Gas Production Kinetics and in vitro Degradability of Tannin-Containing Legumes, Alfalfa and Their Mixtures.

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Gas production kinetics and in vitro degradability of tannin-containing legumes, alfalfa and their mixtures.

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\textit{Abbreviations:} A, Asymptotic gas production; ADF, acid detergent fibre; ADL, acid detergent lignin; ALF, alfalfa; B, time at half asymptote of gas production; BFT, birdsfoot trefoil; C, constant determining the sharpness of the curve; CH\textsubscript{4}, methane; CO\textsubscript{2}, Carbon dioxide; CP, crude protein; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; dOM, Organic matter digestibility; N, nitrogen; aNDF, neutral detergent fibre; NH\textsubscript{3}, ammonia; OM, organic matter; PF, Partitioning factor; PEG, Polyethylene glycol; RMax, maximum rate of gas production; Tmax, time at which maximum gas production rate occurs; SF, sainfoin; VFA, volatile fatty acid.
Abstract

The aim of this study was to determine in vitro ruminal degradability and gas production kinetics of sainfoin (*Onobrachis viciifolia;* SF), birdsfoot trefoil (*Lotus corniculatus;* BFT), alfalfa (*Medicago sativa* L.; ALF) and their binary or trinary mixtures using the gas production technique. The proportions in the mixtures represented: (1) those selected by lambs in a free-choice experiment (70:30 and 50:35:15 ratios for binary and trinary combinations, respectively), or (2) equal proportions (50:50 or 33:33:33 ratios for binary or trinary mixtures, respectively). Organic matter digestibility was greater in ALF and BFT than in SF (0.791 and 0.796 vs 0.751; P<0.05) and this variable decreased as the proportion of SF in the binary mixtures increased. ALF showed greater (P<0.05) gas production rates ($R_{\text{Max}}$ =17.7 ml h$^{-1}$) than BFT (16.5 ml h$^{-1}$) or SF (12.9 ml h$^{-1}$), reaching half of the asymptote of gas production (Parameter $B = 7.3, 7.0$ and $9.5$ h, respectively) and maximum gas production rates at earlier times (2.4, 2.6 and 3.0 h, respectively; P<0.05). The potential gas production (Parameter $A$) was ALF (210.6 ml) > SF (198.3 ml) > BFT (187.6 ml) (P<0.05), and gas production rates decreased relative to pure ALF as the proportions of SF or BFT increased in the mixtures (P<0.05). The presence of two or three species in the substrate did not lead to positive associative effects. Nevertheless, lambs’ preferred mixtures exhibited greater gas production rates and lower times to reach half potential gas production than mixtures formed with equal parts of each of the species (P<0.05). Thus, mixing alfalfa with sainfoin and/or birdsfoot trefoil in a diet at a 70:30 ratio may allow sheep to maintain fermentability values as high as pure alfalfa while ingesting a diverse diet with some bioactives (e.g., condensed tannins) that provide benefits to the internal environment such as reduced bloat and ammonia formation in the rumen, as well as advantages related to dietary diversity in generalist herbivores like improvements in food intake due to reductions in sensory-specific satiety.
Key Words: in vitro gas production kinetics, tannin-containing legumes, alfalfa, sainfoin, condensed tannins, forage mixtures.

1. Introduction

Alfalfa (Medicago sativa L.) is one of the most high-yielding and nutritious forages available, used widely for beef and dairy cattle production around the world. Nevertheless, its use in pure stands has been limited by the associated risk of bloat (Berg et al., 2000). In addition, the inefficient protein use observed in ruminants consuming pure alfalfa may lead to nitrogen (N) losses via urinary excretion, being detrimental to the environment (Julier et al., 2003; Getachew et al., 2006).

In contrast to alfalfa, legume species containing moderate levels of condensed tannins (CT) such as sainfoin (Onobrichis vicifolia) or birdsfoot trefoil (Lotus corniculatus) are non-bloating (Howarth et al., 1978) and show an increased efficiency of N utilization by ruminants (Barry and McNabb, 1999). Condensed tannins are polyphenolic compounds that limit plant protein degradation in the rumen and increase the pool of high-quality protein that reaches the small intestine (Koenig and Beauchemin, 2018). Thus, the use of tannin-containing legumes in association with alfalfa may represent an effective alternative to reduce N pollution by shifting the site of N excretion from urine to more stable forms of N in feces (Wang et al., 2006; Aufrère et al., 2013). In addition, the presence of CT in legumes has been shown to reduce methanogenesis in both in vitro (Niderkorn et al., 2012) and in vivo (Ramírez-Restrepo and Barry, 2005) studies. Nevertheless, associations between alfalfa and tannin-containing legumes need to be achieved in a context where dry matter degradability and ruminal fermentation rates are not constrained. Otherwise, intake and productivity could be negatively compromised when animals ingest such
mixtures. Alternatively, combinations of legumes may lead to associative effects that enhance productivity and reduce environmental impacts.

Previous studies report that high concentrations of CT may depress fiber digestion (McAllister et al., 2005), although the effect of condensed tannins on ruminal digestion may vary depending on their concentration in the diet and on their chemical structure (Wang et al., 2015; Mueller-Harvey et al., 2017). Differences between content and molecular structure of CT in birdsfoot trefoil and sainfoin may have differential effects on rumen fermentation, with potential synergies or antagonisms when these legumes are consumed together. Alternatively, the differential effects of CT may vary when tannin-containing legumes are ingested as the sole forage source or diluted with non-tannin containing legumes such as alfalfa.

