.6 (0.009)	3.7 (0.095)	15.7 (0.011)	-5.9 (0.547)
ALF-BFT	5.8 (0.013)	8.3 (0.002)	5.9 (0.013)	5.6 (0.387)	-8.0 (0.390)
SF-BFT	2.0 (0.340)	3.4 (0.138)	3.4 (0.121)	11.6 (0.076)	4.8 (0.706)

[a–d]LSmeans in a column with different letters differ ($P < 0.05$).

[1]DMD = in vivo Dry matter digestibility.

[2]NDFD = Neutral detergent fiber digestibility.

[3]ADFD = Acid detergent fiber digestibility.

[4]DDMI = Digestible dry matter intake.

[5]FO = Fecal Output.

[6]Indicate that these are preplanned contrasts between 2-way, 3-way choices and single diets.

[7]Associative effects (%): 100 × [(observed value − Estimated value)/Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

ALF (ALF-SF and ALF-BFT > ALF; $P < 0.05$). In fact, significant positive associative effects were observed for choices containing alfalfa and condensed tannin-containing legumes (Table 4). When both condensed tannin-containing legumes were consumed along with alfalfa (3-way choice), DMD was greater than for the BFT ($P = 0.005$), ALF ($P < 0.001$), or ALF-BFT ($P = 0.048$) treatments (Table 4) and similar to the single and 2-way choice treatments containing sainfoin (e.g., SF, SF-ALF, and SF-BFT; $P > 0.10$). When lambs were allowed to choose between 2 or 3 legume species, DMD was 2.4 and 4.3 percent units greater than treatments receiving single species (70.9% and 72.6% vs. 68.1%, respectively; $P < 0.01$), but no significant differences in DMD were detected for lambs receiving 2-way or 3-way choices of the legumes (Table 4).

NDFD and ADFD followed similar trends to those described for DMD, with values for SF being greater than for BFT ($P < 0.001$) or ALF ($P = 0.001$; Table 4). Similarly, when legumes were offered in 2- and 3-way choices, NDFD values were on average greater than values observed in single diets (52.4% and 53.5% vs. 49.5, respectively; $P = 0.001$; Table 4). In addition, some positive associative effects were detected for NDFD and ADFD, particularly when alfalfa was offered in a choice with condensed tannin-containing legumes in 2- and 3-way choices.

On average across diets, DDMI in ALF and SF was greater than DDMI in BFT ($P = 0.003$; Table 4), particularly during the first 3 d of the experiment, which caused a treatment by day interaction ($P < 0.001$). Overall, DDMI for the 3- and 2-way choices were 20% and 15% greater ($P = 0.002$ and $P = 0.001$) than for single diets (27.0 and 25.8 vs. 22.5 g/kg BW, respectively). In contrast, no significant differences were detected between 2- and 3-way choices. The observed DDMI values for ALF-SF-BFT and ALF-SF were 15% greater than the calculated values from the weighted average of the individual legume components, indicating the presence of significant positive associative effects in these choices (Table 4).

BUN and Fecal Nitrogen Excretion

The proportion of Fecal N/Intake N was SF > BFT and ALF ($P = 0.008$ and $P = 0.010$, respectively) and no differences were observed between BFT and ALF treatments ($P = 0.932$; Table 5). The

Table 5. Fecal nitrogen concentration (%) and excretion (g/d), proportion of the consumed nitrogen excreted through the feces (fecal N/intake N ratio), and BUN of legumes presented as single forage or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT), and sainfoin (SF)

