"Relationship between sainfoin proanthocyanidins and *in vitro* fermentation depending on time of harvest and level of inclusion in the diet."

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Abstract

The aim of the current study was to evaluate the effect of the presence of active proanthocyanidins (PAC) from sainfoin, either fresh or dehydrated included in the concentrate, on *in vitro* ruminal fermentation parameters, by including polyethylene glycol to block PAC. Total gas and methane productions, ammonia (NH_3-N), and volatile fatty acids (VFA) were analyzed in fresh and in dehydrated sainfoin included in concentrates at different rates (0%, 20% and 40%), and ruminal biohydrogenation (BH) only in the latter. Active PAC from fresh and dehydrated sainfoin reduced the production of gas and methane ($P < 0.01$), with no effect on total VFA ($P > 0.05$). The presence of active PAC reduced NH_3-N content in fresh sainfoin ($P < 0.01$), whereas the lower PAC contents in concentrates including dehydrated sainfoin only elicited a tendency ($P = 0.06$). The presence of sainfoin PAC in the concentrate decreased the BH extent and promoted the *trans*-11 BH pathway ($P < 0.05$). The inclusion of dehydrated sainfoin in the concentrate decreased the branched-chain fatty acids (FA; $P < 0.01$) and increased most of the *trans*-monounsaturated FA, C18:3 n-3, and BH intermediates ($P < 0.05$). In conclusion, the use of sainfoin in the diet of small ruminants can be a useful strategy to reduce gas and methane productions. In addition, the inclusion of dehydrated sainfoin in the concentrate produced changes in the ruminal FA profile that could promote a healthier meat FA profile.

Keywords: *Onobrychis viciifolia*; polyethylene glycol; gas production; fatty acids; sheep; ruminal biohydrogenation.

Abbreviations: *A*, potential gas production; ADFom, acid detergent fiber exclusive of residual ash; a.s.l., above sea level; BCFA, branched-chain fatty acid; BH, biohydrogenation; BI, biohydrogenation intermediates; *c*, rate of gas production; C2:C3, acetic/propionic acid ratio; CH_4, methane; CP, crude protein; DM, dry matter; FA, fatty acid; FID, flame ionization detector; IVOMD, *in vitro* organic matter degradation; lignin (sa), lignin determined by solubilization of cellulose with sulfuric acid; MUFA, monounsaturated fatty acids; NDFom, neutral detergent fiber exclusive of residual ash; OM, organic matter; *P*, cumulative gas production; PAC, proanthocyanidins; PEG, polyethylene glycol; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; s.e.m., standard error of the mean; VFA, volatile fatty acids.

1. Introduction

The current concern about climate change and the increasing interest in healthy ruminant products force farmers to look for new feeding strategies while ensuring a good quality of meat and milk. In response to these needs, the use of locally grown forages in ruminant diets could simultaneously foster greater sustainability and feed autonomy, besides of a potential improvement of the fatty acid (FA) profile of edible products (Moorby and Fraser, 2021; Santos-Silva et al., 2023).

Sainfoin (*Onobrychis viciifolia*) is a forage legume with a high-medium crude protein (CP) concentration and with a medium content of proanthocyanidins (PAC) (Rufino-Moya et al., 2022), also known as condensed tannins. Two thirds of the annual sainfoin production are collected in the first cut, thus this forage is often preserved as hay, dehydrated or ensiled to be used by ruminants during feed shortage periods (Hayot Carbonero et al., 2011) or included in fattening diets (Baila et al., 2023b). In addition to the effect of forage *per se* on ruminal fermentation characteristics, sainfoin PAC can change the ruminal microbial population (Mannelli et al., 2019; Vasta et al., 2019) and, consequently, modulate the ruminal biohydrogenation (BH) pathways in different ways (Frutos et al., 2020). Among others, these changes may reduce methane, gas, and ammonia (NH_3-N) productions, thus resulting in greater energy efficiency and lower environmental impact (Waghorn, 2008), as well as changes in the concentration of polyunsaturated FA (PUFA) and FA intermediates (C18:3, C18:2, and C18:1 isomers) in the rumen, which may be deposited on ruminant products (Álvarez-Rodríguez et al., 2022; Woods and Fearon, 2009). However, literature is not conclusive concerning the effects of PAC, which depend, *inter alia*, on the concentration and structure of the PAC, the type of diet or the animal for which it is intended (Patra and Saxena, 2011; Niderkorn et al., 2020; Menci et al., 2021). Besides, the preservation can change the content and fractions of PAC (Wang et al., 2015; Huang et al., 2016), being the extent of the effects dependent on the method of preservation (Scharenberg et al., 2007).

Previous studies assessed the effect of using i) fresh sainfoin harvested at different dates in the diet of ewes rearing a suckling lamb and ii) dehydrated sainfoin in the concentrate of finishing lambs. Briefly, while no effects were observed on the productive performance of ewes and lambs (Baila et al., 2022, 2024), changes were found in the fatty acid profile of milk in the first study (Baila et al., 2023b) and in the ruminal BH and fatty acid profile of lamb meat in the second study (Baila et al, 2023a). Therefore, we hypothesized that sainfoin PAC would have different effects on ruminal fermentation depending on the time of harvest, and the level and form of inclusion in the diet. Hence, the aim of the current study was to evaluate the effect of the PAC from fresh and dehydrated sainfoin on *in vitro* fermentation parameters through the addition of polyethylene glycol (PEG) to inhibit the action of PAC. In addition, the study also evaluated the effects on ruminal BH on the diets with dehydrated sainfoin.

2. Material and methods

All procedures were carried out in accordance with the guidelines for experimental animal protection (European Union Directive 2010/63) and were approved by the Animal Care and Use Committee of the Research Centre (Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) (CEEA, protocol no. 2017–07).

Two assays were conducted separately to assess the *in vitro* fermentation of fresh sainfoin and dehydrated sainfoin included in a finishing concentrate. In both assays, the effect of PAC was evaluated using PEG as a blocking agent for PAC activity.

2.1. Incubated feedstuffs

Sainfoin (*Onobrychis viciifolia* cv Reznos) was grown at CITA Research Institute in Zaragoza (41° 42′ N, 0° 49′ W, altitude 216 m.a.s.l., mean annual temperature 15 ± 7.3 °C and mean rainfall 296 ± 78 mm, Ebro Valley, NE Spain). It was sown in autumn 2018 at a seeding rate of 100 kg/ha, and irrigation was applied every 15–21 days in spring. Sainfoin was harvested on a weekly basis between 10^{th} April (vegetative stage) and 8^{th} May (start of flowering stage) to feed ewes rearing suckling lambs (Baila et al., 2023b). Finally, the remaining sainfoin was harvested at flowering stage to be dehydrated, pelleted and included at different rates in finishing concentrates for lambs, as described in detail in Baila et al. (2024).

Assay 1: *Effects of the presence of active PAC of fresh sainfoin and the week of harvest (concerning different vegetative stages)*. Fresh samples of sainfoin without (SF treatment) or with the addition of PEG (SF+PEG treatment) were used to assess the effects of the presence of active PAC and the week of harvest (1 to 4) on *in vitro* fermentative parameters, and gas and methane production. For that, fresh samples of sainfoin from each week were lyophilized and ground through a 0.2 mm sieve. The chemical composition evolved during the 4 weeks, with neutral detergent fiber (NDFom) and acid detergent fiber (ADFom) contents increasing and CP content decreasing from week 1 to 3; PAC contents were highest in weeks 1 and 2 and decreased thereafter (Fig. 1). More details of chemical composition are presented in supplementary Table S1 (Baila et al., 2022).

Assay 2: *Effects of the presence of active PAC in increasing doses of dehydrated sainfoin included in the finishing concentrate of lambs.* Three finishing concentrates for lambs including

0% (0SF), 20% (20SF), and 40% (40SF) dehydrated sainfoin were evaluated without the addition of PEG (SF treatment) or with the addition of PEG (SF+PEG treatment) to study the effects of increasing amounts of active PAC, on *in vitro* fermentation parameters, gas, and methane production. Additionally, the biohydrogenation (BH) was evaluated to assess the potential effect on lamb meat FA profile. The concentrates were formulated to be iso-energetic (18.3 MJ gross energy/kg DM) and iso-proteic (174 g/kg DM), and as the percentage of sainfoin increased and cereals was reduced, the contents of ADF, total PAC, and C18:3 n-3 increased, while that of starch decreased (Fig. 4). Further details on the chemical composition of the sainfoin pellets and the concentrates can be found in Baila et al. (2024) (Table S2 and Table S3).

2.2. Animals and sampling of ruminal digesta

Four adult Rasa Aragonesa wethers (56 ± 1.3 kg body weight) fitted with a ruminal cannula (5 cm-inner diameter; Bar Diamond, ID, USA) were used to obtain the ruminal digesta for *in vitro* assays. The wethers received a diet at maintenance levels, composed of alfalfa hay and barley grain in a proportion of 70:30 distributed in two equal meals at 8:00 h and 14:00 h. The collection of ruminal fluid, as well as the preparation of buffer solution and rumen inoculum, were conducted following the procedures explained in Rufino-Moya et al. (2019) as a modification of the method proposed by Menke and Steingass (1988). Briefly, ruminal fluid from the four wethers was mixed homogeneously, and a buffer solution was added in a proportion of 1:2 (v/v, ruminal fluid:buffer solution) to obtain the rumen inoculum.

2.3. Experimental and sampling procedures

Gas production was determined with the Ankom system (Ankom Technology Corporation, Fairport, NY, USA), which consists of 310-mL capacity bottles fitted with pressure and temperature sensors. Freeze-dried samples (≈500 mg) were incubated with 60 mL of rumen inoculum in a water bath at 39 °C. To measure the effect of PAC, PEG (molecular weight: 4000; Merck, Darmstadt, Germany) was added to the rumen inoculum at a concentration of 2.3 g/L (McSweeney et al., 1999). Three runs were conducted on three separate days, and each sample was incubated in triplicate in each run. Two bottles without substrate, with and without PEG, were used as negative controls (blanks). Gas production was recorded for 72 h in Assay 1 (fresh sainfoin) and 48 h in Assay 2 (dehydrated sainfoin included in concentrates), and the results were corrected by gas production of the blanks.