Therefore, the aim of this study was to determine in vitro ruminal degradability and gas production kinetics of birdsfoot trefoil, sainfoin and alfalfa as single species, binary or trinary mixtures in order to better understand the significance of associations CT-containing legumes-alfalfa relative to single-species. The proportion of legumes in the mixture was designed such that the different species contributed in equal amounts to the mixture or in amounts that represented the selection displayed by lambs during 2- or 3-way choices in cafeteria tests. Thus, our second objective was to compare the gas production kinetics of preferred proportions to equal proportions (i.e., indifferent preference value) of legume mixtures.

2. Materials and methods

Gas production kinetics and extent of degradation was determined using the gas production technique described by Theodorou et al. (1994) and modified by Mauricio et al. (1999).
2.1 Substrates and experimental design:

Samples of CT-containing legumes (birdsfoot trefoil; BTF, cv. Langille and sainfoin; SF, cv. Shoshone), and CT-free alfalfa (ALF, cv. DK), were collected on June 07, 2015 on three monoculture plots of 0.17 ha each (spatial replications) seeded in August 2014 at the Utah State University Intermountain Irrigated Pasture Project research facility in Lewiston, northern Utah (41 56’ N 111 52’W). Birdsfoot trefoil and alfalfa were cut at late bud stage and sainfoin in late flowering stage using a flail harvester (Rem Manufacturing Ltd., Swift Current, SK, Canada) at around 10 cm from ground level. Immediately after harvesting, samples of 250 g (particle size 2-4 cm) from each species were frozen at -20 °C and then freeze dried at -60 °C (Labconco Corporation Kansas City, MO, USA) until constant weight and ground to pass a 1-mm screen with a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Ground material of these legumes were combined in eleven different ratios (treatments). Treatments were: 1) ALF, 2) BFT, 3) SF (single forages), binary and trinary mixtures with proportions of species selected by lambs during a free-choice experiment (Lagrange and Villalba, 2016): 4) 70:30 ratios of: ALF/BFT (A70-B30), 5) ALF/SF (A70-S30), 6) SF/BFT (S70-B30), and 7) 50:35:15 ratio of ALF/SF/BFT (A50-S35-B15). Finally, treatments involved the equal part combinations (i.e., “no preference”) of the legumes: 50:50 ratios for binary (8) A50-B50; 9) A50-S50; 10) S50-B50) and 11) 33:33:33 ratio for trinary mixtures (A33-S33-B33).

Five hundred milligrams of each one of these mixtures were weighted in small aluminum cups and placed in 125 ml serum flasks (Wheaton, Boston, USA) by triplicate. A total of 36 flasks (11 treatments x 3 replicates) plus 3 blanks were incubated in each batch.
2.2 Inoculum:

Rumen fluid was taken 2 h post-feeding from a rumen-cannulated Angus cow on an *ad libitum* diet of tall fescue hay (Utah State University Institutional Animal Care and Use Committee, Approval # 2470). Rumen fluid pH was measured with a potentiometer (HI 991002, Hanna Instruments, Woonsocket, RI, USA) and averaged 6.9 ± 0.3.

2.3 In vitro fermentation procedure and gas production measurements:

Forty ml of buffer medium prepared according to Menke (1988), were slowly added to each 125 ml serum flasks while flushing simultaneously with CO₂ for five seconds. Flasks were subsequently sealed with 20 mm butyl rubber stoppers and aluminum crimp caps (Wheaton Cia, Boston, USA), and stored overnight at 4°C. On the next day, 20 ml of rumen fluid were injected into the flasks directly through the rubber stopper, using a 25 ml syringe with a 18 gauge needle, 1:2 (v:v) rumen fluid : buffer medium ratio. This time was considered time zero where the incubation process started. pH of the buffer and ruminal fluid mixture at this time averaged 7.0 ± 0.1. The rumen fluid was kept at 39°C until all flasks were filled and shaked frequently in order to keep adequate environmental conditions for the microorganisms. The portion of gas displaced by the added liquid into the flask was allowed to escape prior to removing the needle from the stopper. Then, flasks were shaked and placed in a preheated incubator (Percival, Boone, IA, USA) at 39°C.

Head-space gas pressure in the flasks was read with an USB output pressure transducer, (type PX409-015GUSBH, Omega Engineering Inc., Stamford, CT, USA) connected to a PC that enabled to chart, log, display, and output data coming from the transducer (Mauricio et al., 1999). Readings were taken at regular intervals of 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h during the incubation period, inserting through the flasks stoppers a 23-gauge needle which was attached to
the pressure transducer through a luer fitting-type connector. After the last reading, flasks were opened and the pH of the solution measured. Flasks were placed into a fridge at 4°C to slow down the incubation and their contents immediately filtered.

