RESEARCH ARTICLE

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

Pablo G. Toral, Gonzalo Hervás, Hajer Missaoui, Sonia Andrés, Francisco J. Giráldez, Saida Jellali and Pilar Frutos

Instituto de Ganadería de Montaña (CSIC-ULE), Finca Marzanas s/n. 24346 Grulleros (León). Spain

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.

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 Δ^9-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 viciifolia*) 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.*, 2016) has found greater 18:2n-6 and 18:3n-3 concentrations in the muscle. However, no information was given about changes in *trans* 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 ºC, 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 Ultrasonics 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 ºC.

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 ºC, then freeze-dried and stored again at −80 ºC 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 ºC 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

5983-2:2009) and ether extract (Ankom Technology Method 2; Ankom Technology Corp. Macedon, NY, USA, https://ankom.com/procedures.aspx). The NDF, acid detergent fibre (ADF) and acid detergent lignin concentrations were sequentially determined using an Ankom^{2000} fiber analyzer (Ankom Technology Methods 13, 12 and 8, respectively; Ankom Technology Corp.); the former was assayed with sodium sulfite and α-amylase, and both NDF and ADF were expressed with residual ash. Total tannin content was assayed following the Folin-Ciocalteu method in combination with polyvinyl-polypyrrolidone, with tannic acid (Merck, Damstadt, Germany) as the reference standard (Makkar, 2003).

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 a 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 ºC (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

Table 1. Chemical composition and fatty acid profile of the alfalfa and sainfoin hays[1].

Item[2]	Alfalfa	Sainfoin	SED[3]	*p*-value
Chemical composition (g/kg DM)				
Organic matter	878	914	3.4	0.009
Crude protein	189	182	4.6	0.258
Neutral detergent fibre	321	377	19.7	0.106
Acid detergent fibre	247	295	12.6	0.064
Acid detergent lignin	44	54	3.7	0.130
Ether extract	26	23	5.2	0.603
Total tannins[4]	5	35	1.6	0.003
FA profile (g/100 g total FA)				
12:0	0.7	0.2	0.03	0.003
14:0	2.4	1.9	0.14	0.059
16:0	25.0	23.3	0.54	0.082
18:0	5.6	4.2	0.85	0.252
cis-9 18:1	2.3	3.6	0.39	0.080
cis-9 *cis*-12 18:2	14.0	14.8	0.58	0.334
cis-9 *cis*-12 *cis*-15 18:3	41.1	42.7	2.34	0.561

[1] n = 2. [2] DM: dry matter. FA: fatty acid. [3] SED: standard error of the difference. [4] Expressed as tannic acid equivalents.

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 *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 *cis*-9 *trans*-11 CLA nor total CLA concentrations differed between treatments ($p > 0.10$). Surprisingly, although sainfoin hay led to a lower accumulation of *trans*-11 *cis*-15 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 *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 *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 (*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.

Ruminal fermentation

As shown in Table 3, gas production and DMD after 24 h of incubation were significantly lower for sainfoin, compared with the alfalfa hay (−15 and −6%, respectively; $p<0.05$). Similarly, the concentration of ammonia and the production of total VFA were also lower with the tannin-rich legume (−19 and −9%, respectively; $p<0.10$); the molar proportions of butyrate, valerate and isoacids being those that better reflected these changes ($p<0.10$).

Discussion

This *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

Table 2. Fatty acid profile of the ruminal digesta in alfalfa and sainfoin hay incubations with rumen inocula from sheep.

FA (g/100 g total FA)[1]	Incubation time (h)	Alfalfa	Sainfoin	SED[2]	p-value
cis-9 cis-12 cis-15 18:3	0	7.98	8.74	0.443	0.229
	6	6.59	7.20	0.288	0.171
	24	3.22	4.57	0.479	0.107
cis-9 cis-12 18:2	0	35.95	38.73	1.993	0.298
	6	21.01	24.75	0.929	0.057
	24	4.98	8.44	1.106	0.089
trans-11 cis-15 18:2	0	0.08	0.07	0.008	0.201
	6	0.63	0.50	0.020	0.025
	24	0.36	0.62	0.073	0.072
cis-9 trans-11 18:2	0	0.09	0.08	0.017	0.743
	6	0.61	0.61	0.050	0.992
	24	0.49	0.61	0.105	0.383
Total CLA	0	0.34	0.26	0.054	0.279
	6	1.36	1.26	0.054	0.188
	24	1.84	2.19	0.175	0.187
trans-11 18:1	0	0.79	0.54	0.227	0.391
	6	6.80	5.44	0.386	0.071
	24	11.59	12.89	0.400	0.082
trans-10 18:1	0	0.07	0.06	0.010	0.343
	6	0.29	0.26	0.025	0.340
	24	0.53	0.66	0.047	0.117
cis-9 18:1	0	14.70	15.90	0.652	0.207
	6	10.06	11.48	0.348	0.055
	24	5.82	8.16	1.001	0.145
18:0	0	14.00	11.61	1.875	0.331
	6	21.40	19.60	0.471	0.062
	24	35.00	26.42	3.689	0.146
10-oxo-18:0	0	0.08	0.08	0.023	0.924
	6	0.14	0.13	0.012	0.432
	24	0.40	0.28	0.006	0.002
∑OBCFA	0	3.21	3.09	0.093	0.322
	6	3.99	3.89	0.180	0.644
	24	5.90	5.76	0.137	0.396
∑SFA	0	36.18	32.24	2.785	0.293
	6	45.73	42.46	1.107	0.098
	24	62.51	53.26	3.293	0.107
∑MUFA	0	18.17	18.99	0.542	0.267
	6	23.18	22.53	0.759	0.483
	24	25.63	29.40	1.542	0.134
∑PUFA	0	45.37	48.53	2.254	0.296
	6	30.70	34.66	0.637	0.025
	24	11.28	16.76	1.742	0.088