After the incubation, the bottles were placed in ice for 5–10 minutes to stop fermentation and then tempered at room temperature (10–15 minutes). A sample of gas of each treatment was collected in a Vacutainer® at atmospheric pressure with a syringe attached to a manometer tube and conserved at 4 ºC until CH_4 determination. The pH of the fermentation fluid was measured with a pH-meter (Crison Instruments S.A., Barcelona, Spain). The entire bottle content was filtered through a pre-weighed bag (50 µm; Ankom Technology, Macedon, NY, USA) to obtain the *in vitro* organic matter degradation (IVOMD). To determine the NH_3-N content in the fermentation fluid, 2.5 mL of liquid was mixed with HCl 0.1 N in a proportion of 1:1 (v/v). For volatile fatty acid (VFA) determination, 0.5 mL of liquid was added to 0.5 mL of deproteinizing solution [5 mL of 85% (v/v) ortho-phosphoric acid and 0.125 mL of 4-methylvaleric acid (Sigma Aldrich, Saint Louis, MO, USA) as internal standard, dissolved in 250 mL of distilled water] and 1 mL of distilled water. Tubes with samples of NH_3-N and VFA were stored at −20 °C until the analysis. To study the BH in Assay 2, one bottle per diet and per run were collected and immediately frozen at -80 °C to be freeze-dried and then stored at -80 °C until future FA methyl esters analysis.

To study the kinetics of fermentation, gas production was recorded hourly during the incubation using the Ankom system. The gas produced in batch cultures was adjusted to the model described by France et al. (2000):

$$P = A \times (1 - e^{-ct})$$

where P is the cumulative gas production (mL) at time (h), A is the potential gas production (mL) and c is the rate of gas production (h^{-1}).

2.4. Analytical methods

The chemical analysis of dry matter (DM) (index nº. 934.01), ash (index nº. 942.05), CP (Dumas Procedure, index nº.968.06; Model NA 2100, CE Instruments, Thermoquest S.A., Barcelona, Spain) were carried out following the techniques described in AOAC (2000). Contents of NDFom, ADFom, and acid detergent lignin (lignin (sa)) were determined according to the method described by Mertens (2002) using the Ankom 200/220 fiber analyzer (Ankom Technology Corporation). For the IVOMD estimation, the bags with sample were washed twice with distilled water and were dried at 103 °C to a constant weight to obtain the DM content. Thereafter, the sample was placed in a muffle at 550 °C to obtain the ashes. The organic matter (OM) of bag content was obtained as DM-ashes, and the IVOMD was calculated.

The methane (CH_4) was determined through an Agilent 7890B gas chromatograph (Agilent Technology, California-USA) with a PAL3 autosampler equipped with a flame ionization detector (FID), and a HPPlot Q column (15 m × 320 µm × 20 µm, Agilent Technology, CA, USA), and using helium as carrier gas (5.6 mL / min). The temperature was set at 40 ºC for the injector and oven and 350 ºC for the detector. The injection volume was 300 µL. Methane identification was based on the retention time relative to the standard and methane production was calculated by the model proposed by Cattani et al. (2016) for the Ankom Gas Production System:

$$CH_4 = -0.0064 \times [CH_4 \textit{ in the head space} \times (\textit{head space volume} + \textit{gas production})]^2 + 0.9835 \times [CH_4 \textit{ in the head space} \times (\textit{head space volume} + \textit{gas production})]$$

Content of NH_3-N was determined in an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) using the colorimetric method of Berthelot reaction, and read at 625 nm, as described by Chaney and Marbach (1962). The concentrations of VFA were determined using a Bruker Scion 460 gas chromatograph (Bruker, Billerica, MA, USA) equipped with a CP–8400 autosampler, FID, and a BR–SWax capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness, Bruker, Billerica, MA, USA). The helium was used as carrier gas at 1 mL/min. The temperature program of the oven was 100 ºC, followed by a 6 ºC/min increase to 160 ºC. The injection volume was 1 µL with a split ratio of 1:50. The identification of the individual VFA was based on retention time comparisons with commercially available standards of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, and 4-methyl-valeric acids at 99% purity (Sigma-Aldrich, Saint Louis, MO, USA).

Fatty acid determination

For the analysis of the FA profile of sainfoin and ruminal fluid carried out in Assay 2, 500 mg of freeze-dried sainfoin samples were analyzed following the method described by Rufino-Moya et al. (2022), while 250 mg of freeze-dried ruminal fluid were directly *trans*-esterified according to the method described in Alves et al. (2013) and analyzed according to Alves et al. (2017). The FA concentration of both sainfoin and ruminal fluid was determined in a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, MA, USA) with a FID equipped with a CP-8400 autosampler and a SP-2560 capillary column (100 m × 0.25 mm ID × 0.20; Sigma Aldrich, Saint Louis, MO, USA). The identification of the FA was performed with standard FA mixtures GLC-532, GLC-401, GLC-643, and GLC-642 (Nu-Chek Prep, Inc., Elysian, MN, USA) and compared with the retention times described in literature for sainfoin forage (Kramer et al., 1997; Alves and Bessa, 2009; Bravo-Lamas et al., 2016) and for ruminal fluid (Alves et al. 2013; Alves and Bessa 2014). The quantification was performed as described in ISO 12966-4:2015 and expressed as g of FA per 100 g of total FA. Total FA concentrations were expressed as mg of FA per g of sample using C19:0 (methyl-nonadecanoate N-19-M from Nu-Chek Prep, Inc., Elysian, MN, USA) as the internal standard. Calculations concerning the BH extent of C18 dietary FA and the BH completeness (%) in rumen were performed following the procedures described in Alves et al. (2017).

2.5. Statistical Analyses

Data were analyzed using SAS statistical software (SAS V.9.3) and carried out separately for each assay. The fermentation kinetic parameters (*A* and c) were estimated through a non-linear regression model using the SAS NLIN program and, together with the fermentation parameters (pH, gas, CH_4, NH_3-N, VFA, and IVOMD), were analyzed with a mixed model (MIXED procedure). The degrees of freedom were adjusted with the Kenward−Roger correction. In Assay 1: the presence of PAC (through the addition or non-addition of PEG; SF+PEG and SF), the week of harvest (1, 2, 3 and 4), and their interaction were considered as fixed effects and the run as random effect. In Assay 2: the inclusion of sainfoin in the concentrate (at 0%, 20%, and 40%) and the presence of PAC (through the addition or non-addition of PEG; SF+PEG and SF) and their interaction were used as fixed effects and the run as random effect.

When significant, the group statement was included in the model to adjust the variance heterogeneity. The least-squares means and their associated standard errors were obtained and Tukey's correction was used for pairwise comparisons. The effects were considered significant at $P < 0.05$, while $0.05 \leq P < 0.1$ results were treated as a tendency.

3. Results

Assay 1: *Effect of the presence of active PAC and the week of harvest of fresh sainfoin.*

The *in vitro* fermentation parameters according to the presence of active PAC and week are shown in Table 1 and the dynamics of gas production are plotted in Fig. 2. The presence of active PAC decreased the total, potential (*A*), and rate (*c*) of gas production, CH_4 production, NH_3-N content ($P \leq 0.01$), and IVOMD ($P \leq 0.05$) without affecting total VFA ($P > 0.10$). The week only affected total gas and the dynamics of gas production, IVOMD and total VFA ($P \leq 0.01$), with values decreasing as the week progressed ($P < 0.05$). The interaction between the presence of active PAC and the week affected the proportions of VFA ($P \leq 0.03$; Fig. 3), except for the acetic acid, which was lower in the presence of active PAC ($P < 0.001$). The presence of active PAC led to higher proportions of acetic, iso-butyric, butyric, and iso-valeric in weeks 1 and 2 ($P < 0.05$), but had no effect thereafter. These four VFA remained almost stable in the SF+PEG treatment, while in the SF treatment acetic acid decreased and the other VFA increased between week 1 and 4 ($P < 0.05$). Propionic and valeric acids also increased as the weeks progressed ($P < 0.05$).

Assay 2*: Effect of the presence of active PAC and the inclusion level of dehydrated sainfoin in the concentrate.*

In vitro fermentation parameters are presented in Table 2 and the fermentation dynamics in Fig. 5. No interaction between the inclusion of sainfoin in the concentrate and the presence of PAC was observed ($P > 0.05$); therefore, the results are presented separately for the main effects. The presence of active PAC increased the final pH ($P < 0.05$) but decreased total gas production, *A*, *c*, the production of methane ($P < 0.001$), the CH_4:gas ratio, the IVOMD ($P < 0.05$), and tended to reduce NH_3-N content (P = 0.07) but did not affect total VFA ($P > 0.05$). Regarding the percentages of VFA (Fig. 6), the percentages of acetic, propionic, and butyric were not affected ($P > 0.10$) but the presence of active PAC reduced iso-butyric, valeric and iso-valeric acids ($P < 0.001$).

Regardless of its level, the inclusion of sainfoin in the concentrate decreased total gas production and *A* but increased *c* ($P < 0.05$; Table 2; Fig. 5B). Methane production decreased when sainfoin was included in the concentrate, being lower in 40SF than 0SF ($P < 0.05$). As the level of inclusion of sainfoin in the concentrate increased, the IVOMD decreased ($P < 0.001$), without affecting total VFA and NH_3-N ($P > 0.05$). However, NH_3-N production tended to be lower in both concentrates with sainfoin ($P = 0.07$). Only the percentages of acetic and butyric acids were affected by the inclusion of sainfoin ($P < 0.05$), with greater percentage of acetic acid and lower percentage of butyric acid in 40SF concentrate than in the other concentrates ($P < 0.05$).

The effects of the presence of active PAC and the rate of inclusion of sainfoin in the concentrate on the ruminal FA profile are shown in Table 3. The presence of active PAC decreased C10:0, C12:0, C15:0, C17:0, C18:0, and C20:0 and, consequently, total saturated FA (SFA; $P < 0.001$ to $P < 0.05$), but no effect was observed on the branched-chain FA (BCFA; $P > 0.10$). Regarding monounsaturated FA (MUFA), the presence of PAC mainly affected the *cis*-MUFA, increasing the total and several individual *cis*-MUFA, including C18:1 c9 ($P < 0.05$). On the contrary, for *trans*-MUFA, the presence of PAC only increased C18:1 t11 and resulted in a lower C18:1 t10/C18:1 t11 ratio ($P < 0.05$). The presence of PAC also led to higher total PUFA, C18:2 n-6, and C18:3 n-3 ($P < 0.01$).

