Effects of a tannin-rich legume (Onobrychis viciifolia) on in vitro ruminal biohydrogenation and fermentation

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Abstract

There is still controversy surrounding the ability of tannins to modulate the ruminal biohydrogenation (BH) of fatty acids (FA) and improve the lipid profile of milk or meat without conferring a negative response in the digestive utilization of the diet. Based on this, an in vitro trial using batch cultures of rumen microorganisms was performed to compare the effects of two legume hays with similar chemical composition but different tannin content, alfalfa and sainfoin (Onobrychis viciifolia), on the BH of dietary unsaturated FA and on the ruminal fermentation. The first incubation substrate, alfalfa, was practically free of tannins, while the second, sainfoin, contained 3.5% (expressed as tannic acid equivalents). Both hays were enriched with sunflower oil as a source of unsaturated FA. Most results of the lipid composition analysis (e.g., greater concentrations of 18:2n-6, cis-9 18:1 or total polyunsaturated FA in sainfoin incubations) showed the ability of this tannin-containing legume to inhibit the BH process. However, no significant differences were detected in the accumulation of cis-9 trans-11 conjugated linoleic acid, and variations in trans-11 18:1 and trans-11 cis-15 18:2 did not follow a regular pattern. Regarding the rumen fermentation, gas production, ammonia concentration and volatile FA production were lower in the incubations with sainfoin (−17, −23 and −11%, respectively). Thus, although this legume was able to modify the ruminal BH, which might result in improvements in the meat or milk lipid profile, the present results were not as promising as expected or as obtained before with other nutritional strategies.

Additional key words: condensed tannin; conjugated linoleic acid; fatty acid; lipid; rumen; sheep; sainfoin.

Introduction

The growing epidemic of diet-related chronic diseases has stimulated the search for nutritional strategies that modulate the lipid profile of ruminant-derived products (Shingfield et al., 2008; Vasta & Luciano, 2011). The goal is to increase the content of naturally occurring bioactive fatty acids (FA) offering the potential for enhancing health or reducing the risk of disease, such as the cis-9 trans-11 isomer of conjugated linoleic acid (CLA; Palmquist et al., 2005).

In this regard, some studies, particularly in vitro (Vasta et al., 2009; Buccioni et al., 2011), have reported that tannins are able to interfere with FA metabolism in the rumen, and more specifically with the saturation of trans-11 18:1. This 18:1 isomer acts as a precursor for the endogenous synthesis of cis-9 trans-11 CLA in the body tissues of ruminants and humans, through Δ⁹-desaturation (Palmquist et al., 2005). However, other works (Minieri et al., 2014; Carreño et al., 2015) have suggested a general inhibition of the BH process, leading to the accumulation of dietary unsatu-
rated FA (e.g., 18:2n-6 and 18:3n-3). In addition, given the great variation in the chemical composition and activity of different tannins (Buccioni et al., 2011; Carreño et al., 2015), there is still controversy surrounding their ability to modulate the BH without conferring a negative response in the digestive utilization of the diet.

Furthermore, most studies about the use of tannins in ruminant nutrition have been performed using commercial extracts (e.g., Buccioni et al., 2011; Toral et al., 2013; Carreño et al., 2015), whereas it would be of particular interest to find a forage containing these phenolic compounds that allowed its direct utilization by ruminants.

Interestingly, the few available studies on the use of sainfoin (Onobrichys vicifolia) have reported promising results attributable to its condensed tannin content, but very little is known about its impact on ruminal BH (Khiaosa-Ard et al., 2009). In this respect, a very recent work in growing lambs fed sainfoin silage (Girard et al., 2009) has found greater 18:2n-6 and 18:3n-3 concentrations in the muscle. However, no information was given about changes in \( \alpha \) 18:1 isomers or in other intermediate metabolites with potentially bioactive properties.

On this basis, an in vitro study was carried out to compare the effects of alfalfa hay with those of sainfoin hay on the BH of dietary unsaturated FA and on the ruminal fermentation.