Diets	Fecal N[1], %	Fecal N, g/d	Intake N[2], g/d	Fecal N/intake N, %	BUN[3], mg/dL
ALF	2.27[d]	7.6	27.9	26.7[bc]	19.2[b]
BFT	2.51[bc]	5.5	20.4	26.6[bc]	22.6[a]
SF	2.66[ab]	5.8	18.5	31.5[a]	16.1[c]
ALF-SF	2.55[abc]	7.4	28.9	25.5[c]	18.6[bc]
ALF-BFT	2.40[cd]	7.1	27.9	23.7[c]	22.2[a]
SF-BFT	2.69[a]	6.8	23.2	29.4[ab]	20.6[ab]
ALF-SF-BFT	2.56[abc]	7.0	28.5	24.2[c]	21.5[ab]
SEM	0.06	1.0	3.0	1.2	1.0
P					
Diet effect	0.001	0.747	0.087	0.001	0.001
2-species choice vs. singles[4]	0.209	0.346	0.084	0.044	0.140
3-species choice vs. singles	0.257	0.559	0.084	0.006	0.065
3- vs. 2-species choices	0.807	0.932	0.606	0.156	0.383
Associative effects[5]			%—(*P* value)		
ALF-SF-BFT	5.2 (0.083)	4.4 (0.809)	19.8 (0.199)	−14.6 (0.007)	16.0 (0.022)
ALF-SF	6.0 (0.059)	6.1 (0.738)	16.4 (0.287)	−10.0 (0.071)	2.1 (0.673)
ALF-BFT	2.2 (0.505)	1.4 (0.941)	8.6 (0.563)	−11.1 (0.060)	10.7 (0.104)
SF-BFT	2.8 (0.336)	19.0 (0.402)	21.6 (0.287)	−2.3 (0.649)	15.1 (0.039)

[a–d]LSmeans in a column with different letters differ ($P < 0.05$).

[1]Fecal N = Fecal nitrogen.

[2]Intake N = Intake nitrogen.

[3]BUN = Blood urea nitrogen.

[4]Indicate that these are preplanned contrasts between 2-way, 3-way choices and single diets.

[5]Associative effects (%): 100 × [(observed value − Estimated value)/Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

treatment ALF-SF was not different from ALF ($P = 0.471$), but the proportion of Fecal N/Intake N for the ALF-BFT treatment tended to be lower than that observed for ALF ($P = 0.088$) and significant negative associative effects were detected when these 2 species were combined (Table 5).

The lowest and greatest values for BUN among single diets were observed for SF and BFT, respectively ($P < 0.05$; Table 5). The addition of sainfoin to alfalfa in ALF-SF did not reduce the BUN values observed for ALF ($P = 0.703$), but ALF-BFT increased BUN relative to pure ALF ($P = 0.033$), even with proportions of birdsfoot trefoil in the diet as low as 30%. Thus, BUN from ALF-BFT was greater than in the ALF-SF ($P = 0.013$) treatment. The observed values in SF-BFT and ALF-SF-BFT were significantly greater than the estimated values from their single components, indicating the presence of positive associative effects for BUN concentration in these treatments (Table 5).

DISCUSSION

Voluntary Intake and In Vivo Digestibility in Single Diets

Despite the presence of CT and the greater fiber concentration of the SF diet, lambs fed SF did not show any reduction in DMI relative to lambs fed ALF. It is likely that the 10% difference in NDF concentration observed between SF and ALF treatments was not high enough to induce a detrimental effect on DMI in SF diets. Similarly, the CT concentration observed in the SF diet (3.1% DM basis) was below the range of 6% to 12% mentioned by Aerts et al. (1999) or the threshold of 5.5% reported by Min et al. (2003) for causing feed intake reductions in ruminants fed tanniniferous forages. Consistent with our results, Aufrére et al. (2008) observed similar intakes in sheep fed fresh alfalfa or sainfoin when the concentration of CT in the tannin-containing legume was between 2.5% and 3.5% DM. In contrast, when CT content in sainfoin was around 6%, DMI in sheep was reduced by almost 20% relative to fresh alfalfa diets (Aufrére et al., 2013).

On the other hand, DMI in the BFT treatment was 25% lower than in ALF and tended to be lower than in the SF treatment (16% reduction). It is likely that the high concentration of CP in this forage (the highest out of the 3 legumes tested) accounted for the lower values of DMI observed in the BFT treatment. High intakes of readily degradable sources of N lead to increments in the concentration of ammonia in the peripheral circulation once the liver detoxification threshold is surpassed (Lobley and Milano, 1997), causing reductions in food intake in order to maintain blood ammonia concentration below toxic levels (Provenza, 1995). This response is mediated through aversive postingestive feedback, which may occur very quickly within a meal (Villalba and Provenza, 1997). It is known than cattle are able to adjust their daily DMI to maintain blood ammonia nitrogen levels within a physiological limit of 2 mg/L (Nicholson et al., 1992). A restriction in DMI due to high concentration of CP in BFT is supported by the greater concentrations of BUN observed in the BFT than in the ALF or SF treatments.