2.4 Gas production kinetics:

Gas volume estimates were generated for each incubation time from the gas pressure values previously registered by the pressure transducer using the equation reported by Frutos et al. (2002; Eq. (1)). Gas volumes were corrected for the amount of substrate organic matter (OM) incubated and gas released from blanks (ruminal fluid plus buffer medium without substrate). Organic matter in the substrate was determined by ashing substrates at 550°C for 6 h (Thiex and Novotny, 2012). Corrected gas production estimates for each incubation time were then added in order to construct the gas production profiles of each treatment and gas production parameters were obtained using the Groot et al. (1996)’s single phasic model (Eq. (2)),

Head-space gas volume (ml) = 5.3407*gas pressure (psi)  \hspace{1cm} (1)

\[
G = \frac{A}{1 + (B^t/t^c)}
\hspace{1cm} (2)
\]

where $G$ represents the amount of gas produced per unit of organic matter incubated at time $t$ after the beginning of the incubation, $A$ is the asymptotic gas production (ml g\(^{-1}\) OM); $B$ (h) is the time after starting incubation at which half of the asymptotic amount of gas has been formed, representing the speed of gas production, and $C$ is a constant determining the sharpness of the switching characteristics of the curve; as the value of $C$ increases, the curve becomes sigmoidal with an increasing slope. The maximum rate of gas production ($R_{Max}$) and the time at which it occurs ($T_{Max}$) were calculated according to the following equations (Bauer et al., 2001).
\[ R_{\text{Max}} \text{ (mL h}^{-1} \text{)} = (A \cdot B^C \cdot C \cdot T_{\text{Max}}^{(-C-1)}) / ((1 + B^C \cdot T_{\text{Max}}^{-C})^2) \]  

(3)

\[ T_{\text{Max}} \text{ (h)} = B^*(((C-1)/(C+1))^{1/C}) \]  

(4)

\( R_{\text{Max}} \) is obtained when the microbial population is big enough such that it no longer limits the fermentation process of the substrate and digestion is not reduced by chemical or structural barriers of the potentially digestible material at this point (Groot et al., 1996).

2.5 Substrate disappearance:

Organic matter disappearance at 48 h incubation (organic matter degradability; dOM), was determined by filtering the flasks contents with 50 μm porosity (10 x 5 cm) ankon filter bags (Ankon Technology, Macedon, NY), previously oven dried and weighted. Bags were then washed with deionized water and dried in an air-forced oven at 60°C to constant weight. Residual dry matter values were obtained by weighting bags with the digestion residues and extracting the empty dry bag weights. Dry matter degradation was calculated then by difference between the substrate and residue dry weights and corrected by the residual material measured in the blanks. Organic matter degradation (dOM) was determined by ashing the fermentation residues (see below). Finally, substrate disappearance allows for the calculation of a partitioning factor (PF) (Blümmel et al., 1997) which relates the amount of organic matter degraded \textit{in vitro} to the gas volume produced by such amount, providing an estimate of fermentation efficiency.

2.6 Chemical analyses:

Forages were analyzed for dry matter (DM), crude protein (CP), neutral (aNDF) and acid (ADF) detergent fiber, ADL (acid detergent lignin), condensed tannin (CT) content and ash. DM was determined using a two-step process. First, a partial drying using a forced-air drying oven at 60°C for 48 h, and secondly 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 measuring the N content of the samples using a Leco FP-528 nitrogen combustion analyzer (AOAC, 2000; method 990.03) and applying the 6.25 conversion factor. aNDF (Mertens, 2002), ADF (AOAC, 2000; method 973.18) and ADL (Robertson and Van Soest, 1981) determinations were modified by using Whatman 934-AH glass micro-fiber filters with 1.5 μm particle retention and a California Buchner funnel in place of fritted glass crucible. Ash was determined burning samples at 550°C for 6 h (Thiex and Novotny, 2012). Organic matter (OM) was calculated by difference between dry matter and ash. Analyses of total condensed tannins in the legumes were conducted in triplicate, 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.

2.7 Statistical analyses:

The experimental design was a completely randomized block design with three plots (spatial replications) and eleven treatments (different forage mixtures). Substrates and blanks were run twice per plot (experimental units), each run was conducted on a different week with three serum flasks (measurement units) per treatment, totaling six runs in six consecutive weeks with 36 flasks/run.

Gas production parameters were estimated using PROC NLIN in SAS/STAT (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows) with A=200, B=20 and C=1, as initial values. The estimated gas production parameters, maximum rate of digestion ($R_{\text{Max}}$; Eq. (3)), time at which maximum rate occurs ($T_{\text{Max}}$; Eq. (4)), organic matter degradation (dOM) and partitioning factor (PF) were compared using a mixed model in which treatment was the main factor, plot and run as random factors. Analyses were performed using PROC GLIMMIX in SAS. Plot variation was
found non-significant and therefore dropped from the mixed model. Least square means (LSMeans) were compared pairwise using Tukey’s multiple comparison test when F-ratios were significant (P<0.05) and reported along with their standard errors (SEM). Differences among LSMeans with P<0.05 were considered statistically significant. A tendency was considered when 0.10> P> 0.05.

In order to explore the potential associative effects in the legume mixtures, observed to estimated values for gas production parameters and organic matter degradability were calculated as: 100 x [(Observed value – Estimated value)/Estimated value]. The estimated value was the weighted average of the observed values for the single substrates. 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 (e.g. A70-S30 observed value vs ALF*0.7 + SF*0.3 observed values).

In addition, preplanned contrasts were performed to compare the average of gas production parameters for single species vs binary mixtures, single species vs trinary mixtures or trinary vs binary mixtures. A difference between the average of singles, binary or trinary mixtures groups or between observed and estimated values (associative effects) was considered significant when P values were < 0.05. Inspections of studentized residuals revealed no deviations from homoscedasticity of variance or normality.