Material and methods

All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 (BOE, 2013) for the protection of animals used for experimental purposes.

Batch cultures of rumen microorganisms

The study was conducted in vitro using batch cultures of rumen microorganisms and the gas production technique, as outlined previously (Frutos et al., 2004a). Incubation substrates (treatments) were two hays with a similar chemical composition, except for their tannin content: sainfoin hay and alfalfa hay.

Three ruminally cannulated (40 mm internal diameter) sheep (body weight = 59.4 ± 4.77) were used as donors of rumen inocula. Animals were housed in individual tie stalls and offered the same alfalfa hay used as incubation substrate at a rate of 48 g dry matter (DM)/kg metabolic weight and day, which corresponded to approximately their estimated maintenance energy requirements (INRA, 2007). Ewes had continuous access to clean drinking water and a vitamin-mineral block.

After an adaptation period of 15 days, rumen fluid inocula were obtained via the cannula in two non-consecutive days (runs), before the morning feeding. The inocula were immediately taken in thermal flasks to the laboratory where they were strained through a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain) while bubbled with CO\(_2\).

Two independent samples of each legume hay (replicates) were dried in a force-air oven at 45 °C, ground using a hammer-mill fitted with a 1 mm screen and incubated (500 mg) in 125-mL sealed serum flasks with 50 mL of a mix (1:4) of strained rumen fluid and phosphate-bicarbonate buffer (Goering & Van Soest, 1970). The incubated hays were supplemented with 20 g of sunflower oil (Carrefour S.A., Madrid, Spain)/kg DM, which was dissolved in ethanol 96%, dispersed with an ultrasonic device (UP200H, Hielscher Ultrasonic GmbH, Teltow, Germany) and added (200 µL) into the flasks just before the incubation started.

For each of the two runs, five flasks of each replicate and two blanks (i.e., buffered rumen fluid without substrate and with 200 µL of ethanol 96%) were incubated under anaerobic conditions for up to 24 h in an incubator set at 39.5 °C.

Measurements and samplings

The reaction in one flask per replicate and run was stopped at 0, 6 and 24 h post-inoculation by placing the flask into ice-water for approximately 5-10 min. The incubation residue was frozen at −80 °C, then freeze-dried and stored again at −80 °C until FA analysis.

Gas production was determined in the remaining flasks (2 per replicate and run) by measuring head-space gas pressure at 6, 12 and 24 h post-inoculation. Pressure values, corrected for the quantity of organic matter incubated and gas released from the blanks, were used to generate gas volume estimates using a predictive equation (Hervás et al., 2005). After 24 h of incubation, the reaction was stopped, and centrifuged samples (at 976 × g for 10 min) were collected for ammonia and volatile fatty acid (VFA) determinations and stored at −30 °C until analysis. Dry matter disappearance (DMD) was estimated by filtering residues using pre-weighed sintered glass crucibles (100-160 µm; Pyrex, Stone, UK).

Chemical analyses

Hay samples were analysed for DM (ISO 6496:1999), ash (ISO 5984:2002), crude protein (ISO
Fatty acid methyl esters (FAME) of lipid in hays and sunflower oil were prepared in a 1-step extraction-transesterification procedure using chloroform and 20 mL/L sulphuric acid in methanol (Shingfield et al., 2003). Methyl esters were separated and quantified with as gas chromatograph (Agilent 7890A GC System, Santa Clara, CA, USA) equipped with a flame-ionization detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2-μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2 μL sample volume at a split ratio of 1:50 was determined using the temperature gradient program described in Shingfield et al. (2003). Peaks were identified based on retention time comparisons with commercially available standard FAME mixtures (from Nu-Chek Prep., Elysian, MN, USA; and Sigma-Aldrich, Madrid, Spain).