The inclusion of sainfoin in the concentrate had no effect on the total percentage of SFA ($P > 0.10$), but reduced the percentages of C12:0, C13:0, C15:0, C16:0, and C17:0, but not in a clear dose-dependent manner ($P < 0.001$ to $P < 0.05$). Irrespective of the inclusion level, sainfoin in the concentrate decreased the percentages of BCFA, *iso*-BCFA, and *anteiso*-BCFA ($P < 0.01$ to $P < 0.05$), while increased the total and the major individual *trans*-MUFA ($P < 0.001$ to $P < 0.05$). Nevertheless, sainfoin inclusion did not affect total and *cis*-MUFA, total

PUFA, and C18:2 n-6 ($P > 0.10$), but increased C18:2 t11,c15/t10,c15 and C18:3 n-3 contents in 40SF compared to 0SF ($P < 0.05$).

Table 4 shows the effects of the presence of active PAC and the rate of inclusion of sainfoin in the BH calculations. The presence of active PAC increased only as a tendency the sum of BH intermediates (BI; P = 0.65) and significantly the C18:1t11/BI (P < 0.05) and decreased C18:1t10/BI (P<0.001), the BH extent of C18:1 c9, C18:2 n-6, and C18:3 n-3 ($P < 0.01$), resulting in lower BH completeness ($P < 0.001$). Irrespective of the inclusion level, sainfoin in the concentrate increased BI, and BH extent of C18:3 n-3, and decreased the BH completeness ($P < 0.01$).

4. Discussion

4.1. Effect on gas and methane production and degradation

The presence of active PAC of sainfoin has been shown to affect *in vitro* fermentation by decreasing gas, methane, and NH_3-N productions, the extent of the impacts depending on the accession and the dose included (Hatew et al., 2015, Hatew et al., 2016; Niderkorn et al., 2012). In both assays presented here, the presence of active PAC from either fresh sainfoin fed as the sole diet or as dehydrated sainfoin included in concentrates, despite the lower PAC content of the later, always reduced the total, potential, and rate of gas production, as well as methane production, as reported previously in sainfoin hay, extracts or fresh forage (Theodoridou et al., 2011; Calabrò et al., 2012; Niderkorn et al., 2012; Hatew et al., 2016). Additionally, the results confirmed the activity of PAC in the concentrates, despite the potential damage that they could have suffered during the pelleting process due to high temperatures (Wang et al., 2015). There was a decrease in IVOMD due to the effect of PAC, which is consistent with Jayanegara et al. (2012), who showed that CH_4 reduction in the presence of PAC is mainly associated with a lower apparent digestion of the substrate. The literature shows unconclusive results about the effect of PAC on ruminal degradation. Some studies found that the presence of PAC decreased the IVDMD (Niderkorn et al., 2012, 2020; Rufino-Moya et al., 2019), while other studies did not find any differences (Theodoridou et al., 2011; Rufino-Moya et al., 2021). The differences in gas production and IVOMD due to the week of harvest observed here could be related to the differences in NDFom, ADFom, lignin (sa), CP, and PAC contents (Baila et al., 2022), which would compromise the fermentation of soluble carbohydrates.

The decrease of total and potential gas production, and total CH_4 when dehydrated sainfoin was included in the concentrates, regardless of the level on inclusion, could be related to the decrease of IVOMD as a result of a greater content of fiber fractions, being the lignin (sa) content twice in 20SF, and three times in 40SF,and the lower starch content in both diets with dehydrated sainfoin.

4.2. Effect on fermentation end-products

The presence of active PAC in the diet promotes a lower protein degradation (Mueller-Harvey, 2006; Waghorn, 2008), which was evidenced in the current work by a decrease in NH_3-N and iso-acid contents, in agreement with previous findings with sainfoin PAC (Huyen et al., 2016; Toral et al., 2016; Brinkhaus et al., 2017). Consistent with this assumption, the presence of active PAC decreased ruminal NH_3-N production in fresh sainfoin, in line with previous studies (Hatew et al., 2016; Rufino-Moya et al., 2019; Niderkorn et al., 2020). However, when the dehydrated sainfoin was included in the concentrate, only a tendency towards a lower NH_3-N was observed with active PAC, which could be related to the lower PAC content in the concentrates.

The effect of active PAC on total VFA is not clear as previous studies reported no effect, an increase or a decrease depending on the accession (Calabrò et al., 2012; Niderkorn et al., 2012; Rufino-Moya et al., 2019). In the current experiment, the presence of active PAC had no effect on total VFA on either fresh or dehydrated sainfoin. The differences between the studies, as pointed out above, could be due to the time of incubation, accession, chemical structure, and biological activity of PAC, and the species of the donor animal (Frutos et al., 2004; Hatew et al., 2015). Despite the lack of effect on total VFA, differences on the VFA profile appeared due to PAC in fresh and dehydrated sainfoin, although in different patterns probably related with the lower PAC content. While active PAC from dehydrated sainfoin only decreased minor VFA (iso-acids and valeric acid), those of fresh sainfoin reduced all individual VFA, except for acetic acid, during the first two weeks when PAC content was higher. Besides, differences in NDFom, ADFom, CP, and PAC due to the week of harvest (Baila et al., 2022) also led to differences in total VFA and most individual VFA as a consequence of the higher IVOMD produced by the promotion of soluble carbohydrates fermentation. The consistency in the reduction of valeric acid percentages in both studies has been previously described before when studying sainfoin PAC (Niderkorn et al., 2020) and it has been associated with altered ruminal H_2 pathways, affecting directly the formation of this VFA. The percentage of propionic acid was only affected in Assay 1 due to the reduction of the amylolytic fermentation pathway in a highly fibrous diet incubated for a long period (i.e. 72 h).

In Assay 2, the concentrate with 40% sainfoin had higher fiber content, which favors the development of cellulolytic microorganisms resulting in a greater proportion of acetic acid, as had been recorded in the present study. In addition, those differences are consistent with the effect observed in the rumen of lambs fed with these concentrates during all the fattening process, although in the in vivo study, acetic values were similar between both sainfoin-containing concentrates (Baila et al., 2024).

4.2. Effect on ruminal BH

The presence of PAC in the diet can modify the ruminal BH, modulating the saturation of dietary PUFA and the production of some intermediate FA in the rumen (Frutos et al., 2020).

Thus, ruminal BH plays an important role by actively contributing to the final composition of the FA profile of ruminant-derived products, improving the presence of FA with potential beneficial effect on consumers health (Woods and Fearon, 2009; Ferlay et al., 2017; Álvarez-Rodríguez et al., 2022). Due to the great relevance of its implications, BH is a topic that has attracted much research interest over the last 20 years (Toral et al., 2024). Nevertheless, the effects of PAC on ruminal BH are highly variable, depending on many factors such as the concentration, dosage, and chemical structure of PAC (Buccioni et al., 2011; Guerreiro et al., 2021; Valenti et al., 2021), and therefore need to be studied in depth for each individual assay.

The effect of active PAC as an inhibitory factor on microbial growth and rumen function and therefore, affecting ruminal concentrations of BCFA (Fievez et al., 2012; Costa et al., 2017), was not observed in the present study. We suggest that the low content of active PAC in the concentrates, due to the dehydrating and pelleting process, may not be sufficient to affect BCFA. However, PAC modulated BH, as shown by the reduction in saturation (lower SFA, including the final BH product, C18:0), the increase in total PUFA and the three major dietary unsaturated FA (i.e., C18:1 c9, C18:2 n-6, and C18:3 n-3), and the tendency towards a higher BI content. The decrease of dietary PUFA, together with the promotion of *trans*-11 BH pathway, as observed in the present study, are two of the main objectives to be achieved in the study of ruminal BH modulation (Palmquist, 2006; Chilliard et al., 2007; Scollan et al., 2017). According to our findings, Toral et al. (2016) observed an increase of C18:2 n-6, and total PUFA of sainfoin hay substrate compared to alfalfa hay in an *in vitro* assay, which could be a consequence of a lower BH of dietary FA during the first steps of the BH process. Similarly, Campidonico et al. (2016) found greater concentrations of total PUFA and C18:3 n-3 in lambs fed with sainfoin and red clover silages (as two PAC–containing legumes) compared to those receiving timothy (a grass without PAC).

Besides, the greater percentage of several *cis*-MUFA due to the presence of PAC and the lack of effect on *trans*-MUFA (except for the increase of the *trans*-11 isomer) also suggests a reduction of the isomerization process occurring during the first-intermediate phase of ruminal BH (Frutos et al., 2020). The explanation for this lower ruminal isomerization seems to lie in an adaptive mechanism of bacteria against the toxicity exerted by certain compounds on their membrane permeability (Eberlein et al., 2018).

The inclusion of sainfoin in the concentrate produced a less clear effect on BH compared to the changes generated by fresh sainfoin active PAC. The inclusion of forage in the diet is a strategy to improve the FA profile of edible products, as it has been shown to promote the *trans*-11 BH pathway instead of the *trans*-10 BH pathway (increased by concentrate-rich diets) (Griinari et al., 1998). While the former leads to a more beneficial FA profile for human health (Vahmani et al., 2020), the latter is considered as non-desirable as it has been linked to negative health (Aldai et al., 2013; Ferlay et al., 2017) and productive implications (Griinari et al., 1998; Baumgard et al., 2000; Dewanckele et al., 2020). Nevertheless, in the present study, an increase of both *trans*-10 and *trans*-11 was observed, although some changes in the rumen FA profile suggest a greater predominance of pathways involving the BH of C18:3 n-3 with sainfoin inclusion, as the greater BH extent of C18:3 n-3 *per se*, and the tendency towards a higher formation of CLA c9,t11. In addition, the increase in the formation of BI together with the tendency to present higher C18:1 t11/BI ratio, indicates that the increase in BI was more related to this isomer when sainfoin was included in the concentrate, regardless of the inclusion level.