3. Results

3.1 Chemical composition of substrates

Chemical composition of the substrates assayed in the study is shown in Table 1. The greatest content of CP was observed in BFT, followed by ALF and then SF. In contrast, fiber
concentration (NDF and ADF) was greater in SF than in BFT and ALF. Condensed tannin contents were on average 2.5 X greater in SF than in BFT. Alfalfa is a non-tannin containing legume, confirmed by the very low values of CT revealed in the assay (Table 1).

Table 1. Chemical composition (g/kg dry matter [SEM]) of the forages used in the study.

<table>
<thead>
<tr>
<th>Species</th>
<th>CP</th>
<th>NDF</th>
<th>ADF</th>
<th>ADL</th>
<th>Ash</th>
<th>OM</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>168.0 (4.0)</td>
<td>364.0 (4.7)</td>
<td>306.7 (5.8)</td>
<td>65.0 (1.3)</td>
<td>89.8 (1.8)</td>
<td>910.2 (1.8)</td>
<td>1.9 (0.1)</td>
</tr>
<tr>
<td>Birdsfoot Trefoil</td>
<td>189.3 (2.0)</td>
<td>385.0 (2.0)</td>
<td>334.0 (4.0)</td>
<td>70.8 (2.9)</td>
<td>146.8 (3.1)</td>
<td>853.2 (3.1)</td>
<td>12.9 (0.7)</td>
</tr>
<tr>
<td>Sainfoin</td>
<td>131.7 (3.4)</td>
<td>438.0 (10.2)</td>
<td>391.0 (5.3)</td>
<td>86.2 (4.3)</td>
<td>73.9 (4.8)</td>
<td>926.1 (4.8)</td>
<td>31.0 (1.4)</td>
</tr>
</tbody>
</table>

CP= crude protein; NDF= neutral-detergent fiber; ADF= acid-detergent fiber; ADL= acid detergent lignin; OM= organic matter and CT= Condensed tannin content.

3.2 Organic matter disappearance:

Regarding single substrates, ALF and BFT degradabilities were similar (P=0.999) and greater than SF (P<0.001) for both species, respectively (Table 2). The substitution of sainfoin for alfalfa significantly reduced the extend of degradation in the A50-S50 mixture, however A70-S30 did not differ from ALF (P>0.05). Similarly, the replacement of sainfoin for birdsfoot trefoil only reduced BFT degradability significantly at the higher proportion of sainfoin in the mixture (S70-B30). On average across all the mixtures, either 2- or 3-way combinations did not differ significantly from single forages and no associative effect were observed in any of the mixtures (P>0.05).

3.3 Gas production kinetics:

Cumulative gas production profiles, rate of gas production curves and parameters describing the cumulative gas production for each substrate are presented in Fig. 1 and Table 2, respectively. The mono-phasic model of Groot et al. (1996), fitted the gas production data obtained during the fermentation process (R² mean value = 0.999). All parameters were found different among substrates (P<0.01). The asymptotic gas production (A) was ALF > SF (P<0.001) > BFT (P<0.001).
Table 2. Organic matter degradation and gas production kinetics (LSmeans) of Alfalfa, Sainfoin and B. Trefoil mixtures incubated as single forages or in 2- and 3-way combinations.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>dOM</th>
<th>A (ml/g OM)</th>
<th>B (h)</th>
<th>C</th>
<th>R_{Max} (ml/h)</th>
<th>T_{Max} (h)</th>
<th>PF (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALF</td>
<td>0.791</td>
<td>210.6 a</td>
<td>7.3 de</td>
<td>1.48 ab</td>
<td>17.7 a</td>
<td>2.41 c</td>
<td>4.19 d</td>
</tr>
<tr>
<td>BFT</td>
<td>0.797</td>
<td>187.6 f</td>
<td>7.0 e</td>
<td>1.57 a</td>
<td>16.5 bc</td>
<td>2.66 abc</td>
<td>4.75 a</td>
</tr>
<tr>
<td>SF</td>
<td>0.751</td>
<td>198.3 cd</td>
<td>9.5 c</td>
<td>1.45 b</td>
<td>12.9 h</td>
<td>3.00 ab</td>
<td>4.58 abc</td>
</tr>
<tr>
<td>A70-S30</td>
<td>0.775</td>
<td>203.3 bc</td>
<td>7.7 cd</td>
<td>1.48 ab</td>
<td>16.1 cd</td>
<td>2.56 abc</td>
<td>4.37 cd</td>
</tr>
<tr>
<td>A50-S50</td>
<td>0.766</td>
<td>206.0 ab</td>
<td>8.7 b</td>
<td>1.43 b</td>
<td>14.7 ef</td>
<td>2.62 abc</td>
<td>4.42 bcd</td>
</tr>
<tr>
<td>A70-B30</td>
<td>0.785</td>
<td>197.1 cde</td>
<td>7.0 e</td>
<td>1.53 ab</td>
<td>17.3 ab</td>
<td>2.51 bc</td>
<td>4.46 bcd</td>
</tr>
<tr>
<td>A50-B50</td>
<td>0.801</td>
<td>190.4 ef</td>
<td>7.7 cd</td>
<td>1.57 a</td>
<td>15.2 de</td>
<td>2.90 abc</td>
<td>4.77 a</td>
</tr>
<tr>
<td>S70-B30</td>
<td>0.768</td>
<td>194.4 def</td>
<td>8.8 b</td>
<td>1.45 b</td>
<td>13.7 fg</td>
<td>2.73 abc</td>
<td>4.68 ab</td>
</tr>
<tr>
<td>S50-B50</td>
<td>0.780</td>
<td>187.8 f</td>
<td>8.0 c</td>
<td>1.52 ab</td>
<td>14.3 ef</td>
<td>2.83 abc</td>
<td>4.77 a</td>
</tr>
<tr>
<td>A50-S35-B15</td>
<td>0.781</td>
<td>197.1 cde</td>
<td>8.2 c</td>
<td>1.50 ab</td>
<td>14.8 e</td>
<td>2.78 abc</td>
<td>4.59 abc</td>
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<tr>
<td>A33-S33-B33</td>
<td>0.787</td>
<td>193.4 def</td>
<td>8.9 b</td>
<td>1.50 ab</td>
<td>13.3 gh</td>
<td>3.06 a</td>
<td>4.80 a</td>
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<tr>
<td>S.E.M</td>
<td>0.007</td>
<td>2.5</td>
<td>0.2</td>
<td>0.03</td>
<td>0.6</td>
<td>0.18</td>
<td>0.13</td>
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<tr>
<td>P-value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.002</td>
<td>&lt; 0.001</td>
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<tr>
<td>2-way vs singles</td>
<td>0.848</td>
<td>0.030</td>
<td>0.802</td>
<td>0.876</td>
<td>0.006</td>
<td>0.988</td>
<td>0.112</td>
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<tr>
<td>3-way vs singles</td>
<td>0.423</td>
<td>0.010</td>
<td>&lt; 0.001</td>
<td>0.989</td>
<td>&lt; 0.001</td>
<td>0.027</td>
<td>0.001</td>
</tr>
<tr>
<td>3- vs 2-way choices</td>
<td>0.283</td>
<td>0.294</td>
<td>&lt; 0.001</td>
<td>0.880</td>
<td>&lt; 0.001</td>
<td>0.015</td>
<td>0.017</td>
</tr>
</tbody>
</table>