Lipid in 200 mg of freeze-dried rumen samples was extracted with a mixture of hexane and isopropanol (3:2, vol/vol; Shingfield et al., 2003) and converted to FAME by sequential base-acid catalysed transesterification (Toral et al., 2010). Total FAME profile was determined by gas chromatography using the same chromatograph and temperature gradient program applied for the analysis of feeds, but isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170 °C (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with the same FAME mixtures used for the analysis of feeds, other commercially available standards (from Nu-Chek Prep.; Sigma-Aldrich; and Larodan, Solna, Sweden), cross referencing with chromatograms reported in the literature (e.g., Shingfield et al., 2003; Toral et al., 2010), and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives (Toral et al., 2010).

The ammonia concentration was determined by colorimetry, and VFA by gas chromatography using crotonic acid as the internal standard (Frutos et al., 2004a).

Statistical analysis

Data were subjected to statistical analysis with the MIXED procedure of the SAS software package (version 9.4, SAS Institute Inc., Cary, NC, USA). Hay composition data were analysed by one-way ANOVA. The statistical model for ruminal BH and fermentation data included the fixed effects of the legume hay. Replicates were nested within the hay, and the interaction between this component and the incubation run was considered as the random effect. Each incubation run was considered as a block. Differences were declared significant at \( p<0.05 \) and considered a trend towards significance at \( p<0.10 \). Least square means are reported.

Results

Diet composition

The chemical composition of the two studied hays, including their FA profile, is reported in Table 1. Both of them supplied very similar amounts of crude protein and ether extract, but the concentrations of organic matter and ADF were slightly greater in the sainfoin than in the alfalfa hay (\( p<0.10 \)). The major difference between the composition of the two legumes lied in their total tannin content (\( p<0.01 \)), as foreseen in the experimental design, since this value was very low in the alfalfa hay (0.5%, expressed in tannic acid equivalents) and of 3.5% in the sainfoin. Differences in lipid profiles were minor, although the alfalfa showed a little higher proportions of 12:0, 14:0 and 16:0, and lower of cis-9 18:1, than the sainfoin hay (\( p<0.10 \)).

Sunflower oil contained (% total FA; data not shown in tables): 16:0 (6.5), 18:0 (3.9), cis-9 18:1 (24.8) and 18:2n-6 (61.2).

Ruminal biohydrogenation

Table 2 reports the concentration of selected FA in the in vitro ruminal digesta, with particular attention to 18-carbon BH intermediates. A much higher number of FA (\( n = 78 \)) was identified and quantified in the digesta samples, but only those with greater relevance
for the aim of the study are reported. Before describing in detail the effects of the type of legume on the rumen FA profile, it must be noted that no differences were detected between the alfalfa and the sainfoin at the beginning of the incubation (i.e., at 0 h; \( p > 0.10 \)).

The most abundant unsaturated FA in the sunflower oil, 18:2n-6, tended to accumulate in the \textit{in vitro} ruminal digesta with the tannin-rich hay (+18 and +69% compared with the alfalfa at 6 and 24 h post-inoculation, respectively; \( p < 0.10 \)). On the other hand, the numerical differences in the proportion of the major polyunsaturated fatty acid (PUFA) in the forages, 18:3n-3, did not attain statistical significance at any incubation time (\( p > 0.10 \)). Neither \textit{cis}-9 \textit{trans}-11 CLA nor total CLA concentrations differed between treatments (\( p > 0.10 \)). Surprisingly, although sainfoin hay led to a lower accumulation of \textit{cis}-9 \textit{trans}-11 18:2 than the alfalfa at 6 h post-inoculation (‒21%; \( p < 0.05 \)), the opposite trend was observed at 24 h (+72%; \( p < 0.10 \)). The same irregular pattern was followed by \textit{trans}-11 18:1 concentrations, showing a tendency toward lower or greater values in sainfoin incubation at 6 h or 24 h (‒20 and +11%, respectively; \( p < 0.10 \)). In relation to \textit{trans}-10 18:1, no significant effects of the type of hay were detected at any time point. The proportion of the other reported 18:1 isomer, oleic acid (\textit{cis}-9 18:1), tended to be significantly greater after 6 h of incubation with the tannin-rich legume (\( p < 0.10 \)).