Lastly, the fact that most of the effects observed on ruminal BH due to sainfoin inclusion are related to changes in BCFA percentages, contrarily to that observed due to sainfoin PAC, shows a great impact of forage on ruminal populations, even being an *in vitro* trial. As is well-known, forage inclusion improves the environment for cellulolytic bacteria, while higher concentrations of starch in the diet promote the development of amylolytic populations, which have been related to *anteiso*-BCFA production (Fievez et al., 2012). The lower percentage of *anteiso*-BCFA with sainfoin inclusion was also observed *in vivo* (Baila et al., 2024).

5. Conclusions

The presence of active PAC from sainfoin reduces gas and methane productions and IVOMD, regardless of whether the sainfoin used is fresh or dehydrated included in the concentrate. However, when sainfoin is dehydrated and fed as ingredient in the concentrates, the activity of PAC is not enough to reduce the NH_3-N production and only reduces the minor VFA. The week of harvest involves a phenological evolution of forage, which leads to a lower gas production, total VFA, and IVOMD as the maturation of sainfoin advances, but does not affect NH_3-N formation.

The presence of even low quantities of active PAC seems enough to reduce the BH extent of dietary PUFA and the BH completeness while promoting the *trans*-11 BH pathway. The ruminal BH of concentrates with dehydrated sainfoin also lead to higher percentages of many of the *trans*-MUFA, including the *trans*-11 isomer, and lower BH completeness; with the level of inclusion of sainfoin having little effect. The use of sainfoin in the diet of small ruminants can reduce methane production while improving the ruminal FA profile, which could be reflected in healthier edible animal products.

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CRediT authorship contribution statement

Clàudia Baila-Bigné: Writing – original draft, Formal analysis, Data curation. **Sandra Lobón:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization **Mireia Blanco:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Isabel Casasús:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Margalida Joy:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Aldai, N., de Renobales, M., Barron, L.J.R., Kramer, J.K.G., 2013. What are the trans fatty acids issues in foods after discontinuation of industrially produced trans fats? Ruminant products, vegetable oils, and synthetic supplements. Eur. J. Lipid Sci. Technol. 115, 1378–1401. https://doi.org/10.1002/ejlt.201300072.

Álvarez-Rodríguez, J., Urrutia, O., Lobón, S., Ripoll, G., Bertolín, J.R., Joy, M., 2022. Insights into the role of major bioactive dietary nutrients in lamb meat quality: a review. J. Anim. Sci. Biotechnol. 13, 20. https://doi.org/10.1186/s40104-021-00665-0.

Alves, S.P., Bessa, R.J.B., 2009. Comparison of two gas–liquid chromatograph columns for the analysis of fatty acids in ruminant meat. J. Chromatogr. A. 1216, 5130–5139. https://doi.org/10.1016/j.chroma.2009.04.079.

Alves, S.P., Bessa, R.J.B., 2014. The *trans*-10, *cis*-15 18:2: A missing intermediate of trans-10 shifted rumen biohydrogenation pathway? Lipids 49, 527–541. https://doi.org/10.1007/s11745-014-3897-4.

Alves, S.P., Francisco, A., Costa, M., Santos-Silva, J., Bessa, R.J.B., 2017. Biohydrogenation patterns in digestive contents and plasma of lambs fed increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and vegetable oils. Anim. Feed Sci. Technol. 225, 157–172. https://doi.org/10.1016/j.anifeedsci.2017.01.018.

Alves, S.P., Santos-Silva, J., Cabrita, A.R.J., Fonseca, A.J.M., Bessa, R.J.B., 2013. Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. PLoS One 8, e58386. https://doi.org/10.1371/journal.pone.0058386.

Azuhnwi, B.N., Boller, B., Martens, M., Dohme-Meier, F., Ampuero, S., Günter, S., Kreuzer, M., Hess, H.D., 2011. Morphology, tannin concentration and forage value of 15 Swiss accessions of sainfoin (*Onobrychis viciifolia* Scop.) as influenced by harvest time and cultivation site. Grass Forage Sci. 66, 474–487. https://doi.org/10.1111/j.1365-2494.2011.00811.x.

Baila, C., Joy, M., Blanco, M., Casasús, I., Bertolín, J.R., Lobón, S., 2022. Effects of feeding sainfoin proanthocyanidins to lactating ewes on intake, milk production and plasma metabolites. Animal 16, 100438. https://doi.org/10.1016/j.animal.2021.100438.

Baila, C., Lobón, S., Blanco, M., Casasús, I., Ripoll, G., Joy, M., 2024. Sainfoin can be included up to 40% in the concentrate of finishing lambs without impairing their performance, rumen fermentation, and carcass quality. Anim. Feed Sci. Technol. 312, 115975. https://doi.org/10.1016/j.anifeedsci.2024.115975.

Baila, C., Joy, M., Bertolín, J.R., Blanco, M., Casasús, I., Lobón, S., 2023a. Effect of sainfoin proanthocyanidins on milk fatty acids from ewes rearing suckling lambs. Animal 17, 100862. https://doi.org/10.1016/j.animal.2023.100862.

Baila, C., Joy, M., Bertolín, J.R., Alves, S., Bessa, R., Blanco, M., Lobón, S., 2023b. Inclusion of sainfoin in the concentrate of finishing lambs: fatty acid profiles of rumen, plasma, and muscle. J. Agric. Food Chem. 71, 17947–17958. https://doi.org/10.1021/acs.jafc.3c05902J.

Baumgard, L.H., Corl, B.A., Dwyer, D.A., Sæbø, A., Bauman, D.E., 2000. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, 179–184. https://doi.org/10.1152/ajpregu.2000.278.1.R179.

Bravo-Lamas, L., Barron, L.J.R., Kramer, J.K.G., Etaio, I., Aldai, N., 2016. Characterization of the fatty acid composition of lamb commercially available in northern Spain: Emphasis on the trans-18: 1 and CLA content and profile. Meat Sci. 117, 108–116. https://doi.org/10.1016/j.meatsci.2016.02.043.

Brinkhaus, A.G., Wyss, U., Arrigo, Y., Girard, M., Bee, G., Zeitz, J.O., Kreuzer, M., Dohme-Meier, F., 2017. In vitro ruminal fermentation characteristics and utilisable CP supply of sainfoin and birdsfoot trefoil silages and their mixtures with other legumes. Animal 11, 580–590. https://doi.org/10.1017/S1751731116001816.

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. https://doi.org/10.1017/S1751731111000759.

Buccioni, A., Pauselli, M., Viti, C., Minieri, S., Pallara, G., Roscini, V., Rapaccini, S., Marinucci, M.T., Lupi, P., Conte, G., 2015. Milk fatty acid composition, rumen microbial population, and animal performances in response to diets rich in linoleic acid supplemented with chestnut or quebracho tannins in dairy ewes. J. Dairy Sci. 98, 1145–1156. https://doi.org/10.3168/jds.2014-8651.

Calabrò, S., Guglielmelli, A., Iannaccone, F., Danieli, P.P., Tudisco, R., Ruggiero, C., Piccolo, G., Cutrignelli, M.I., Infascelli, F., 2012. Fermentation kinetics of sainfoin hay with and without PEG. J. Anim. Physiol. Anim. Nutr. 96, 842–849. https://doi.org/10.1111/j.1439-0396.2011.01260.x.

Campidonico, L., Toral, P.G., Priolo, A., Luciano, G., Valenti, B., Hervás, G., Frutos, P., Copani, G., Ginane, C., Niderkorn, V., 2016. Fatty acid composition of ruminal digesta and longissimus muscle from lambs fed silage mixtures including red clover, sainfoin, and timothy. J. Anim. Sci. 94, 1550–1560. https://doi.org/10.2527/jas.2015-9922.

Cattani, M., Maccarana, L., Rossi, G., Tagliapietra, F., Schiavon, S., Bailoni, L., 2016. Dose-response and inclusion effects of pure natural extracts and synthetic compounds on in vitro methane production. Anim. Feed Sci. Technol. 218, 100–109. https://doi.org/10.1016/j.anifeedsci.2016.05.014.

Chaney, A.L., Marbach, E.P., 1962. Modified Reagents for Determination of Urea and Ammonia. Clin. Chem. 8, 130–132. https://doi.org/10.1093/clinchem/8.2.130.

Costa, M., Alves, S.P., Cabo, Â., Guerreiro, O., Stilwell, G., Dentinho, M.T., Bessa, R.J.B., 2017. Modulation of in vitro rumen biohydrogenation by *Cistus ladanifer* tannins compared with other tannin sources. J. Sci. Food Agric. 97, 629–635. https://doi.org/10.1002/jsfa.7777.

Dewanckele, L., Toral, P.G., Vlaeminck, B., Fievez, V., 2020. Invited review: Role of rumen biohydrogenation intermediates and rumen microbes in diet-induced milk fat depression: An update. J. Dairy Sci. 103, 7655–7681. https://doi.org/10.3168/jds.2019-17662.

Ferlay, A., Bernard, L., Meynadier, A., Malpuech-Brugère, C., 2017. Production of trans and conjugated fatty acids in dairy ruminants and their putative effects on human health: A review. Biochimie 141, 107–120. https://doi.org/10.1016/j.biochi.2017.08.006.

Fievez, V., Colman, E., Castro-Montoya, J.M., 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. https://doi.org/10.1016/j.anifeedsci.2011.12.008.

France, J., Dijkstra, J., Dhanoa, M.S., Lopez, S., Bannink, A., 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations. Br. J. Nutr. 83, 143–150. https://doi.org/10.1017/S0007114500000180.

Frutos, P., Hervás, G., Giráldez, F.J., Mantecón, A.R., 2004. Tannins and ruminant nutrition, Review. Span. J. Agric. Res. 2, 191–202. https://doi.org/10.5424/sjar/2004022-73.

Frutos, P., Hervás, G., Natalello, A., Luciano, G., Fondevila, M., Priolo, A., Toral, P.G., 2020. Ability of tannins to modulate ruminal lipid metabolism and milk and meat fatty acid profiles. Anim. Feed Sci. Technol. 269, 114623. https://doi.org/10.1016/j.anifeedsci.2020.114623.