[1] FA: fatty acid. CLA: conjugated linoleic acid. OBCFA: odd- and branched-chain fatty acid. SFA: saturated fatty acids (n = 25). MUFA: monounsaturated fatty acids (n = 30). PUFA: polyunsaturated fatty acids (n = 23). [2] SED: standard error of the difference.

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 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

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

Item[1]	Alfalfa	Sainfoin	SED[2]	*p*-value
Gas production (mL/g OM)	239	203	1.5	0.002
DMD (%)	60.8	57.0	0.59	0.023
Ammonia (mg/L)	326	263	13.6	0.044
Total VFA (mmol/L)	52.6	47.8	1.21	0.058
Molar proportions (%)				
Acetate	69.5	72.7	0.24	0.005
Propionate	20.2	20.9	0.66	0.428
Butyrate	6.1	4.3	0.41	0.051
Valerate	1.8	0.8	0.08	0.008
Isobutyrate + isovalerate	2.4	1.3	0.04	0.001

[1] OM: organic matter. DMD: dry matter disappearance. VFA: volatile fatty acids. [2] SED: standard error of the difference.

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 impared 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 Δ^9-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 & Luciano, 2011). Regarding the vaccenic acid (*trans*-11 18:1; another desirable FA due to its mentioned role as a substrate for Δ^9-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 18:1 (Vasta *et al.*, 2009; Bucconi *et al.*, 2011). However, similar differences in *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-

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 *trans*-10 18:1, whose potential specific role for human health and animal performance is still unclear (Shingfield *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 *trans*-11 18:1, particularly in dairy cows (Shingfield *et al.*, 2008; Toral *et al.*, 2012). Nevertheless, this shift in *trans*-11 18:1/ *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 *et al.*, 2008; Fievez *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 *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 *et al.*, 2008; Toral *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 fermen-

tation 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 *et al.*, 2004a; Rodríguez *et al.*, 2011; Lobón *et al.*, 2015) and have been attributed to their inhibition of bacterial attachment to feed particles, microbial growth and enzyme activity (McAllister *et al.*, 1994; Smith *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 *et al.*, 2008; Doce *et al.*, 2009; Rodríguez *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 *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 *et al.* (2011) reported similar findings in an *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 *et al.*, 2004a; Tiemann *et al.*, 2008; Romero-Pérez *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 *et al.*, 2009) that tannins impair valerate and isoacid 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 viciifolia*, 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 tanning-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 choice in ruminant feeding.

References

Addis M, Cabiddu A, Pinna G, Decandia M, Piredda G, Pirisi A, Molle G, 2005. Milk and cheese fatty acid composition in sheep fed Mediterranean forages with reference to conjugated linoleic acid *cis*-9, *trans*-11. J Dairy Sci 88: 3443-3454. http://dx.doi.org/10.3168/jds.S0022-0302(05)73028-9

BOE, 2013. Royal Decree 53/2013, of 8 February, on the protection of animals used for experimental purposes. Boletín Oficial del Estado, pp. 11370–11421. Spanish Government Publishing, Madrid.