The concentration of CT present in birdsfoot trefoil at the moment of being harvested for this study (13 g/kg) apparently was not high enough to reduce the degradation of CP in the rumen. In support of this, it has been suggested that the minimum concentrations of CT in birdsfoot trefoil to reduce the degradation of dietary protein and the production of ruminal ammonia through the formation of indigestible complexes is 20 g/kg DM (Aerts et al., 1999). In fact, previous studies using birdsfoot trefoil with less than 2% CT have shown that ruminal effective N degradability (Marichal et al., 2010) and ruminal concentrations of ammonia nitrogen (NH_3-N) (Williams et al., 2011; Christensen, 2015) were similar for birdsfoot trefoil and alfalfa diets with comparable concentrations of ruminal degradable protein. In contrast, sainfoin showed greater amounts of undegradable crude protein after 8 and 24 h of in vitro incubations than birdsfoot trefoil (Scharenberg et al., 2007a), suggesting that the greater concentration of CT in sainfoin, as shown in this study (31.2 g/kg), was one of the reasons for preventing dietary protein from being degraded to ammonia in the rumen.

The lower DMD observed in this study for ALF and BFT may be due to the lower NDF and ADF digestibilities in these treatments than in SF. In a previous in vitro study, conducted with the same forages used in the present study (Lagrange et al., 2019), alfalfa and birdsfoot trefoil showed lower fiber concentrations and greater rates of fermentation and gas production (CH_4 and CO_2) at early incubation times than sainfoin. It is likely that ALF and BFT diets with a lower content of cell wall components, compounded with greater fermentation rates, increased passage rates of digesta through the rumen, which allowed for potentially digestible cell wall components and other forage constituents to escape ruminal digestion, explaining the observed reductions in fiber digestibility (Allen, 1996; Van Soest, 2018). Other studies (Aufrère et al., 2008; Chung et al., 2013) also observed a greater DMD for sainfoin than for alfalfa diets. The combination of high DM intakes and lower forage digestion in the ALF treatment resulted in lambs showing the greatest fecal outputs out of the three single species tested in the study, excreting 42.6% and 47.3% more feces than lambs eating SF or BFT, respectively.

Voluntary Intake, Preference, and In Vivo Digestibility in Diverse Diets

It was clear that lambs were selective when they were presented with 2- and 3-way choices. In support of this, the nutritional composition of the ingested forages (Table 2) was greater than the composition of the forages on offer (Table 1). This pattern appeared to increase with the increment in availability of alternatives, particularly for NDF, ADF, and ADL (single legumes > 2-way > 3-way choices).

Herbivores manifest partial preferences, even when nutrients in single forages are adequate and toxins are not a concern (Provenza, 1996). A diverse diet allows herbivores to incorporate plants into their diets, that even when less nutritious, provide chemicals (i.e., flavors, antioxidants, and compounds with medicinal properties) that enhance animal nutrition, health, and welfare (Provenza et al., 2003; Villalba and Provenza, 2007). Consistent with this notion, lambs selected a diverse diet when offered choices among the 3 legumes used in the present study, and they preferred the species that showed greater DMI values when fed as single diets. For instance, lambs fed ALF-SF or ALF-BFT treatments preferred alfalfa to the alternative legume in a 70:30 ratio, but this combination did not constrain overall DMI as lambs offered those choices showed DMI values comparable to lambs receiving just alfalfa. Similarly, when lambs had to choose between all three species (ALF-SF-BFT), they selected a diet with proportions of the species: ALF > SF > BFT (53:33:14) that did not constrain DMI relative to the ALF treatment. Finally, when lambs had to choose between sainfoin or birdsfoot trefoil (SF-BFT treatment), they preferred sainfoin to birdsfoot trefoil in a 70:30 ratio, and DMI of the combination did not differ from intake values observed for the SF treatment. The lower preference manifested for birdsfoot trefoil could be a consequence of the high concentration of CP present in this species, as described above. In support of this, by selecting 30% of birdsfoot trefoil in SF-BFT, lambs increased their BUN concentration relative to lambs consuming the SF treatment, suggesting that an excess of N prevented further incorporation of birdsfoot trefoil into the SF-BFT diet.