**Associative Effects:**

<table>
<thead>
<tr>
<th>Substrates (%)</th>
<th>dOM</th>
<th>A (ml/g OM)</th>
<th>B (h)</th>
<th>C</th>
<th>R_{Max} (ml/h)</th>
<th>T_{Max} (h)</th>
<th>PF (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A70-S30 (%)</td>
<td>-0.5 ns</td>
<td>-1.7 ns</td>
<td>-2.9 ns</td>
<td>1.0 ns</td>
<td>-0.3 ns</td>
<td>0.9 ns</td>
<td>1.4 ns</td>
</tr>
<tr>
<td>A50-S50 (%)</td>
<td>-0.7 ns</td>
<td>0.8 ns</td>
<td>3.2 ns</td>
<td>-2.1 ns</td>
<td>-3.9 *</td>
<td>-2.4 ns</td>
<td>0.5 ns</td>
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<tr>
<td>A70-B30 (%)</td>
<td>-1.0 ns</td>
<td>-3.2 ***</td>
<td>-3.7 ns</td>
<td>1.8 ns</td>
<td>0.3 ns</td>
<td>1.5 ns</td>
<td>2.4 ns</td>
</tr>
<tr>
<td>A50-B50 (%)</td>
<td>0.7 ns</td>
<td>-4.4 ***</td>
<td>7.5 ***</td>
<td>3.1 ns</td>
<td>-10.7 ***</td>
<td>16.2 **</td>
<td>6.5 ***</td>
</tr>
<tr>
<td>S70-B30 (%)</td>
<td>0.3 ns</td>
<td>-0.3 ns</td>
<td>0.1 ns</td>
<td>-2.5 ns</td>
<td>-1.9 ns</td>
<td>-5.0 ns</td>
<td>1.0 ns</td>
</tr>
<tr>
<td>S50-B50 (%)</td>
<td>0.6 ns</td>
<td>-2.6 **</td>
<td>-2.7 ns</td>
<td>0.6 ns</td>
<td>-2.1 ns</td>
<td>1.4 ns</td>
<td>2.2 ns</td>
</tr>
<tr>
<td>A50-S35-B15 (%)</td>
<td>0.1 ns</td>
<td>-2.9 **</td>
<td>2.3 ns</td>
<td>1.1 ns</td>
<td>-6.8 ***</td>
<td>6.5 ns</td>
<td>4.1 *</td>
</tr>
<tr>
<td>A33-S33-B33 (%)</td>
<td>0.8 ns</td>
<td>-2.7 **</td>
<td>12.7 ***</td>
<td>0.2 ns</td>
<td>-15.2 ***</td>
<td>15.0 **</td>
<td>6.3 ***</td>
</tr>
</tbody>
</table>