Glasser, F., Doreau, M., Maxin, G., Baumont, R., 2013. Fat and fatty acid content and composition of forages: A meta-analysis. Anim. Feed Sci. Technol. 185, 19–34. https://doi.org/10.1016/j.anifeedsci.2013.06.010.

Griinari, J.M., Dwyer, D.A., Mcguire, M.A., Bauman, D.E., Palmquist, D.L., Nurmela, K.V.V., 1998. Trans-Octadecenoic Acids and Milk Fat Depression in Lactating Dairy Cows. J. Dairy Sci. 81, 1251–1261. https://doi.org/10.3168/jds.S0022-0302(98)75686-3.

Guerreiro, O., Alves, S.P., Costa, M., Duarte, M.F., Jerónimo, E., Bessa, R.J.B., 2021. Effects of increasing doses of condensed tannins extract from *Cistus ladanifer l.* On in vitro ruminal fermentation and biohydrogenation. Animals 11, 1–13. https://doi.org/10.3390/ani11030761.

Hatew, B., Hayot Carbonero, C., Stringano, E., Sales, L.F., Smith, L.M.J., Mueller-Harvey, I., Hendriks, W.H., Pellikaan, W.F., 2015. Diversity of condensed tannin structures affects rumen in vitro methane production in sainfoin (*Onobrychis viciifolia*) accessions. Grass Forage Sci. 70, 474–490. https://doi.org/10.1111/gfs.12125.

Hatew, B., Stringano, E., Mueller-Harvey, I., Hendriks, W.H., Carbonero, C.H., Smith, L.M.J., Pellikaan, W.F., 2016. Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on in vitro ruminal methane production and fermentation characteristics. J. Anim. Physiol. Anim. Nutr. 100, 348–360. https://doi.org/10.1111/jpn.12336.

Hayot Carbonero, C., Mueller-Harvey, I., Brown, T.A., Smith, L., 2011. Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. Plant Genet. Res. 9, 70–85. https://doi.org/10.1017/S1479262110000328.

Huang, Q.Q., Jin, L., Xu, Z., Acharya, S., McAllister, T.A., Hu, T.M., Iwaasa, A., Schellenberg, M., Peng, K., Wang, Y., 2016. Effects of conservation method on condensed tannin content, ruminal degradation, and in vitro intestinal digestion of purple prairie clover (*Dalea purpurea* Vent.). Can. J. Anim. Sci. 96, 524–531.

Huyen, N.T., Fryganas, C., Uittenbogaard, G., Mueller-Harvey, I., Verstegen, M.W.A., Hendriks, W.H., Pellikaan, W.F., 2016. Structural features of condensed tannins affect in vitro ruminal methane production and fermentation characteristics. J. Agric. Sci. 154, 1474–1487. https://doi.org/10.1017/S0021859616000393.

Jayanegara, A., Leiber, F., Kreuzer, M., 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. J. Anim. Physiol. Anim. Nutr. 96, 365–375. https://doi.org/10.1111/j.1439-0396.2011.01172.x.

Kramer, J.K.G., Fellner, V., Dugan, M.E.R., Sauer, F.D., Mossoba, M.M., Yurawecz, M.P., 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 32, 1219–1228. https://doi.org/10.1007/s11745-997-0156-3.

Lobón, S., Blanco, M., Sanz, A., Ripoll, G., Bertolín, J.R., Joy, M., 2017. Meat quality of light lambs is more affected by the dam's feeding system during lactation than by the inclusion of quebracho in the fattening concentrate. J. Anim. Sci. 95, 4998–5011. https://doi.org/10.2527/jas2017.1595.

Mannelli, F., Daghio, M., Alves, S.P., Bessa, R.J.B., Minieri, S., Giovannetti, L., Conte, G., Mele, M., Messini, A., Rapaccini, S., 2019. Effects of chestnut tannin extract, vescalagin and gallic acid on the dimethyl acetals profile and microbial community composition in rumen liquor: an in vitro study. Microorganisms 7, 202. https://doi.org/10.3390/microorganisms7070202.

McSweeney, C.S., Palmer, B., Bunch, R., Krause, D.O., 1999. Isolation and characterization of proteolytic ruminal bacteria from sheep and goats fed the tannin-containing shrub legume *Calliandra calothyrsus*. Appl. Environ. Microbiol. 65, 3075–3083. https://doi.org/10.1128/AEM.65.7.3075-3083.1999.

Menci, R., Coppa, M., Torrent, A., Natalello, A., Valenti, B., Luciano, G., Priolo, A., Niderkorn, V., 2021. Effects of two tannin extracts at different doses in interaction with a green or dry forage substrate on in vitro rumen fermentation and biohydrogenation. Anim. Feed Sci. Technol. 278, 114977. https://doi.org/10.1016/j.anifeedsci.2021.114977.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217–1240. https://doi.org/10.1093/jaoac/85.6.1217.

Moorby, J.M., Fraser, M.D., 2021. New feeds and new feeding systems in intensive and semi-intensive forage-fed ruminant livestock systems. Animal 15, 100297. https://doi.org/10.1016/j.animal.2021.100297.

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

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 tannins. Anim. Feed Sci. Technol. 178, 48–56. https://doi.org/10.1016/j.anifeedsci.2012.09.014.

Niderkorn, V., Barbier, E., Macheboeuf, D., Torrent, A., Mueller-Harvey, I., Hoste, H., 2020. In vitro rumen fermentation of diets with different types of condensed tannins derived from sainfoin (*Onobrychis viciifolia* Scop.) pellets and hazelnut (*Corylus avellana L.*) pericarps. Anim. Feed Sci. Technol. 259, 114357. https://doi.org/10.1016/j.anifeedsci.2019.114357.

Palmquist, D.L., St-Pierre, N., Mcclure, K.E., 2004. Nutritional models tissue fatty acid profiles can be used to quantify endogenous rumenic acid synthesis in lambs 1,2, J. Nutr. 134, 2407–2414. https://doi.org/10.1093/jn/134.9.2407.

Patra, A.K., Saxena, J., 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91, 24–37. https://doi.org/10.1002/jsfa.4152.

Rufino-Moya, P.J., Blanco, M., Lobón, S., Bertolín, J.R., Armengol, R., Joy, M., 2019. The inclusion of concentrate with quebracho is advisable in two forage-based diets of ewes according to the in vitro fermentation parameters. Animals 9, 451. https://doi.org/10.3390/ani9070451.

Rufino-Moya, P. J, Blanco, M., Bertolín, J.R., Joy, M., 2019. Effect of the method of preservation on the chemical composition and in vitro fermentation characteristics in two legumes rich in condensed tannins. Anim. Feed Sci. Technol. 251, 12–20. https://doi.org/10.1016/j.anifeedsci.2019.02.005.

Rufino-Moya, P.J., Blanco, M., Bertolín, J.R., Joy, M., 2019. Methane production of fresh Sainfoin, with or without PEG, and fresh alfalfa at different stages of maturity is similar but the fermentation end products vary. Animals 9, 197. https://doi.org/10.3390/ani9050197.

Rufino-Moya, P.J., Blanco, M., Joy, M., 2021. Condensed tannins of sainfoin and their effect on ruminal fermentation. influence of phenological stage and preservation. A review. ITEA 117, 227–246. https://doi.org/10.12706/itea.2020.026.

Sackmann, J.R., Duckett, S.K., Gillis, M.H., Realini, C.E., Parks, A.H., Eggelston, R.B., 2003. Effects of forage and sunflower oil levels on ruminal biohydrogenation of fatty acids and conjugated linoleic acid formation in beef steers fed finishing diets 1, J. Anim. Sci. 81, 3174–3181. https://doi.org/10.2527/2003.81123174x.

Santos-Silva, J., Alves, S.P., Francisco, A., Portugal, A.P., Dentinho, M.T., Almeida, J., da Silva, J.L.R., Fialho, L., Cachucho, L., Jerónimo, E., Barradas, A., Rodrigues, A., Rodrigues, N., Teixeira, R.F.M., Domingos, T., Bessa, R.J.B., 2023. Forage based diet as an alternative to a high concentrate diet for finishing young bulls - Effects on growth performance, greenhouse gas emissions and meat quality. Meat Sci. 198, 109098. https://doi.org/10.1016/J.MEATSCI.2023.109098.

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

Shingfield, K.J., Chilliard, Y., Toivonen, V., Kairenius, P., Givens, D.I. *Trans* fatty acids and bioactive lipids in ruminant milk. Bioactive components of milk. Ed.: Zsuzsanna Bösze Springer Editors, Netherlands. Vol. 606. p: 3–65, 2008. https://doi.org/10.1007/978-0-387-74087-4.

Theodoridou, K., Aufrère, J., Niderkorn, V., Andueza, D., Le Morvan, A., Picard, F., Baumont, R., 2011. In vitro study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetation cycles. Anim. Feed Sci. Technol. 170, 147–159. https://doi.org/10.1016/j.anifeedsci.2011.09.003.

Toral, P.G., Angulo, G.H., Missaoui, H., Llorente, S.A., García, F.J.G., Jellali, S., Frutos, P., 2016. Effects of a tannin-rich legume (*Onobrychis viciifolia*) on in vitro ruminal biohydrogenation and fermentation. Span. J. Agric. Res. 14, 12. https://doi.org/10.5424/sjar/2016141-8989.

Toral, P.G., Hervás, G., Frutos, P., 2024. Invited review: Research on ruminal biohydrogenation: Achievements, gaps in knowledge, and future approaches from the perspective of dairy science. J. Dairy Sci. 107, 10115–10140. https://doi.org/10.3168/jds.2023-24591.

Valenti, B., Campidonico, L., Natalello, A., Lanza, M., Salami, S.A., Priolo, A., Serra, A., Pauselli, M., Luciano, G., 2021. Fatty acid metabolism in lambs supplemented with different condensed and hydrolysable tannin extracts. PLoS One 16, e0258265. https://doi.org/10.1371/journal.pone.0258265.

Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C., Mele, M., 2019. Invited review: Plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: Experimental evidence and methodological approaches. J. Dairy Sci. 102, 3781–3804. https://doi.org/10.3168/JDS.2018-14985

Vasta, V., Pennisi, P., Lanza, M., Barbagallo, D., Bella, M., Priolo, A., 2007. Intramuscular fatty acid composition of lambs given a tanniniferous diet with or without polyethylene glycol supplementation. Meat Sci. 76, 739–745. https://doi.org/10.1016/j.meatsci.2007.02.015.