Buccioni A, Minieri S, Rapaccini S, Antongiovanni M, Mele M, 2011. Effect of chestnut and quebracho tannins on fatty acid profile in rumen liquid- and solid-associated bacteria: an in vitro study. Animal 5: 1521-1530. http://dx.doi.org/10.1017/S1751731111000759

Cabiddu A, Molle G, Decandia M, Spada S, Fiori M, Piredda G, Addis M, 2009. Responses to condensed tannins of flowering sulla (*Hedysarum coronarium* L.) grazed by dairy sheep. Part 2: Effects on milk fatty acid profile. Livest Sci 123: 230-240. http://dx.doi.org/10.1016/j.livsci.2008.11.019

Carreño D, Hervás G, Toral PG, Belenguer A, Frutos P, 2015. Ability of different types and doses of tannin extracts to modulate in vitro ruminal biohydrogenation in sheep. Anim Feed Sci Technol 202: 45-51. http://dx.doi.org/10.1016/j.anifeedsci.2015.02.003

Doce RR, Hervás G, Belenguer A, Toral PG, Giráldez FJ, Frutos P, 2009. Effect of the administration of young oak (*Quercus pyrenaica*) leaves to cattle on ruminal fermentation. Anim Feed Sci Technol 150: 75-85. http://dx.doi.org/10.1016/j.anifeedsci.2008.08.005

Fievez V, Colman E, Castro-Montoya JM, Stefanov I, Vlaeminck B, 2012. Milk odd- and branched-chain fatty acids as biomarkers of rumen function - An update. Anim Feed Sci Technol 172: 51-65. http://dx.doi.org/10.1016/j.anifeedsci.2011.12.008

Frutos P, Hervás G, Giráldez FJ, Mantecón AR, 2004a. An in vitro study on the ability of polyethylene glycol to inhibit the effect of quebracho tannins and tannic acid on rumen fermentation in sheep, goats, cows, and deer. Aust J Agric Res 55: 1125-1132. http://dx.doi.org/10.1071/AR04058

Frutos P, Hervás G, Giráldez FJ, Mantecón AR, 2004b. Review. Tannins and ruminant nutrition. Span J Agric Res 2: 191-202. http://dx.doi.org/10.5424/sjar/2004022-73

Girard M, Dohme-Meier F, Silacci P, Ampuero Kragten S, Kreuzer M, Bee G, 2016. Forage legumes rich in condensed tannins may increase n-3 fatty acid levels and sensory quality of lamb meat. J Sci Food Agr (in press). http://dx.doi.org/10.1002/jsfa.7298

Goering MK, Van Soest PJ, 1970. Forage fiber analysis (apparatus, reagents, procedures and some applications). Agriculture handbook, No 379. Agricultural Research Service, USDA, Washington, USA.

Hervás G, Frutos P, Giráldez FJ, Mora MJ, Fernández B, Mantecón AR, 2005. Effect of preservation on fermentative activity of rumen fluid inoculum for *in vitro* gas production techniques. Anim Feed Sci Technol 123-124: 107-118. http://dx.doi.org/10.1016/j.anifeedsci.2005.05.004

INRA, 2007. Alimentation des bovins, ovins et caprins. INRA, Versailles, France.

Jenkins TC, Wallace RJ, Moate PJ, Mosley EE, 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci 86: 397-412. http://dx.doi.org/10.2527/jas.2007-0588

Khiaosa-Ard R, Bryner SF, Scheeder MRL, Wettstein HR, Leiber F, Kreuzer M, Soliva CR, 2009. Evidence for the inhibition of the terminal step of ruminal alpha-linolenic acid biohydrogenation by condensed tannins. J Dairy Sci 92: 177-188. http://dx.doi.org/10.3168/jds.2008-1117

Lobón S, Molino F, Legua MA, Eseverri P, Cespedes MA, Joy M, 2015. Efecto del forraje y de la inclusión de concentrado en la dieta sobre la producción de gas y metano en ovino. Pastos y forrajes en el siglo XXI. 54 Reunión Científica de la Sociedad Española para el Estudio de los Pastos (SEEP), Palma de Mallorca (Spain), April 14-17. pp: 243-250.

Makkar HPS, 2003. Quantification of tannins in tree and shrub foliage. A laboratory manual. Kluwer Acad. Publ., Dordrecht, The Netherlands. http://dx.doi.org/10.1007/978-94-017-0273-7

McAllister TA, Bae HD, Jones GA, Cheng KJ, 1994. Microbial attachment and feed digestion in the rumen. J Anim Sci 72: 3004-3018. https://www.animalsciencepublications.org/publications/jas/abstracts/72/11/3004

Minieri S, Buccioni A, Rapaccini S, Pezzati A, Benvenuti D, Serra A, Mele M, 2014. Effect of Quebracho tannin extract on soybean and linseed oil biohydrogenation by solid associated bacteria: an in vitro study. Ital J Anim Sci 13: 604-608. http://dx.doi.org/10.4081/ijas.2014.3267

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

Palmquist DL, Lock AL, Shingfield KJ, Bauman DE, 2005. Biosynthesis of conjugated linoleic acid in ruminants and

humans. In: Advances in food and nutrition research; Taylor SL (ed.). pp: 179-217. Elsevier Academic Press, San Diego, USA. http://dx.doi.org/10.1016/S1043-4526(05)50006-8