$dOM$: Coefficient of organic matter digestibility; $A$: Asymptotic gas production (ml/g OM); $B$: time to half of the asymptote (h); $C$: Constant determining the sharpness of the curve; $R_{Max}$: maximum gas production rate (ml h$^{-1}$); $T_{Max}$: time at which $R_{Max}$ occurs (h); $PF$: Partitioning Factor (mg OM disappeared/ml gas produced); * Associative effects (%): 100 x [(observed value – Estimated value)/Estimated value]. Estimated value was the weighted average of the observed values for the pure substrates. Means in a column with different letters differ significantly (P<0.05); ns: non-significant; * P<0.05; ** P<0.01; *** P<0.001.
The inclusion of the tannin-containing legumes (SF or BFT) in the 2- or 3-way mixtures, reduced (P<0.05) the asymptotic gas production compared with single ALF (Table 2). However, mixtures with BFT (A70-B30 and A50-B50) had significant lower A than mixtures with SF (A70-S30 and A50-S50). In fact, significant negative associative effects were observed for mixtures containing ALF and BFT (Table 2), to the point that no differences were observed in the asymptotic gas production between the A50-B50 mix and the BFT treatment (190.4 vs 187.6 ml/g OM, respectively).

Similarly, maximum gas production rates were reached faster (T\text{max}: 2.4 vs 3.0 h; P=0.016) and they were greater (R\text{Max}: 17.7 vs 12.9 ml/h; P<0.001) for ALF than for SF, respectively, with A70-S30 and A50-S50 presenting intermediate values between ALF and SF treatments (Table 2). However, after T\text{max} was reached, ALF gas production rates deaccelerated faster than in SF such that after 8 h of incubation, gas production rate profiles looked very similar among all ALF-SF mixtures (Fig. 1a), and by 18 h the SF rate was greater than that of ALF maintaining this trend towards the end of the incubation period. Consistent with this trend, the ALF treatment required less time than SF to reach half of the potential gas production (parameter B: 7.3 vs 9.5 h; P<0.001, respectively) and that time was extended as the proportion of SF in the mixture increased.

Maximum gas production rate was also greater for ALF than for BFT (R\text{Max}: 17.7 vs 16.5 ml/h, respectively; P=0.023), but in contrast with SF, gas production rates in BFT began to deaccelerate rapidly after 12 h of incubation, and gas production almost reached its asymptotic value after 18 h (Fig. 1b). Similar to ALF, the rates of gas production in BFT were greater than in SF only at early incubation times (e.g., between 2 – 8 h; Fig. 1c), decreasing R\text{Max} (P<0.05) as the proportion of SF increased in the BFT-SF mix (Table 2).
Fig. 1. Cumulative gas production and rate of gas production profiles from different mixtures of a) Alfalfa and Sainfoin; b) Alfalfa and B. trefoil; c) Sainfoin and B. Trefoil; d) Alfalfa, Sainfoin and B. Trefoil. Bars represent standard errors of the mean (SEM).
In general, no significant differences in gas production parameters were observed between the average of binary mixtures and the average of single substrates, except for the potential gas production ($A$: 198.8 vs 196.5 ml/g OM) and maximum rate of gas production ($R_{Max}$: 15.7 vs 15.2 ml/h) for single forages vs 2-way mixtures, respectively (Table 2), likely driven for the negative associative effects observed for these parameters in the ALF-BFT mixtures.

When both tannin-containing legumes were incubated with alfalfa, regardless of their proportions in the 3-way mixtures, reduced the rates of gas production at the beginning of the incubation process relative to ALF ($R_{Max}$: 17.7, 14.8 and 13.3 ml/h for ALF, A50-S35-B15 and A33-S33-B33, respectively), and extended the time to reach half of the potential gas production (P<0.05). The delays in gas production increased with increments in the proportion of tannin-containing legumes in the mixture ($T_{max}$: 2.4, 2.8 and 3.1 h; Parameter $B$: 7.3, 8.2 and 8.9 h; for ALF, A50-S35-B15 and A33-S33-B33, respectively; P<0.05). In fact, the gas profile for the A33-S33-B33 mixture was very similar to the profile observed for pure sainfoin (SF), while A50-S35-B15 showed intermediate values between the singles substrates (Fig. 1d).

Some negative associative effects were present specially for $R_{Max}$ (P<0.001) when equal proportions of alfalfa and birdsfoot trefoil were combined (A33-S33-B33) as in the binary mixture (50A-50B) and in $T_{max}$ and parameter $B$ where the time was longer for observed than for estimated values (P<0.001). In fact, the average of both trinary mixtures (A50-S35-B15 and A33-S33-B33) showed a lower gas production rate ($R_{Max}$: 14.1 vs 15.7 ml/h; P<0.001) and potential gas production ($A$: 195.3 vs 198.8 ml/g OM; P<0.05) than the average of the three single substrates respectively, and a greater $T_{Max}$ (2.9 vs 2.7 h; P<0.05) and parameter $B$ (8.6 vs 7.9 h; P<0.001).
4. Discussion

4.1 Organic matter disappearance:

The greater OM degradability in ALF and BFT than in SF is likely attributable to the lower concentrations of ADF in the former forages, in line with the more advanced stage of maturity of sainfoin at the time of being sampled. BFT and ALF were cut at the early flowering stage, while SF was harvested in the late flowering stage of the first growth cycle. Our results are consistent with prior research (Niderkorn et al., 2011), showing greater values of in vitro degradability in ALF than in SF.