Vasta, V., Priolo, A., Scerra, M., Hallett, K.G., Wood, J.D., Doran, O., 2009. Δ9 desaturase protein expression and fatty acid composition of longissimus dorsi muscle in lambs fed green herbage or concentrate with or without added tannins. Meat Sci. 82, 357–364. https://doi.org/10.1016/j.meatsci.2009.02.007.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci. Technol. 147, 116–139. https://doi.org/10.1016/j.anifeedsci.2007.09.013.

Wang, Y., McAllister, T.A., Acharya, S., 2015. Condensed tannins in Sainfoin: composition, concentration, and effects on nutritive and feeding value of sainfoin forage. Crop Sci. 55, 13–22. https://doi.org/10.2135/cropsci2014.07.0489

Woods, V.B., Fearon, A.M., 2009. Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: A review. Livest. Sci. 126, 1–20. https://doi.org/10.1016/j.livsci.2009.07.002.

Table 1

Effect of presence of active proanthocyanidins (PAC)[1] and week of harvest of sainfoin on the pH, production of gas and methane (CH_4), the kinetics of fermentation, *in vitro* organic matter degradation (IVOMD), ammonia (NH_3-H), and total volatile fatty acids (VFA) after 72 h of incubation of sainfoin (Assay 1).

	PAC [1]			Week					*P*-value [2]	
	SF	SF+PEG	s.e.m. [3]	1	2	3	4	s.e.m. [3]	PAC	Week
Final pH	6.51	6.48	0.028	6.46	6.49	6.52	6.52	0.040	0.338	0.707
Total gas production, mL/g DM	154	163	5.1	163^{a}	165^{a}	158^{ab}	149^{b}	5.7	0.006	0.009
Potential gas production (*A*), mL	170	182	4.8	180^{a}	182^{a}	173^{ab}	168^{b}	5.3	0.001	0.010
Rate of gas production (*c*), mL/h	0.17	0.19	0.006	0.19^{ab}	0.18^{bc}	0.17^{c}	0.19^{a}	0.007	<0.001	0.031
Total CH_4 production, mL/g DM	9.36	10.6	0.624	10.25	10.28	9.96	9.32	0.694	0.008	0.383
CH_4:gas	0.061	0.064	0.0021	0.063	0.062	0.063	0.063	0.0024	0.076	0.965
IVDMO, %	82.0	82.8	0.63	88.3^{a}	83.7^{b}	80.7^{c}	76.8^{d}	0.69	0.031	<0.001
NH_3-N, mg/L	234	288	24.1	255	282	253	254	26.8	0.002	0.551
Total VFA, mM	76.3	79.2	3.22	82.2^{a}	80.7^{ab}	75.4^{bc}	72.7^{c}	3.51	0.150	0.004

Within a parameter and effect, means with different superscript (a, b, c, or d) differ at $P < 0.05$.

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

[2] The interaction was never significant.

[3] Standard error of the mean.

Table 2

Effect of presence of active proanthocyanidins (PAC)[1] and inclusion of dehydrated sainfoin (SF)[2] in the finishing concentrate of lambs on the pH, production of gas and methane (CH_4), the kinetics of fermentation, *in vitro* organic matter degradation (IVOMD), ammonia (NH_3-N), and volatile fatty acids (VFA) after 48 h of incubation (Assay 2).

	PAC [1]			SF [2]				*P*-value [3]	
	SF	SF+PEG	s.e.m. [4]	0SF	20SF	40SF	s.e.m. [4]	PAC	SF
Final pH	6.35	6.32	0.012	6.31[b]	6.33[ab]	6.34[a]	0.013	0.001	0.011
Total gas production, mL/g DM	190	207	5.1	206[a]	195[b]	194[b]	5.4	<0.001	0.007
Potential gas production (*A*), mL	188	209	5.5	208[a]	193[b]	194[b]	5.9	<0.001	0.003
Rate of gas production (*c*), mL/h	0.12	0.15	0.004	0.13[b]	0.14[a]	0.14[a]	0.004	<0.001	<0.001
Total CH_4 production, mL/g DM	10.0	12.1	0.72	11.8[a]	11.1[ab]	10.2[b]	0.77	<0.001	0.034
CH_4:gas	0.05	0.06	0.003	0.06	0.06	0.05	0.003	0.038	0.249
IVDMO, %	92.5	92.8	0.18	94.6[a]	92.7[b]	90.8[c]	0.19	0.010	<0.001
NH_3-N, mg/L	197	225	21.4	235	194	203	22.5	0.06	0.068
Total VFA, mM	71.7	75.9	3.38	75.6	73.1	75.6	3.56	0.069	0.545

Within a parameter and effect, means with different superscript (a, b, c, or d) differ at $P < 0.05$.

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

[2] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the finishing concentrate.

[3] The interaction was never significant.

[4] Standard error of the mean.

Table 3

Effect of the presence of active proanthocyanidins (PAC)[1] and the inclusion of dehydrated sainfoin (SF)[2] in the finishing concentrate on fatty acid (FA) profile in ruminal fluid of the *in vitro* trial (Assay 2).

	PAC [1]			SF [2]				*P*-value [3]	
Item	SF	SF+ PEG	s.e.m [4]	0SF	20SF	40SF	s.e.m [4]	PAC	SF
Total FA, mg/g DM	32.2	33.7	2.6	30.6	34.8	33.5	2.77	0.423	0.218
SFA[6], % FA [5]	82.6	86.7	0.58	85.3	84.1	84.5	0.67	<0.001	0.341
C10:0	0.27	0.81	0.053	0.53	0.55	0.55	0.057	<0.001	0.923
C11:0	0.11	0.10	0.01	0.11	0.12	0.1	0.011	0.206	0.264
C12:0	0.36	0.40	0.018	0.41[a]	0.35[b]	0.37[b]	0.188	0.008	0.006
C13:0	0.20	0.19	0.013	0.15[a]	0.14[b]	0.14[ab]	0.007	0.241	0.031
C14:0	1.84	1.96	0.134	2.01	1.75	1.94	0.144	0.269	0.167
C15:0	1.78	1.94	0.091	1.94[a]	1.82[b]	1.82[b]	0.091	<0.001	0.001
C16:0	30.7	30.8	0.38	31.2[a]	30.1[b]	31.0[a]	0.4	0.687	0.005
C17:0	0.84	0.93	0.047	0.92[a]	0.88[ab]	0.87[b]	0.047	<0.001	0.041
C18:0	41.1	44.3	1.18	42.2	43.2	42.7	1.25	0.005	0.629
C20:0	0.67	0.75	0.037	0.72	0.69	0.72	0.037	<0.001	0.161
C22:0	0.16	0.16	0.021	0.18	0.17	0.13	0.025	0.666	0.643
BCFA[7], % FA	5.15	5.11	0.315	5.68[a]	4.90[b]	4.80[b]	0.329	0.829	0.007
iso-BCFA	2.19	2.14	0.153	2.35[a]	2.08[b]	2.07[b]	0.157	0.487	0.015
iso-C13:0	0.2	0.19	0.013	0.21[a]	0.18[b]	0.18[ab]	0.013	0.241	0.035
iso-C14:0	0.34	0.33	0.022	0.37[a]	0.33[b]	0.32[b]	0.023	0.34	0.008
iso-C16:0	0.75	0.72	0.059	0.83[a]	0.70[ab]	0.68[b]	0.063	0.469	0.036
iso-C17:0	0.90	0.91	0.062	0.95[a]	0.88[b]	0.89[b]	0.062	0.836	0.011
anteiso-BCFA	2.11	2.03	0.135	2.41[a]	1.94[b]	1.86[b]	0.15	0.561	0.014
anteiso-C15:0	0.97	0.93	0.063	1.14[a]	0.87[ab]	0.84[b]	0.075	0.63	0.022
anteiso-C17:0	1.14	1.10	0.087	1.28[a]	1.07[b]	1.02[b]	0.091	0.472	0.007
MUFA[8], % FA	12.5	10.7	0.53	11.1	11.6	12.1	3.57	0.33	0.18
cis-MUFA	6.56	5.28	0.328	6.29	5.56	5.92	0.369	0.004	0.259
C16:1 c7/t3 [9]	0.09	0.07	0.005	0.08	0.07	0.09	0.007	0.024	0.058
C16:1 c9	0.06	0.04	0.011	0.06	0.05	0.05	0.011	0.035	0.242
C18:1 c9 [10]	3.70	2.71	0.179	3.22	2.97	3.43	0.204	<0.001	0.202
C18:1 c11	0.37	0.27	0.028	0.35	0.31	0.3	0.031	0.002	0.255
C18:1 c12	0.14	0.10	0.013	0.12	0.13	0.11	0.014	0.006	0.262
C18:1 t16/c14 [11]	0.03	0.02	0.014	0.04	0.03	0.01	0.016	0.325	0.259
C18:1 c15	0.12	0.11	0.007	0.12	0.1	0.12	0.008	0.235	0.093
C18:1 c16	0.05	0.05	0.004	0.05	0.05	0.05	0.005	0.785	0.468
trans-MUFA	5.82	5.28	0.241	4.68[b]	5.91[a]	6.05[a]	0.271	0.056	0.002
C18:1 t5	0.04	0.04	0.008	0.05	0.03	0.04	0.009	0.292	0.359
C18:1 t6/t7/t8 [12]	0.27	0.27	0.013	0.24[b]	0.26[ab]	0.32[a]	0.016	0.842	0.013
C18:1 t9	0.23	0.22	0.014	0.19[b]	0.23[a]	0.26[a]	0.015	0.26	<0.001
C18:1 t10	0.29	0.32	0.017	0.26[b]	0.33[a]	0.33[a]	0.018	0.103	0.01
C18:1 t11	4.4	3.87	0.229	3.45[b]	4.45[a]	4.51[a]	0.251	0.028	0.003

C18:1 t12	0.33	0.32	0.013	0.28^{b}	0.36^{a}	0.35^{a}	0.015	0.31	0.004
C18:1 t15	0.24	0.24	0.019	0.21^{b}	0.25^{a}	0.25^{a}	0.02	0.616	0.009
C18:1 t10/C18:1 t11	0.07	0.08	0.007	0.08	0.07	0.07	0.008	<0.001	0.441
PUFA[13], % FA	2.92	2.20	0.096	2.61	2.45	2.62	0.118	<0.001	0.559
C18:2 n-6	1.92	1.28	0.096	1.74	1.52	1.55	0.116	<0.001	0.372
C18:2 t11,c15/t10,c15 [14]	0.05	0.04	0.004	0.04^{b}	0.04^{ab}	0.06^{a}	0.005	0.065	0.02
C18:3 n-3	0.69	0.57	0.025	0.58^{b}	0.59^{b}	0.72^{a}	0.03	0.004	0.011
CLA [15]	0.26	0.31	0.025	0.26	0.29	0.29	0.028	0.078	0.393
CLA c9,t11	0.07	0.08	0.004	0.07	0.08	0.09	0.005	0.320	0.078
CLA t10,c12	0.18	0.23	0.023	0.19	0.22	0.21	0.026	0.100	0.643

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

[2] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the finishing concentrate.