An in vitro study (Lagrange et al., 2019) using the same forages used in this study shows that fermentation rates and total gas production were similar between alfalfa and substrates representing the 70:30 ratio of alfalfa:sainfoin or alfala:birdsfoot trefoil selected by lambs in the present study. In contrast, fermentation rates and gas production declined when substrates were composed of equal proportions (50:50 ratio) of the same binary choices (i.e., indifferent preference). Similarly, substrates representing the 3-way choice selected by lambs in this study (50:35:15 ALF:SF:BFT ratio) showed greater in vitro fermentation parameters than a mixture composed of equal proportions of the 3 legumes (33:33:33 ALF:SF:BFT). Thus, when lambs had ad libitum access to more than 1 legume, they selected a diverse diet in proportions that yielded fermentation rates (and DMI) similar to those observed for ALF. Thus, instead of just selecting the forage that offered the greatest fermentation rates and one of the greatest intake values (alfalfa), lambs incorporated tannin-containing legumes into their diet in proportions that did not reduce those parameters. This behavior provided the benefit of incorporating bioactive compounds like CT into the diet, which contributed to reduce the incidence of bloat (Howarth et al., 1978; McMahon et al., 1999) and improved the efficiency of N utilization (Barry and McNabb, 1999; Min et al., 2003; Chung et al., 2013). In addition, a diverse diet prevents reductions in DMI caused by the continuous and frequent exposure to the same orosensorial characteristics of a single diet (i.e., sensory-specific satiety, Provenza, 1996; Scott and Provenza, 1998; Atwood et al., 2001). Finally, interactions among chemicals in a diverse diet may lead to positive associative effects that enhance DMI and improve the nutrition of lambs (Görgülü et al., 1996; Keskin et al., 2004). In support of this idea, the mean DMI value of the 2-species choice was greater and the 3-species choice tended to be greater than the mean value for single diets. Another example of positive associative effects is that 2- and 3-way choices resulted in improvements of DMD, NDFD, and ADFD relative to the ALF treatment, with the 3-way choice yielding the highest synergic effect on digestibility. Likewise, lambs in the ALF-SF treatment had greater (14%) DDMI and lambs in ALF-SF-BFT tended to consume more digestible DM (10%) than lambs in the ALF treatment. Such improved forage digestion with the addition of sainfoin and birdsfoot trefoil to alfalfa reduced FO in the 3-way choice relative to the ALF treatment.

Fecal and Blood Urea Nitrogen

No differences were observed in the ratio of Fecal N/Intake N between BFT and ALF treatments, but SF showed the greatest ratio. This response is likely mediated by the presence of CT, which form insoluble complexes with protein under the mild acidic-neutral conditions of the rumen (Perez-Maldonado et al., 1995; Le Bourvellec and Renard, 2012) and inhibit the proteolytic activity of ruminal bacteria (Jones and McAllister, 1994). Some tannin-bound proteins are released in the abomasum and anterior duodenum at lower pH values and then digested, but the process may be incomplete and some proportion of those proteins bound to tannins may end up in the feces (Waghorn et al., 1987), a process that has been reported for sainfoin (McNabb et al., 1998). Thus, the lower concentrations of CT observed in birdsfoot trefoil compounded with their lower precipitation capacity (McAllister et al., 2005) explain the reduced proportion of N into feces in the BFT relative to the SF treatment.

Greater ruminal protein degradation in lambs fed BFT, in addition to the high CP values observed in the birdsfoot trefoil forage, explains the greatest BUN values observed among the single diets for lambs fed the BFT treatment, since high BUN values result from the absorption of excess ammonia from the rumen (Huntington and Archibeque, 2000). Protein degradation and ruminal ammonia-N concentration have been reported to be greater (Dahlberg et al., 1988) or similar (Christensen, 2015) in birdsfoot trefoil than in nontannin-containing legumes like alfalfa. In contrast, lambs fed SF showed the lowest concentrations of BUN, which suggest lower urinary excretions as there is a positive correlation between BUN and urinary N (Kohn et al., 2005). Thus, it is likely that there was a shift in the partition of N from urine to feces in the SF treatment, a pattern that may contribute to reduce environmental N pollution, as fecal N outputs are considered to be less harmful to the environment than urinary N (de Klein and Eckard, 2008). Urinary N is rapidly converted to ammonia and then oxidized to nitrite, nitrates and to volatile nitrous oxide (Oenema et al., 2005) which is a potent greenhouse gas (Forster et al., 2007). In addition, the runoff and leaching of nitrates into ground water contribute to eutrophication of streams and lakes (Whitehead, 2000; Huang et al., 2014). In contrast, fecal N is converted to ammonium at a much slower rate, retained to the soil, and contributing to accumulation of soil organic matter (de Klein and Eckard, 2008).