The higher content of some dietary unsaturated FA and ruminal BH intermediates observed in sainfoin cultures was not accompanied by relevant changes in 18:0 concentration, which only tended to be lower than in the alfalfa when the reaction was stopped at 6 h post-inoculation (‒8%; \( p < 0.10 \)). The accumulation of 10-oxo-18:0 was impaired by constituents of the sainfoin after 24 h of incubation (\( p < 0.001 \)), but the sum of odd- and branched-chain FA (OBCFA) remained unmodified (\( p > 0.10 \)).

Regarding major FA groups, the proportion of PUFA in the rumen digesta from sainfoin incubations was enhanced (13 and 49% greater compared to the alfalfa incubations at 6 and 24 h, respectively; \( p < 0.10 \)), while that of saturated FA (‒7% at 6 h post-inoculation; \( p < 0.10 \)) was slightly decreased.

### Table 1. Chemical composition and fatty acid profile of the alfalfa and sainfoin hays\(^{(1)}\).

<table>
<thead>
<tr>
<th>Item(^{(2)})</th>
<th>Alfalfa</th>
<th>Sainfoin</th>
<th>SED(^{(3)})</th>
<th>( p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic matter</td>
<td>878</td>
<td>914</td>
<td>3.4</td>
<td>0.009</td>
</tr>
<tr>
<td>Crude protein</td>
<td>189</td>
<td>182</td>
<td>4.6</td>
<td>0.258</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>321</td>
<td>377</td>
<td>19.7</td>
<td>0.106</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>247</td>
<td>295</td>
<td>12.6</td>
<td>0.064</td>
</tr>
<tr>
<td>Acid detergent lignin</td>
<td>44</td>
<td>54</td>
<td>3.7</td>
<td>0.130</td>
</tr>
<tr>
<td>Ether extract</td>
<td>26</td>
<td>23</td>
<td>5.2</td>
<td>0.603</td>
</tr>
<tr>
<td>Total tannins(^{(4)})</td>
<td>5</td>
<td>35</td>
<td>1.6</td>
<td>0.003</td>
</tr>
<tr>
<td>FA profile (g/100 g total FA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>0.7</td>
<td>0.2</td>
<td>0.03</td>
<td>0.003</td>
</tr>
<tr>
<td>14:0</td>
<td>2.4</td>
<td>1.9</td>
<td>0.14</td>
<td>0.059</td>
</tr>
<tr>
<td>16:0</td>
<td>25.0</td>
<td>23.3</td>
<td>0.54</td>
<td>0.082</td>
</tr>
<tr>
<td>18:0</td>
<td>5.6</td>
<td>4.2</td>
<td>0.85</td>
<td>0.252</td>
</tr>
<tr>
<td>\textit{cis}-9 \textit{cis}-18:2</td>
<td>2.3</td>
<td>3.6</td>
<td>0.39</td>
<td>0.080</td>
</tr>
<tr>
<td>\textit{cis}-9 \textit{cis}-12-18:3</td>
<td>14.0</td>
<td>14.8</td>
<td>0.58</td>
<td>0.334</td>
</tr>
<tr>
<td>\textit{cis}-9 \textit{cis}-12 \textit{cis}-15 18:3</td>
<td>41.1</td>
<td>42.7</td>
<td>2.34</td>
<td>0.561</td>
</tr>
</tbody>
</table>

\(^{(1)}\) \( n = 2 \). \(^{(2)}\) DM: dry matter. FA: fatty acid. \(^{(3)}\) SED: standard error of the difference. \(^{(4)}\) Expressed as tannic acid equivalents.