[3] Probability of significant differences due to the presence of PAC in the concentrate.

[4] Standard error of the mean.

[5] Total identified FA.

[6] Saturated FA.

[7] Branched-chain FA.

[8] Monounsaturated FA.

[9] C16:1 c7 and C16:1 t3 might coelute.

[10] C18:1 c9 might coelute with the pair C18:1 t13 and t14.

[11] C18:1 t16 coelutes with C18:1 c14 as a minor isomer.

[12] C18:1 t6, C18:1 t7, and C18:1 t8 might coelute.

[13] Polyunsaturated FA.

[14] C18:2 t11,c15 and C18:2 t10,c15 might coelute.

[15] Conjugated linoleic acid.

Table 4

Effect of the presence of active proanthocyanidins (PAC)[1] and the inclusion of dehydrated sainfoin (SF)[2] in the finishing concentrate on biohydrogenation intermediates (BI) and biohydrogenation (BH) in ruminal fluid of the *in vitro* trial (Assay 2).

	PAC[1]			SF[2]				*P*-value[3]	
	SF	SF+PEG	s.e.m.[4]	0SF	20SF	40SF	s.e.m.[4]	PAC	SF
BI[5]	6.72	6.18	0.225	5.54[b]	6.84[a]	6.97[a]	0.260	0.065	0.002
C18:1t10/BI	0.043	0.052	0.0038	0.048	0.048	0.047	0.0038	<0.001	0.909
C18:1t11/BI	0.65	0.62	0.014	0.62	0.65	0.65	0.015	0.013	0.069
BH extent, %									
C18:1 c9	77.8	83.4	2.00	79.4	81.3	81.2	2.09	0.001	0.378
C18:2 n-6	91.7	94.2	1.08	92.9	93.7	92.4	0.12	<0.001	0.194
C18:3 n-3	77.3	82.5	1.19	68.9[b]	84.0[a]	86.7[a]	1.45	0.009	<0.001
Completeness, %	83.9	85.7	0.49	86.3[a]	84.3[b]	83.9[b]	0.52	<0.001	0.001

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

[2] 0SF: 0% sainfoin; 20SF: 20% sainfoin; 40SF: 40% sainfoin in the finishing concentrate.

[3] The interaction was never significant.

[4] Standard error of the mean.

[5] All C18 FA except C18:0, C18:1 c9, C18:1 c11, C18:2 n-6, and C18:3 n-3.

FIGURE CAPTIONS

Fig. 1. Chemical composition of fresh sainfoin during 4 weeks of harvest (Assay 1) (adapted from Baila et al., 2022).

NDFom: neutral detergent fiber exclusive of residual ash; ADFom: acid detergent fiber exclusive of residual ash; CP: crude protein; PAC: proanthocyanidins

Fig. 2. Fermentation kinetics of fresh sainfoin according to the presence of active proanthocyanidins (PAC[1]; *A*) and week of harvest (B) during 72 hours of incubation (Assay 1).

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

Fig. 3. Effect of the presence of active PAC[1] and week of harvest on the proportions of volatile fatty acids in the *in vitro* assay at 72 h of incubation (Assay 1).

Within a parameter and week or treatment, means with different letter differ at $P < 0.05$.

[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

Fig. 4. Neutral detergent fiber exclusive of residual ash (NDFom), acid detergent fiber exclusive of residual ash (ADFom), lignin (sa), starch, total proanthocyanidins (PAC), and percentages of C16:0, C18:0, C18:1 c9, C18:2 n-6, and C18:3 n-3 of concentrates with different inclusion of dehydrated sainfoin[1] (Assay 2) (adapted from Baila et al., 2024).

[1]0SF: 0% sainfoin; 20SF: 20% sainfoin; 40SF: 40% sainfoin.

Fig. 5. Fermentation kinetics according to the presence of active proantocyanidins[1] (PAC; A) and the inclusion of dehydrated sainfoin[2] (SF; B) in the fattening concentrate during 48 h of incubation (Assay 2).

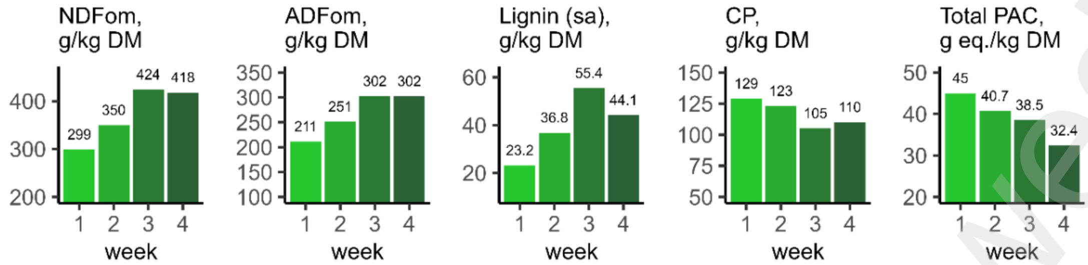
[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).).

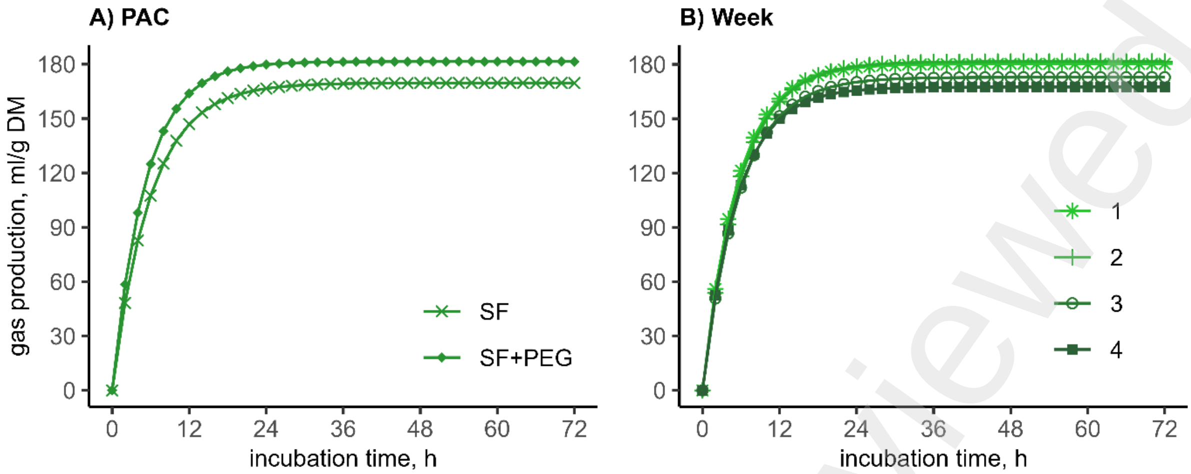
[2] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the finishing concentrate.

Fig. 6. Effect of the presence of active proantocyanidins[1] (PAC; A) and the inclusion of dehydrated sainfoin[2] (SF; B) in the fattening concentrate on the proportions of the proportions of volatile fatty acids in the in vitro assay at 48 h of incubation (Assay 2) .

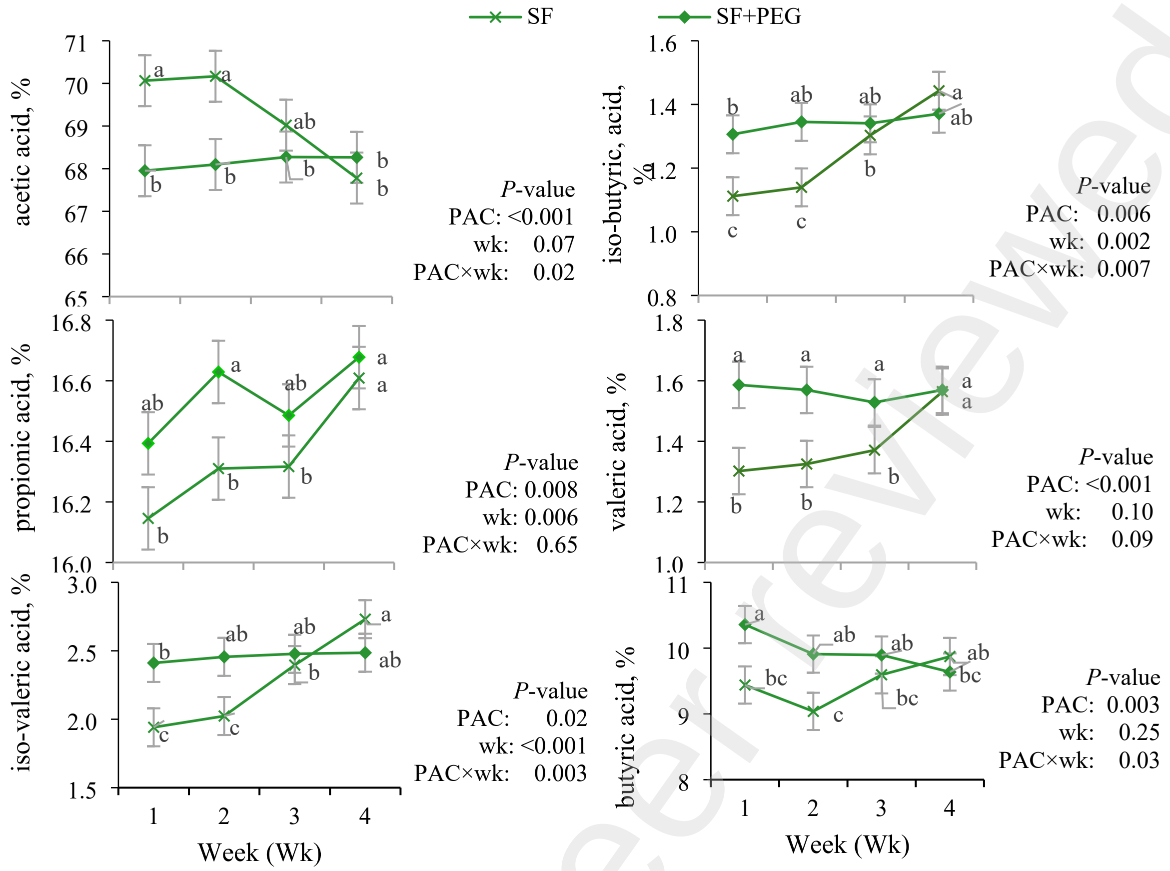
[1] Study of the presence of PAC through the non-addition (SF) or addition of PEG (SF+PEG).