Ingestion of sainfoin and birdsfoot trefoil in this study had different effects on fecal N concentration and BUN when they were ingested in a choice with alfalfa. Lambs consuming 30% of sainfoin in the ALF-SF treatment showed greater concentrations of N in feces than lambs fed ALF, and this parameter tended to be greater in ALF-SF than in ALF-BFT, although the proportion of Fecal N/Intake N or BUN values were similar to lambs in the ALF treatment. In contrast, lambs ingesting a 30% proportion of birdsfoot trefoil in the ALF-BFT treatment had greater BUN values and showed a trend for lower Fecal N/intake N ratios than lambs in ALF. These results suggest that CT in birdsfoot trefoil did not affect the fate of N excretion or that the high concentrations of CP in birdsfoot trefoil just added more highly degradable protein to the rumen.

CONCLUSIONS

Tannin-containing legumes like sainfoin and birdsfoot trefoil have the potential to reduce environmental impacts and enhance the nutrition of ruminants when presented in a diverse diet with other legumes such as alfalfa. Alfalfa fed as a single diet led to one of the highest DMI values for the study, but FO and BUN values were also proportional to such intake values, suggesting potential for increased environmental impacts. Sainfoin fed as a single forage led to greater concentrations of fecal N and reduced concentrations of BUN, whereas BFT increased BUN likely due to the high CP concentration of this forage. When offered choices among all legumes in 2-way choices, lambs mixed alfalfa with 30% sainfoin or birdsfoot trefoil, and when offered 3-way choices they mixed alfalfa with 33% sainfoin and 14% birdsfoot trefoil. Such selection was proportional to the intake and digestion rates of single forages, without reducing overall DMI relative to the pure alfalfa diet. Mixing legumes also led to positive associative effects that increased forage digestibility relative to ALF. Our results suggest that diverse combinations of legumes have the potential to enhance DMI and DMD relative to feeding single species, while allowing for the incorporation of beneficial bioactive compounds like CT into the diet. Some of the benefits of these compounds entail reductions in ruminal ammonia concentration and increases in the proportions of fecal N, an environmentally less harmful form of N than urinary N. In addition, selecting from an array of legumes also provides benefits related to dietary diversity in generalist herbivores, like improvements in animal welfare and reductions in sensory-specific satiety.

LITERATURE CITED

Aerts, A., Barry, T., and McNabb, W. 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agr. Ecosyst. Environ. 75:1–12. doi:10.1016/S0167-8809(99)00062-6

Agreil, C., and M. Meuret. 2004. An improved method for quantifying intake rate and ingestive behaviour of ruminants in diverse and variable habitats using direct observation. Small Rumin. Res. 54:99–113. doi:10.1016/j.smallrumres.2003.10.013

Allen, M. S. 1996. Physical constraints on voluntary intake of forages by ruminants. J. Anim. Sci. 74:3063–3075. doi:10.2527/1996.74123063x

AOAC. 2000. Official methods of analysis. 17th ed. Assoc. Off. Anal. Chem. Arlington, VA.

Atwood, S. B., F. D. Provenza, R. D. Wiedmeier, and R. E. Banner. 2001. Changes in preferences of gestating heifers fed untreated or ammoniated straw in different flavors. J. Anim. Sci. 79:3027. doi:10.2527/2001.79123027x

Aufrère, J., M. Dudilieu, and C. Poncet. 2008. In vivo and in situ measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. Animal 2:1331–1339. doi:10.1017/S1751731108002450

Aufrère, J., M. Dudilieu, D. Andueza, C. Poncet, and R. Baumont. 2013. Mixing sainfoin and lucerne to improve the feed value of legumes fed to sheep by the effect of condensed tannins. Animal 7:82–92. doi:10.1017/S1751731112001097

Barry, T. N., and W. C. McNabb. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Br. J. Nutr. 81:263–272. doi:10.1017/S0007114599000501

Christensen, R. G. 2015. Improvement of Nutrient Utilization Efficiency, Ruminal Fermentation and Lactational Performance of Dairy Cows by Feeding Birdsfoot Trefoil. All Graduate Theses and Dissertations. 4286. https://digitalcommons.usu.edu/etd/4286/

Chung, Y. H., E. J. Mc Geough, S. Acharya, T. A. McAllister, S. M. McGinn, O. M. Harstad, and K. A. Beauchemin. 2013. Enteric methane emission, diet digestibility, and nitrogen excretion from beef heifers fed sainfoin or alfalfa. J. Anim. Sci. 91:4861–4874. doi:10.2527/jas.2013-6498

Cochran, R. C., and M. L. Galyean. 1994. Measurement of in vivo forage digestion by ruminants. In: G.C. Fahey, Jr, editor, Forage quality, evaluation, and utilization. p. 613–643.