### Discussion

This \textit{in vitro} trial was performed using sainfoin and alfalfa hays as incubation substrates because both legume forages have very similar chemical compositions but, while the alfalfa may be considered as virtually free of tannins, the concentration of these phenolic compounds in the sainfoin reached 35 g of tannic acid equivalents.
highlight once again that this value (5%) derives mainly from studies conducted with Lotus species and cannot be extrapolated to other plants (Mueller-Harvey, 2006). In addition, the lack of normalization not only in the analytical methods for tannin determination but equivalents/kg DM. This value can be considered as moderate and is below the widespread 5% level that has too often been considered as the threshold for negative effects in ruminants (Frutos et al., 2004b; Mueller-Harvey, 2006). However, it is important to
also in the standards used to express them can provide very different, and therefore ambiguous, results that make between-experiment comparisons rather complicated (Makkar, 2003; Frutos et al., 2004b).

### Ruminal biohydrogenation

The FA composition of the ruminal digesta in sainfoin incubations, particularly the greater total PUFA concentration, suggests that this tannin-containing plant was able to decrease the extent of ruminal BH compared to the tannin-free alfalfa. This is in agreement with a recent report on muscle FA profile in lambs fed sainfoin silage (Girard et al., 2016). Previous studies in small ruminants receiving dietary tannins from sulla (Hedysarium coronarium) or birdsfoot trefoil (Lotus corniculatus) have also shown similar changes in milk and meat composition (e.g., Priolo et al., 2005; Cabiddu et al., 2009; Girard et al., 2016). In our work, the impaired ruminal BH observed in the sainfoin cultures was more evident on the major PUFA constituent of the sunflower oil, 18:2n-6, whereas numerical differences in 18:3n-3 were not statistically significant and contrasting results were detected for trans-11 cis-15 18:2 (a major metabolite of 18:3n-3 BH; Jenkins et al., 2008) after 6 and 24 h of incubation.

Even though certain types and doses of tannins have been reported to increase cis-9 trans-11 CLA content in the ruminal digesta, changes in the accumulation of this health-promoting FA have seldom been observed (Vasta et al., 2009; Carreño et al., 2015). This is in line with our results and with the fact that the proportion of cis-9 trans-11 CLA in milk and meat is mostly explained by Δ9-desaturation of trans-11 18:1 in body tissues (Palmquist et al., 2005) rather than by increases in its ruminal outflow.

In relation to 18:1 isomers, the response in the concentration of cis-9 18:1 would support the potential inhibitory effect of sainfoin tannins on the metabolism of dietary unsaturated FA, which may favourably modify the lipid profile of ruminant-derived products (Vasta & Lucianu, 2011). Regarding the vaccenic acid (trans-11 18:1; another desirable FA due to its mentioned role as a substrate for Δ9-desaturase in ruminants and humans, Palmquist et al., 2005), the variation possibly due to the effect of sainfoin tannins did not follow a regular pattern. Thus, data collected in 24 h incubations would prove the hypothesis suggested by some authors that these secondary compounds specifically inhibit the last step of BH, increasing the ruminal outflow of trans-11 cis-15 18:2 concentration might point towards an inhibition of other saturation steps. In addition, results of both FA at 6 h post-inoculation (i.e., lower values of trans-11 18:1 and trans-11 cis-15 18:2 in the sainfoin cultures) constitute a good example of the controversy about the impact of tannins on ruminal BH. In any case, responses at both time points may be attributed to the effect of sainfoin on ruminal lipid metabolism, given that the inhibition of trans C18 saturation would accumulate vaccenic acid and trans-11 cis-15 18:2, but a negative influence on the first BH steps would decrease their production. This latter response, linked to a lower or slower disappearance of dietary PUFA, appears to better reflect the in vivo effects reported for other tannin-rich legumes, such as the lower milk proportion of trans-11 18:1 and cis-9 trans-11 CLA together with increases in 18:3n-3 and 18:2n-6 in ewes fed sulla (Addis et al., 2005; Cabiddu et al., 2009). In line with this, Turner et al. (2005) pointed toward a general inhibition of BH, and not only on the last step, when examining the effects of Lotus corniculatus in-