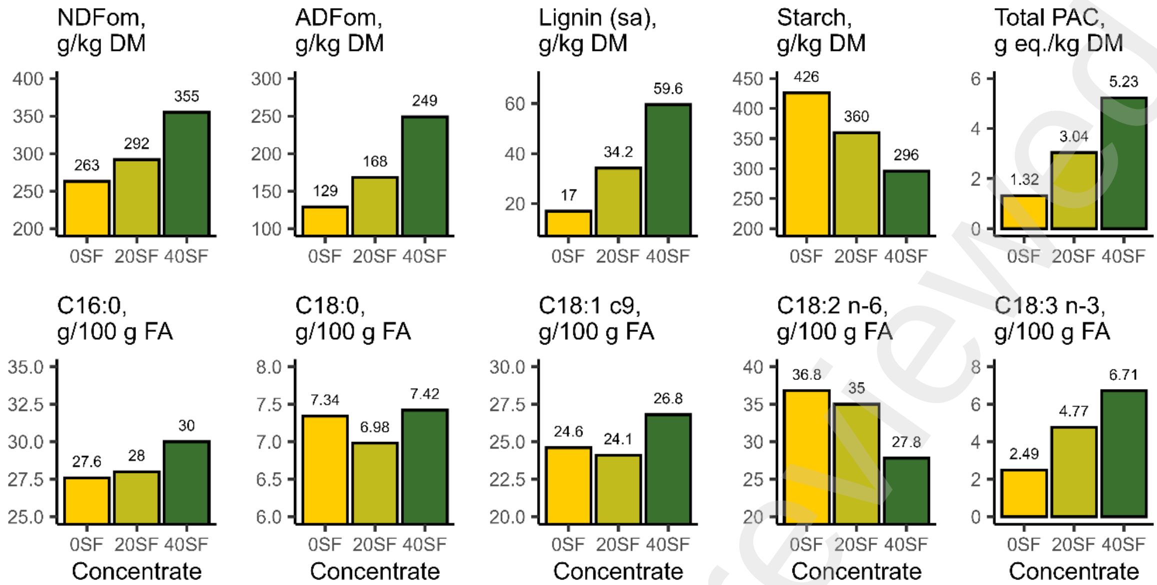
[2] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the finishing concentrate.


NDFom,
g/kg DM
ADFom,
g/kg DM
Lignin (sa),
g/kg DM
CP,
g/kg DM
Total PAC,
g eq./kg DM
299
350
424
418
211
251
302
302
23.2
36.8
55.4
44.1
129
123
105
110
45
40.7
38.5
32.4
week
week
week
week
week

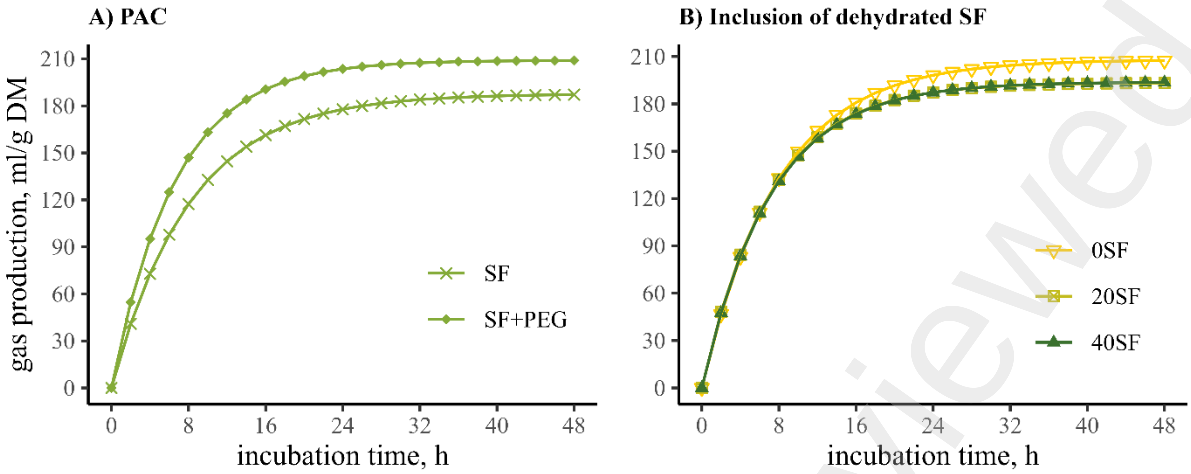


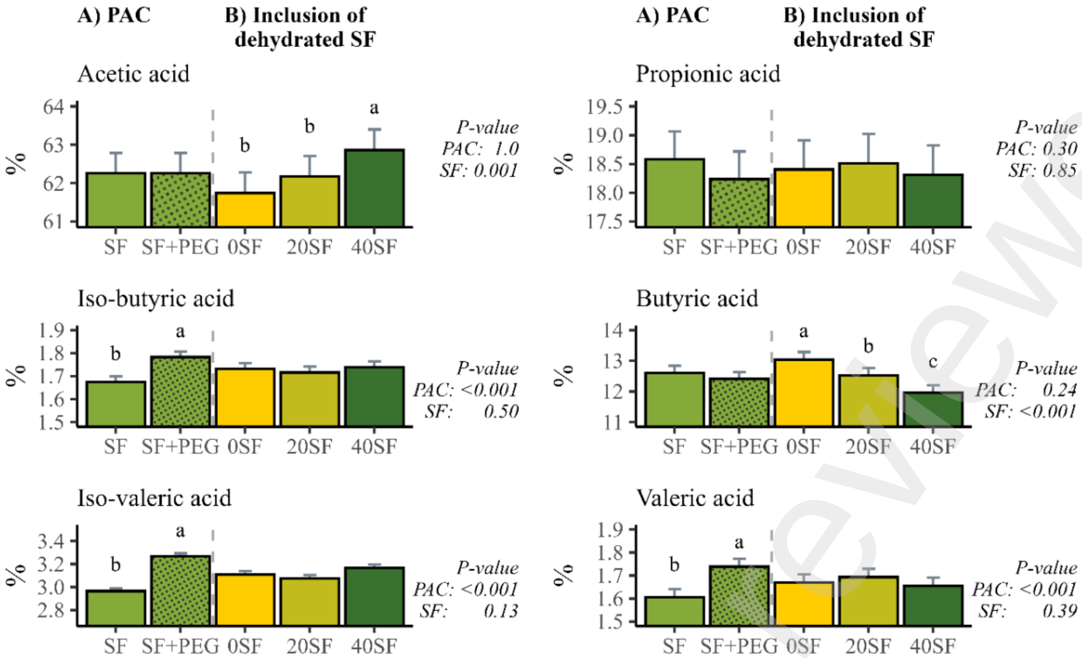
A) PAC
B) Week
gas production, ml/g DM
0
30
60
90
120
150
180
0
12
24
36
48
60
72
incubation time, h
SF
SF+PEG
1
2
3
4


SF
SF+PEG
acetic acid, %
P-value
PAC: <0.001
wk: 0.07
PAC×wk: 0.02
iso-butyric, acid, %
P-value
PAC: 0.006
wk: 0.002
PAC×wk: 0.007
propionic acid, %
P-value
PAC: 0.008
wk: 0.006
PAC×wk: 0.65
valeric acid, %
P-value
PAC: <0.001
wk: 0.10
PAC×wk: 0.09
iso-valeric acid, %
P-value
PAC: 0.02
wk: <0.001
PAC×wk: 0.003
butyric acid, %
P-value
PAC: 0.003
wk: 0.25
PAC×wk: 0.03
Week (Wk)
Week (Wk)



NDFom, g/kg DM
263
292
355
ADFom, g/kg DM
129
168
249
Lignin (sa), g/kg DM
17
34.2
59.6
Starch, g/kg DM
426
360
296
Total PAC, g eq./kg DM
1.32
3.04
5.23
C16:0, g/100 g FA
27.6
28
30
C18:0, g/100 g FA
7.34
6.98
7.42
C18:1 c9, g/100 g FA
24.6
24.1
26.8
C18:2 n-6, g/100 g FA
36.8
35
27.8
C18:3 n-3, g/100 g FA
2.49
4.77
6.71
0SF 20SF 40SF
Concentrate


A) PAC
B) Inclusion of dehydrated SF
gas production, ml/g DM
incubation time, h
incubation time, h
SF
SF+PEG
0SF
20SF
40SF
210
180
150
120
90
60
30
0
0
8
16
24
32
40
48


A) PAC
B) Inclusion of dehydrated SF
Acetic acid
Propionic acid
Iso-butyric acid
Butyric acid
Iso-valeric acid
Valeric acid
%
SF
SF+PEG
0SF
20SF
40SF
P-value
PAC: 1.0
SF: 0.001
PAC: 0.30
SF: 0.85
PAC: <0.001
SF: 0.50
PAC: 0.24
SF: <0.001
PAC: <0.001
SF: 0.13
PAC: <0.001
SF: 0.39