Previous studies found negative correlations between in vitro OM disappearance and CT contents (Frutos et al., 2002) with concentrations of CT generally greater than 50 g/kg. It is likely that the lower content of CT in the legumes of this study compounded with their chemistries (Mueller Harvey et al., 2017) did not influence forage degradability. In fact, we did not find any differences for this parameter between a non-tannin (alfalfa) containing legume and birdsfoot trefoil. Consistent with this notion, previous in vitro (Wang et al., 2007) and in vivo (Theodoridou et al., 2010, Theodoridou et al., 2012) studies did not find any increments in sainfoin OM digestibility when polyethylene glycol (PEG), a binding agent that suppresses tannin activity, was included in the incubation or dosed directly to the rumen, discarding any influence of CT on sainfoin degradability.

Degradability values in mixtures were in general a linear combination of the values found in the pure substrates, but there were some exceptions. For instance, when the proportion of sainfoin in the mix with alfalfa was 0.30, dOM values did not differ from those observed for ALF. However, when proportion of SF in the mix grew to 0.50, there was a significant reduction in dOM relative to ALF. These results suggest that alfalfa might be mixed with sainfoin up to a proportion of 0.30
without negative impacts on organic matter degradability, which help explain the proportions of alfalfa and sainfoin selected by lambs in a cafeteria test (Lagrange and Villalba, 2016). Ruminants select diets based on the association between the taste of a food and its post-ingestive consequences (Provenza, 1995), so it is likely lambs selected a 70:30 ratio of alfalfa/sainfoin to maintain degradability values of the mix as high as pure alfalfa with the benefit of including a tannin-containing legume like sainfoin in the diet.

The addition of SF to BFT (SF-BFT mixtures) also reduced dOM relative to BFT, but only with the highest proportion of sainfoin in the mixture (S70-B30). In this case, lambs offered a free choice of the same two legumes used in this study preferred sainfoin over birdsfoot trefoil in a 70:30 ratio, suggesting that factors other than digestibility might have driven this selection. Considering the high CP content observed in BFT, the proportion selected may represent the need to attenuate the accumulation of NH$_3$ in the rumen through the ingestion of CT from sainfoin (Chung et al., 2013; Copani et al., 2015), particularly given that excesses of NH$_3$ in the rumen fluid are aversive, promoting reductions in food intake (Provenza, 1995; Villalba and Provenza, 1997).

Finally, another important result of this study is that the presence of sainfoin along with alfalfa and birdsfoot trefoil in trinary mixtures resulted in digestibilities values comparable to those observed for pure ALF or BFT. Thus, the selection that lambs performed during preference tests (A50-S35-B15) with the same forages used in this study allowed for an increased dietary diversity with digestibility values comparable to those observed in single legumes that exhibited the greatest values for this parameter.

4.2 Gas production kinetics:

The slower rate of gas production for SF at early incubation times could be due in part to its advanced stage of maturity, with greater concentration of cell walls and lower crude protein
content (Guglielmelli et al., 2011). In support of this, Niderkorn et al. (2011) found that in vitro fermentation of sainfoin led to lower VFA concentration and gas production than alfalfa during the first hours of incubation. We also observed that ALF and BFT - with lower contents of fiber and greater concentration of protein –had greater gas production rates and greater levels of gas produced at shorter periods of time than in the SF treatment.

According to Groot et al., (1996), the initial time of the incubation period is related to the fermentation of the soluble, fast fermentable fraction of the substrate (i.e., soluble carbohydrates) and microbial protein synthesis, whereas the last portion of the incubation period is related to the fermentation of the insoluble but potentially degradable components like the NDF fraction. This is in line with the proportionally greater amounts of gas production observed during the latter incubation times of the SF treatment, which contrasts with the deaccelerating gas production process observed for ALF and especially for BFT. In addition, sainfoin is characterized for a very low fiber digestion at early incubation times (Niderkorn et al., 2011), and previous studies reported a negative impact of CT on gas production for sainfoin substrates (Theodoridou et al., 2011). Thus, it can be concluded that CT in SF may be contributing along with the greater contents of fiber and lower concentration of protein to the reductions in the rate of gas production and potential gas production observed in this study. The influence of CT in BFT might be different from that described for SF since gas production rates at early incubation times were greater for BFT than for SF. The different CT concentrations measured in SF and BFT along with differences in chemical structures (McAllister et al., 2005; Mueller-Harvey, 2006) may be driving the distinct effect of CT on the digestion process.

The asymptotic gas production in BFT was lower than in ALF despite similar organic matter degradabilities and nutritional composition. It is possible that the organic matter degraded
in BFT led to lower production of VFA and gasses, shifting more substrate to microbial synthesis (Blümmel et al., 1997), supported in this study by the greater partitioning factor observed for BFT.

The amount of gas produced by the different forages at the beginning of incubation could be used to predict a ranking of DMI intake across species when presented as single forages or preference when presented in cafeteria tests since gas production is positively correlated with greater digestibility, greater energy content of the forage and potentially reduced fill effect (Blummel et al., 2005). According to our in vitro results, we might expect the greatest dry matter intake for ALF, because of its greater gas production rate at the beginning of incubation, potential gas production and degradability values, followed by BFT and then by SF. When lambs were offered the legumes used in this study (Lagrange and Villalba, 2016), intake values were ALF > SF > BFT. It is likely that other variables like the high concentration of CP in BFT limited the ingestion of this forage since it is known that ruminants reduce intake of forages high in CP content in order to maintain blood ammonia concentration below toxic levels (Provenza, 1995).