Parodi PW, 2009. Has the association between saturated fatty acids, serum cholesterol and coronary heart disease been over emphasized? Int Dairy J 19: 345-361. http://dx.doi.org/10.1016/j.idairyj.2009.01.001

Priolo A, Bella M, Lanza M, Galofaro V, Biondi L, Barbagallo D, Salem HB, Pennisi P, 2005. Carcass and meat quality of lambs fed fresh sulla (*Hedysarum coronarium* L.) with or without polyethylene glycol or concentrate. Small Ruminant Res 59: 281-288. http://dx.doi.org/10.1016/j.smallrumres.2005.05.012

Rodríguez R, Britos A, Rodríguez-Romero N, Fondevila M, 2011. Effect of plant extracts from several tanniferous browse legumes on in vitro microbial fermentation of the tropical grass *Pennisetum purpureum*. Anim Feed Sci Technol 168: 188-195. http://dx.doi.org/10.1016/j.anifeedsci.2011.04.095

Romero-Pérez GA, Ominski KH, McAllister TA, Krause DO, 2011. Effect of environmental factors and influence of rumen and hindgut biogeography on bacterial communities in steers. Appl Environ Microbiol 77: 258-268. http://dx.doi.org/10.1128/AEM.01289-09

Shingfield KJ, Ahvenjärvi S, Toivonen V, Äröla A, Nurmela KVV, Huhtanen P, Griinari JM, 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Anim Sci 77: 165-179. ftp://s173-183-201-52.ab.hsia.telus.net/Inetpub/wwwroot/DairyScience/Resources/NTS/AS77_165.pdf

Shingfield KJ, Chilliard Y, Toivonen V, Kairenius P, Givens DI, 2008. *Trans* fatty acids and bioactive lipids in ruminant milk. Adv Exp Med Biol 606: 3-65. http://dx.doi.org/10.1007/978-0-387-74087-4_1

Smith AH, Zoetendal E, Mackie RI, 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microb Ecol 50: 197-205. http://dx.doi.org/10.1007/s00248-004-0180-x

Tiemann TT, Avila P, Ramírez G, Lascano CE, Kreuzer M, Hess HD, 2008. *In vitro* ruminal fermentation of tanniniferous tropical plants: plant-specific tannin effects and counteracting efficiency of PEG. Anim Feed Sci Technol 146: 222-241. http://dx.doi.org/10.1016/j.anifeedsci.2007.12.009

Toral PG, Shingfield KJ, Hervás G, Toivonen V, Frutos P, 2010. Effect of fish oil and sunflower oil on rumen fermentation characteristics and fatty acid composition of digesta in ewes fed a high concentrate diet. J Dairy Sci 93: 4804-4817. http://dx.doi.org/10.3168/jds.2010-3300

Toral PG, Hervás G, Bichi E, Belenguer A, Frutos P, 2011. Tannins as feed additives to modulate ruminal biohydrogenation: Effects on animal performance, milk fatty acid composition and ruminal fermentation in dairy ewes fed a diet containing sunflower oil. Anim Feed Sci Technol 164: 199-206. http://dx.doi.org/10.1016/j.anifeedsci.2011.01.011

Toral PG, Belenguer A, Shingfield KJ, Hervás G, Toivonen V, Frutos P, 2012. Fatty acid composition and bacterial community changes in the rumen fluid of lactating sheep fed sunflower oil plus incremental levels of marine algae. J Dairy Sci 95: 794-806. http://dx.doi.org/10.3168/jds.2011-4561

Toral PG, Hervás G, Belenguer A, Bichi E, Frutos P, 2013. Effect of the inclusion of quebracho tannins in a diet rich in linoleic acid on milk fatty acid composition in dairy ewes. J Dairy Sci 96: 431-439. http://dx.doi.org/10.3168/jds.2012-5622

Turner SA, Waghorn GC, Woodhard SL, Thomson NA, 2005. Condensed tannins in birdsfoot trefoil *(Lotus corniculatus)* affect the detailed composition of milk from dairy cows. Proc New Zeal Soc Anim Prod 65: 283-289.

Vasta V, Luciano G, 2011. The effects of dietary consumption of plants secondary compounds on small ruminants' products quality. Small Rumin Res 101: 150-159. http://dx.doi.org/10.1016/j.smallrumres.2011.09.035

Vasta V, Makkar HPS, Mele M, Priolo A, 2009. Ruminal biohydrogenation as affected by tannins *in vitro*. Brit J Nutr 102: 82-92. http://dx.doi.org/10.1017/S0007114508137898