### Table 3. Rumen fermentation characteristics after 24 h-incubations of alfalfa and sainfoin hays with rumen inocula from sheep.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Gas production (mL/g OM)</td>
<td>239</td>
<td>203</td>
<td>1.5</td>
<td>0.002</td>
</tr>
<tr>
<td>DMD (%)</td>
<td>60.8</td>
<td>57.0</td>
<td>0.59</td>
<td>0.023</td>
</tr>
<tr>
<td>Ammonia (mg/L)</td>
<td>326</td>
<td>263</td>
<td>13.6</td>
<td>0.044</td>
</tr>
<tr>
<td>Total VFA (mmol/L)</td>
<td>52.6</td>
<td>47.8</td>
<td>1.21</td>
<td>0.058</td>
</tr>
<tr>
<td>Molar proportions (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>69.5</td>
<td>72.7</td>
<td>0.24</td>
<td>0.005</td>
</tr>
<tr>
<td>Propionate</td>
<td>20.2</td>
<td>20.9</td>
<td>0.66</td>
<td>0.428</td>
</tr>
<tr>
<td>Butyrate</td>
<td>6.1</td>
<td>4.3</td>
<td>0.41</td>
<td>0.051</td>
</tr>
<tr>
<td>Valerate</td>
<td>1.8</td>
<td>0.8</td>
<td>0.08</td>
<td>0.008</td>
</tr>
<tr>
<td>Isobutyrate + isovalerate</td>
<td>2.4</td>
<td>1.3</td>
<td>0.04</td>
<td>0.001</td>
</tr>
</tbody>
</table>

take in dairy cows. And similarly, our 24 h incubations seem to indicate that combined increases in dietary PUFA and some intermediate metabolites are also possible. Concerning the \textit{trans}-10 18:1, whose potential specific role for human health and animal performance is still unclear (Shingfield \textit{et al.}, 2008), no variations were detected. The concentration of this 18:1 isomer tends to increase markedly with certain nutritional strategies based on diet supplementation with marine lipids, for example, and it can even exceed that of \textit{trans}-11 18:1, particularly in dairy cows (Shingfield \textit{et al.}, 2008; Toral \textit{et al.}, 2012). Nevertheless, this shift in \textit{trans}-11 18:1/\textit{trans}-10 18:1 ratio, a clear indicator of altered ruminal environment, was not observed in our study.

The fact that the concentration of 10-oxo-18:0, a metabolite resulting from a pathway involving the hydration and subsequent oxidation of dietary unsaturated FA, was lower in the sainfoin incubations would be in line with the lack of differences in the OBCFA, since all these FA have been suggested as indicators of altered rumen environment or microbial biomarkers (Jenkins \textit{et al.}, 2008; Fievez \textit{et al.}, 2012).

Surprisingly, because it would be expected that the inhibitory effect of tannins on ruminal BH would lead to lower 18:0 accumulation in the digesta and numerical results seem to corroborate it, differences were only significant at 6 h of incubation.

Finally, FA sums showed a trend for SFA to be lower and PUFA to be higher in sainfoin incubations. However, two notes of caution should be mentioned: first, FA sums may include individual compounds with antagonistic responses and, while some FA can increase, others can remain unmodified or even decrease in response to the same source of variation. And second, the assumption that all saturates are detrimental for consumer’s health is just a generalization because only 12:0, 14:0 and 16:0 have been unequivocally related to increased cardiovascular disease risk (Shingfield \textit{et al.}, 2008; Parodi, 2009).

Overall, the results show the ability of sainfoin, possibly due to its moderate tannin content, to modify the BH of dietary unsaturated FA, which might improve the nutritional quality of meat and milk fat. However, they are not as promising as expected or as obtained before with other strategies (e.g., lipid supplementation; Shingfield \textit{et al.}, 2008; Toral \textit{et al.}, 2011, 2012).