Dahlberg, E. M., M. D. Stern, and F. R. Ehle. 1988. Effects of forage source on ruminal microbial nitrogen metabolism and carbohydrate digestion in continuous culture. J. Anim. Sci. 66:2071–2083. doi:10.2527/jas1988.6682071x

Dijkstra, J., O. Oenema, J. W. van Groenigen, J. W. Spek, A. M. van Vuuren, and A. Bannink. 2013. Diet effects on urine composition of cattle and N2O emissions. Animal 7 Suppl 2:292–302. doi:10.1017/S1751731113000578

Forster, P., V. Ramaswamy, P. Artaxo, T. Berntsen, R. Betts, D.W. Fahey, J. Haywood, J. Lean, D.C. Lowe, G. Myhre, J. Nganga, R. Prinn, G. Raga, M. Schulz and R. Van Dorland. 2007. Changes in Atmospheric Constituents and in Radiative Forcing. In Climate Change 2007. The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In: Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M.Tignor and H. L. Miller, editors. Cambridge University Press, Cambridge, United Kingdom and NY. http://www.ipcc.ch/pdf/assessment-report/ar4/wg1/ar4-wg1-chapter2.pdf

Getachew, G., E. J. Depeters, W. Pittroff, D. H. Putnam, and A. M. Dandekar. 2006. Review: does protein in alfalfa need protection from rumen microbes? Prof. Anim. Sci. 22:364–373. doi:10.15232/S1080-7446(15)31129-3

Görgülü, M., H. R. Kutlu, E. Demir, O. Öztürkcan, and J. M. Forbes. 1996. Nutritional consequences among ingredients of free-choice feeding Awassi lambs. Small Rumin. Res. 20:23–29. doi:10.1016/0921-4488(95)00777-6

Grabber, J. H., W. E. Zeller, and I. Mueller-Harvey. 2013. Acetone enhances the direct analysis of procyanidin- and prodelphinidin-based condensed tannins in lotus species by the butanol-HCl-iron assay. J. Agric. Food Chem. 61:2669–2678. doi:10.1021/jf304158m

Grosse Brinkhaus, A., G. Bee, P. Silacci, M. Kreuzer, and F. Dohme-Meier. 2016. Effect of exchanging Onobrychis viciifolia and Lotus corniculatus for Medicago sativa on ruminal fermentation and nitrogen turnover in dairy cows. J. Dairy Sci. 99:4384–4397. doi:10.3168/jds.2015-9911

Howarth, R. E., B. P. Goplen, A. C. Fesser, and S. A. Brandt. 1978. A possible role for leaf cell rupture in legume pasture Bloat1. Crop Sci. 18:129–133. doi:10.2135/cropsci1978.0011183X001800010034x

Huang, T., B. Gao, X. K. Hu, X. Lu, R. Well, P. Christie, L. R. Bakken, and X. T. Ju. 2014. Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. Sci. Rep. 4:3950. doi:10.1038/srep03950

Huntington, G. B., and S. L. Archibeque. 2000. Practical aspects of urea and ammonia metabolism in ruminants. J. Anim. Sci. 77:1. doi:10.2527/jas2000.77E-Suppl1y

Jones, D. B. 1931. Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins. United States Department of Agriculture. Circular N0 183.

Jones, G. A., and T. A. McAllister. 1994. Effects of sainfoin (Onobrychis viciifolia Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Env. Microbiol. 60:5.