Mixing sainfoin with alfalfa in a ratio that represented lambs’ preference (A70-S30), reduced the rate of gas production ($R_{Max}$) compared to pure ALF. However, the time to reach maximum rate ($T_{Max}$) and half of potential gas production ($B$) was not modified by the inclusion of SF in the mix. These results suggest that ruminants might take advantage of the extra benefits of incorporating sainfoin to their diets while maintaining high rates of ruminal fermentation. On the other hand, when the proportion of sainfoin in the mix increased to 0.50, both parameter $B$ and $R_{Max}$ were lower than for pure ALF.

Our results suggest that BFT may be mixed with ALF in a proportion of 0.30 without producing any changes in the rate of gas production relative to pure ALF, although the potential gas production may be affected when BFT is at that level in the mix. When the amount of BFT
increased up to 0.50 in the mix, potential gas production and gas production rates declined, resulting in a gas production profile more similar to pure BFT than to the average of values observed by the two singles substrates. This slightly antagonistic effect observed on the gas production rates for the A50-B50 mixture is then translated into lower amounts of total gas production at the end of the incubation period. Additionally, these results also contribute to explain lambs’ preference for ALF over BFT (70:30) when they had *ad libitum* access to both forages. At this ratio, the rate of gas production was not different from pure ALF and lambs incorporated a tannin-containing legume to their diet with the benefits of reduced incidence of bloat described above.

The lambs’ preferred trinary mixture (A50:S35:B15) exhibited better gas production rates which occurred at earlier incubation times than for the equal parts mixture (i.e., indifferent preference value; A33:S33:B33). As in the binary mixtures, the presence of the three species together did not trigger any synergic effects with regards to gas production kinetics, and the combination of these three species in general slowed down the fermentation process relative to the responses observed when the forages were incubated as single species. Moreover, some antagonistic effects on gas production rate were observed when these species were combined at equal proportions (A33:S33:B33). Therefore, the proportion at which these three legume species are combined affects the fermentation process, and combinations do not appear to enhance gas production kinetics in terms of rate or potential gas production. Nevertheless, certain proportions –like those selected by lambs when fed the same forages assayed in the present study- improve fermentation relative to others (i.e., equal proportions) – allowing the animal to take advantage of other benefits of diet mixing, i.e., reduced bloat and NH₃ formation in the rumen or reduced
sensory specific satiety (triggered by ingesting the same food too frequently or in excess), which reduces food intake (Provenza, 1996).

5. Conclusions:

This study shows a greater OM degradability and rate of gas production in alfalfa and birdsfoot trefoil than in sainfoin, attributable to the greater contents of cell walls and lower concentration of protein in sainfoin. The 70:30 alfalfa/sainfoin or alfalfa/birdsfoot trefoil ratio showed greater rate of gas production than mixtures formed with equal proportions of the legumes (i.e., indifferent preference). The presence of the two tannin-containing legumes along with alfalfa in the trinary mixtures did not trigger any positive associative effects on degradability or gas production kinetics. In addition, trinary mixtures were not as fermentable as binary mixtures, which contained a greater proportion of alfalfa. In conclusion, mixing tannin-containing legumes with alfalfa could give ruminants the advantage of maintaining high rates of ruminal fermentation while ingesting beneficial bioactive compounds, as well as benefits related to dietary diversity in generalist herbivores like improvements in food intake due to reductions in sensory-specific satiety.

Declarations of Interest: We declare no conflict of interest.

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References:


https://doi.org/10.1079/BJN19970089.


https://doi.org/10.15232/S1080-7446(15)31129-3.

Grabber, J.H., Zeller, W.E., Mueller-Harvey, I., 2013. Acetone enhances the direct analysis of procyanidin- and prodelphinidin-based condensed tannins in Lotus species by the

https://doi.org/10.1021/jf304158m.


8401(96)01012-7.


Niderkorn, V., Mueller-Harvey, I., Le Morvan, A., Aufrère, J., 2012. Synergistic effects of
mixing cocksfoot and sainfoin on in vitro rumen fermentation. Role of condensed
https://doi.org/10.1016/j.anifeedsci.2012.09.014.

Provenza, F.D., 1995. Postingestive feedback as an elementary determinant of food preference

Provenza, F.D., 1996. Acquired aversions as the basis for varied diets of ruminants foraging on

compounds for improving sustainable productivity in grazing ruminants. Anim. Feed Sci.
Technol. 120, 179–201. https://doi.org/10.1016/j.anifeedsci.2005.01.015

Robertson, J.B., Van Soest, P.J., James, W.P.T. and Theander, O., 1981. The analysis of dietary
fiber in food. Ed. W. P. T. James, and O. Theander. (Marcel Dektier: New York), pp.123-
58.

Dry Matter by Oven Drying for 3 Hours at 105 C. NFTA Reference Methods. National
Forage Testing Association, Omaha, NB.

Theodoridou, K., Aufrère, J., Andueza, D., Le Morvan, A., Picard, F., Pourrat, J., Baumont, R.,
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.
https://doi.org/10.1017/S1751731111001510.

Theodoridou, K., Aufrère, J., Andueza, D., Pourrat, J., Le Morvan, A., Stringano, E., Mueller-
Harvey, I., Baumont, R., 2010. Effects of condensed tannins in fresh sainfoin