**Ruminal fermentation**

Once proven the ability of the sainfoin hay to modify the BH process, this second part was conducted to study whether its tannins may impair ruminal fermentation and compromise its practical application in ruminant feeding. This is of particular relevance because of the potential impact of altered rumen digestion on animal performance.

Reductions in gas production and DMD observed in the sainfoin cultures were consistent with results from other studies examining the impact of tannins (e.g., Frutos \textit{et al.}, 2004a; Rodriguez \textit{et al.}, 2011; Lobón \textit{et al.}, 2015) and have been attributed to their inhibition of bacterial attachment to feed particles, microbial growth and enzyme activity (McAllister \textit{et al.}, 1994; Smith \textit{et al.}, 2005). Nonetheless, it must be considered that the effects of tannins on ruminal fermentation, as well as on BH, are highly variable depending on their type, dosage rate, microbiota adaptation to their consumption, etc. (Tiemann \textit{et al.}, 2008; Doce \textit{et al.}, 2009; Rodriguez \textit{et al.}, 2011). Furthermore, inherent differences in the chemical composition of both forages, which was very similar but not identical, cannot be ignored and might also contribute to explain some subtle differences.

The ammonia concentrations (19% lower with the sainfoin hay) confirmed the well-known inhibition elicited by tannins on the ruminal degradation of dietary proteins, as a result of the high affinity between the hydroxyl radicals of phenolic groups and the carbonyl groups of proteins (Frutos \textit{et al.}, 2004b; Mueller-Harvey, 2006). Depending on the basal diet composition, this effect may be clearly advantageous for the animal, increasing the flow of non-degraded protein to the intestine. Romero-Pérez \textit{et al.} (2011) reported similar findings in an \textit{in vitro} study conducted with rumen inoculum from cows fed alfalfa or sainfoin silages.

On the other hand, the lower production of total VFA (–9%), the major energy source for ruminants, with the tannin-containing hay would entail a nutritional disadvantage. In contrast to the consistent action of tannins on ruminal ammonia concentration, reports on the response of VFA to these secondary compounds are highly variable, with reductions, no effects and even increases in their production (e.g., Frutos \textit{et al.}, 2004a; Tiemann \textit{et al.}, 2008; Romero-Pérez \textit{et al.}, 2011). This discrepancy is even stronger when referred to individual VFA. Moreover, further to the tannin content, differences in the chemical composition of alfalfa and sainfoin hays, particularly related to the cell wall, might also account for different VFA concentrations.

Notwithstanding these variations, there is a general agreement (e.g., Doce \textit{et al.}, 2009) that tannins impair valerate and isocapric acid concentrations, given that these VFA are mostly originated from decarboxylation and deamination of certain amino acids. This was also found in our study when comparing alfalfa and sainfoin cultures and concurs with ammonia changes.
In summary, most results on the lipid profile of the in vitro ruminal digesta (e.g., greater concentrations of 18:2n-6, cis-9 18:1 and total PUFA in sainfoin hay cultures) suggest the ability of *Onobrychis vicifolia*, probably due to its moderate tannin content, to inhibit the ruminal BH of dietary unsaturated FA. The variations in *trans*-11 18:1 do not allow firm conclusions concerning its behaviour. With regard to ruminal fermentation, the tannin-containing sainfoin cultures show lower gas and VFA productions but also a positive lower ammonia concentration.

Thus, although this legume is able to modify the ruminal BH, which might result in improvements in the meat or milk lipid profile, the present results are not as promising as expected or as obtained before with other nutritional strategies. In any event, depending on the pursued objectives, sainfoin might be a forage of other nutritional strategies. In any event, depending on the pursued objectives, sainfoin might be a forage of other nutritional strategies.

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Sainfoin, fatty acid biohydrogenation and ruminal fermentation