Julier, B., F. Guines, J.-C. Emile, and C. Huyghe. 2003. Variation in protein degradability in dried forage legumes. Anim. Res. 52:401–412. doi:10.1051/animres:2003029

Keskin, M., A. Şahin, O. Biçer, and S. Gül. 2004. Comparison of the behaviour of Awassi lambs in cafeteria feeding system with single diet feeding system. Appl. Anim. Behav. Sci. 85:57–64. doi:10.1016/j.applanim.2003.09.002

de Klein, C. A. M., and R. J. Eckard. 2008. Targeted technologies for nitrous oxide abatement from animal agriculture. Aust. J. Exp. Agric. 48:14. doi:10.1071/EA07217

Kohn, R. A., M. M. Dinneen, and E. Russek-Cohen. 2005. Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. J. Anim. Sci. 83:879–889. doi:10.2527/2005.834879x

Le Bourvellec, C., and C. M. Renard. 2012. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. Crit. Rev. Food Sci. Nutr. 52:213–248. doi:10.1080/10408398.2010.499808

Lagrange, S., S. Lobón, and J. J. Villalba. 2019. Gas production kinetics and in vitro degradability of tannin-containing legumes, alfalfa and their mixtures. Anim. Feed Sci. Technol. 253:56–64. doi:10.1016/j.anifeedsci.2019.05.008

Lobley, G. E., and G. D. Milano. 1997. Regulation of hepatic nitrogen metabolism in ruminants. Proc. Nutr. Soc. 56:547–563. doi:10.1079/PNS19970057

Marichal, M. de J., M. Carriquiry, L. Astigarraga, and A.I. Trujillo. 2010. N fractionation, degradability, intestinal digestibility, and adequacy for ruminal microbial activity of cultivated legumes. Livest. Res. Rural Dev. 22: 23. http://www.lrrd.cipav.org.co/lrrd22/2/mari22023.htm

McAllister, T. A., T. Martinez, H. D. Bae, A. D. Muir, L. J. Yanke, and G. A. Jones. 2005. Characterization of condensed tannins purified from legume forages: chromophore production, protein precipitation, and inhibitory effects on cellulose digestion. J. Chem. Ecol. 31:2049–2068. doi:10.1007/s10886-005-6077-4

McMahon, L. R., W. Majak, T. A. McAllister, J. W. Hall, G. A. Jones, J. D. Popp, and K.-J. Cheng. 1999. Effect of sainfoin on in vitro digestion of fresh alfalfa and bloat in steers. Can. J. Anim. Sci. 79:203–212. doi:10.4141/A98-074

McNabb, W. C., J. S. Peters, L. Y. Foo, G. C. Waghorn, and F. S. Jackson. 1998. Effect of condensed tannins prepared from several forages on the in vitro precipitation of ribulose-1,5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1). J. Sci. Food Agric. 77:201–212. doi:10.1002/(SICI)1097-0010(199806)77:2<201::AID-JSFA26>3.0.CO;2-J

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217–1240. https://pubag.nal.usda.gov/pubag/downloadPDF.xhtml?id=26403&content=PDF

Min, B. R., T. N. Barry, G. T. Attwood, and W. C. McNabb. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Technol. 106:3–19. doi:10.1016/S0377-8401(03)00041-5

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86:2010–2037. doi:10.1002/jsfa.2577

Mueller-Harvey, I., G. Bee, F. Dohme-Meier, H. Hoste, M. Karonen, R. Kölliker, A. Lüscher, V. Niderkorn, W. F. Pellikaan, J.-P. Salminen, et al. 2019. Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration, and diet composition. Crop Sci. 59:1–25 doi:10.2135/cropsci2017.06.0369

NAAIC. 2017. Importance of Alfalfa. North American Alfalfa Improvement Conference–[accessed March 30, 2019]. https://www.naaic.org/resource/importance.php.

Nicholson, J. W. G., E. Charmley, and R. S. Bush. 1992. The effect of supplemental protein source on ammonia levels in rumen fluid and blood and intake of alfalfa silage by beef cattle. Can. J. Anim. Sci. 72:853–862. doi:10.4141/cjas92-097

Oenema, O., N. Wrage, G. L. Velthof, J. W. van Groenigen, J. Dolfing, and P. J. Kuikman. 2005. Trends in global nitrous oxide emissions from animal production systems. Nutr. Cycl. Agroecosystems. 72:51–65. doi:10.1007/s10705-004-7354-2

Perez-Maldonado, R. A., B. W. Norton, and G. L. Kerven. 1995. Factors affecting in vitro formation of tannin-protein complexes. J. Sci. Food Agric. 69:291–298. doi:10.1002/jsfa.2740690305

Provenza, F. D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manag. 48:2–17. doi:10.2307/4002498

Provenza, F. D. 1996. Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. J. Anim. Sci. 74:2010–2020. doi:10.2527/1996.7482010x

Provenza, F. D., and J. J. Villalba. 2006. Foraging in domestic herbivores: linking the internal and external milieux. Feed. Domest. Vertebr. Struct. Funct. Bels VL Ed. CABI Publ Oxfs. 210–240. doi:10.1079/9781845930639.0210

Provenza, F. D., J. J. Villalba, L. E. Dziba, S. B. Atwood, and R. E. Banner. 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. Small Rumin. Res. 49:257–274. doi:10.1016/S0921-4488(03)00143-3

Robertson, J. B., P. J. Van Soest, W. P. T. James, and O. Theander. 1981. The analysis of dietary fiber in food. W P T James, O. Theander, editors, Marcel Dekker, NY. p. 123–58.

Scharenberg, A., Y. Arrigo, A. Gutzwiller, C. R. Soliva, U. Wyss, M. Kreuzer, and F. Dohme. 2007a. Palatability in sheep and in vitro nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). Arch. Anim. Nutr. 61:481–496. doi:10.1080/17450390701664355

Scharenberg, A., Y. Arrigo, A. Gutzwiller, U. Wyss, H. D. Hess, M. Kreuzer, and F. Dohme. 2007b. Effect of feeding dehydrated and ensiled tanniferous sainfoin (*Onobrychis viciifolia*) on nitrogen and mineral digestion and metabolism of lambs. Arch. Anim. Nutr. 61:390–405. doi:10.1080/17450390701565081

Scott, L. L., and F. D. Provenza. 1998. Variety of foods and flavors affects selection of foraging location by sheep. Appl. Anim. Behav. Sci. 61:113–122. doi:10.1016/S0168-1591(98)00093-8

Shreve, B., N. Thiex, and M. Wolf. 2006. National forage testing association reference method: dry matter by oven drying for 3 hours at 105 C. NFTA Reference Methods. National Forage Testing Association, Omaha, NB. https://docs.wixstatic.com/ugd/24f64f_76e09765aa4c4550acb4d845e4d446dd.pdf

Stewart, E. K. 2018. Effect of tannin-containing legume hays on enteric methane emissions and nitrogen partitioning in beef cattle. All Graduate Theses and Dissertations. https://digitalcommons.usu.edu/etd/7170/.

Theodoridou, K., J. Aufrère, D. Andueza, A. Le Morvan, F. Picard, J. Pourrat, and R. Baumont. 2012. Effects of condensed tannins in wrapped silage bales of sainfoin (Onobrychis viciifolia) on in vivo and in situ digestion in sheep. Animal 6:245–253. doi:10.1017/S1751731111001510

Theodoridou, K., J. Aufrère, D. Andueza, J. Pourrat, A. Le Morvan, E. Stringano, I. Mueller-Harvey, and R. Baumont. 2010. Effects of condensed tannins in fresh sainfoin (Onobrychis viciifolia) on in vivo and in situ digestion in sheep. Anim. Feed Sci. Technol. 160:23–38. doi:10.1016/j.anifeedsci.2010.06.007

Tilman, D. 1982. Resource competition and community structure. Princeton University Press, Princeton, NJ.

Van Soest, P. J. 2018. Nutritional Ecology of the Ruminant. Cornell University Press, Ithaca, NY.

Villalba, J. J., and F. D. Provenza. 1997. Preference for flavoured foods by lambs conditioned with intraruminal administration of nitrogen. Br. J. Nutr. 78:545–561. doi:10.1079/bjn19970174

Villalba, J. J., and F. D. Provenza. 2007. Self-medication and homeostatic behaviour in herbivores: learning about the benefits of nature's pharmacy. Animal 1:1360–1370. doi:10.1017/S1751731107000134

Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on Lotus corniculatus L. Br. J. Nutr. 57:115–126. doi:10.1079/bjn19870015

Wang, Y., W. Majak, and T. A. McAllister. 2012. Frothy bloat in ruminants: cause, occurrence, and mitigation strategies. Anim. Feed Sci. Technol. 172:103–114. doi:10.1016/j.anifeedsci.2011.12.012

Whitehead, D. C. 2000. Nutrient Elements in Grassland: Soil-plant-animal Relationships. CABI. doi:10.1079/9780851994376.0001

Williams, C. M., J.-S. Eun, J. W. MacAdam, A. J. Young, V. Fellner, and B. R. Min. 2011. Effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms. Anim. Feed Sci. Technol. 166–167:364–372. doi:10.1016/j.anifeedsci.2011.04.025