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Effect of sainfoin proanthocyanidins on milk fatty acids from ewes rearing suckling lambs

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ABSTRACT

Proanthocyanidins (**PAC**) can modulate the fatty acid (**FA**) profile of animal products and make them healthier for human consumption, but their effects are highly variable depending on several factors such as PAC chemical structure or dose. The present experiment aimed to evaluate the effect of PAC on the milk FA profile of Rasa Aragonesa ewes fed fresh sainfoin (PAC-containing forage legume) during the rearing period of suckling lambs (4 weeks postlambing). Twenty lactating ewes rearing a single male lamb were fed fresh sainfoin *ad libitum* plus 200 g/d of barley. Half the ewes were orally dosed with 100 mL of water (Sainfoin Group; n = 10) and the other half with polyethylene glycol (50 g PEG4000/100 mL water, Sainfoin + PEG Group; n = 10) to block PAC effects. Sainfoin and milk samples were collected weekly to determine their FA profile by gas chromatography. Fresh sainfoin contents of C18:3n-3 decreased from week 1 to week 2, C16:0 and C18:0 increased from week 1 to week 3, and C18:2n-6 did not change. Regarding milk FA, there were minor effects of PAC on milk-saturated FA. During the whole study, the presence of PAC increased C18:0, C18:2n-6, C18:3n-3 and total polyunsaturated FA (**PUFA**) n-6 and n-3, and decreased C18:1 t11, branched- and odd-chain FA. However, the decrease of milk concentrations of *trans*-monounsaturated FA, C18:1 t10, and total conjugated linoleic acid (**CLA**) and the increase of total PUFA due to the presence of PAC occurred only in week 1, while CLA c9,t11 was lower during weeks 1 and 2. The canonical analyses confirmed the differences between treatments in the FA profile of milk. Overall, the use of fresh sainfoin in the diet of lactating ewes resulted in a beneficial modification of the concentration of several milk FAs, suggesting some changes in ruminal biohydrogenation.

Implications

Nowadays, there is an increasing interest to produce high-quality animal products rich in polyunsaturated fatty acids, especially n-3. In ruminants, unsaturated fatty acids are biohydrogenated by ruminal microorganisms, becoming less healthy for consumers. However, proanthocyanidins, secondary compounds of plants, have been used to inhibit ruminal biohydrogenation, reducing the detrimental fatty acid saturation process. The use of fresh sainfoin, as a forage containing proanthocyanidins, in lactating ewes' diet resulted, among other effects, in higher polyunsaturated fatty acids and lower C18:1 and C18:2 isomers in milk, suggesting an effect of proanthocyanidins on improving the milk fatty acid profile.

Introduction

The production of healthier products and increasing farm sustainability and self-sufficiency are some of the most important current challenges in animal production. To face some of them, many recent studies have focused on assessing the effect of using local forage in animal diets to reduce the dependence on imported protein sources (Moorby and Fraser, 2021). Moreover, forages have a beneficial fatty acid (**FA**) profile with high polyunsaturated FA n-3 (**PUFA**) n-3 content, especially C18:3n-3, which can improve the FA profile of animal products. However, the FA profile of forage is widely variable (Glasser et al., 2013) and has been rarely analysed prior to their use in animal diets. Furthermore, the mechanisms by which they are transformed and transferred to the final product are only partially known and may depend on forage species (Lourenço et al., 2008).

Sainfoin (*Onobrychis viciifolia*) is a good-quality forage legume grown in the Mediterranean Region. It has a medium–low content

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(10–90 g eq. sainfoin PAC/kg DM) of proanthocyanidins (**PAC**; commonly known as condensed tannins). The PAC are naturally occurring phenolic compounds in some plants that can modify some animal productive parameters. In ruminants, sainfoin PAC have been studied to improve FA profile in meat (Lobón et al., 2017) and milk (Pascual et al., 2019) due to changes in ruminal fermentation. The mechanisms of action by which sainfoin PAC produce these changes in ruminal biohydrogenation (**BH**) have been studied in assays *in vitro* (Toral et al., 2016) and *in vivo* (Campidonico et al., 2016). These modifications to ruminal lipid metabolism can be reflected in the FA profile of meat and milk. In a review about the effect of condensed and hydrolysable tannins on the FA profile of meat and milk, Frutos et al. (2020) reported an increase in PUFA n-3 in milk as a general effect of PAC. However, as ruminal biohydrogenation is a very complex process, with several pathways leading to many intermediate FA, the literature about the effects of PAC on lipid BH metabolism is inconclusive.

A highly appreciated product in certain Mediterranean areas is the suckling lamb commercial category (10–12 kg BW and less than 35 d), which is characterised to be fed exclusively on maternal milk. The quality of this milk depends on the diet fed to the dam, which is reflected in the meat of suckling lambs since they are highly correlated (Lobón et al. 2019). Within this framework, this study aims to evaluate the effect of fresh sainfoin fed to dams, as a PAC-containing forage, on the milk FA profile during the rearing process of a suckling lamb. Sainfoin FA profile evolution was also assessed.

Material and methods

Animal management and experimental design

The experiment was conducted in spring 2019 at the CITA facilities (41°3′N, 0°47′W and 216 m above sea level) in Zaragoza, Spain. Twenty multiparous Rasa Aragonesa (autochthonous breed) ewes and their single suckling male lambs 2–3 d after lambing were allocated in individual indoor pens and randomly assigned to one of two treatments, according to ewe BW (61 ± 6.2 kg), body condition score (3.3 ± 0.57), lambing date (April 6 ± 0.1d) and lamb BW at birth (4.1 ± 0.64 kg). Treatments and management specifications are explained in detail in Baila et al. (2022a). Fresh sainfoin (*Onobrychis viciifolia* cv Reznos) from vegetative to flowering stage onset throughout the experiment was offered *ad libitum* + 200 g of barley divided into two meals (0900 and 1600 h). Before each feed supply, 10 ewes (Sainfoin Group) were orally dosed with 100 mL of water, and the other 10 ewes (Sainfoin + PEG Group) were orally dosed with a solution of polyethylene glycol (**PEG**; 50 g of PEG 4000/100 mL of water), a binding agent that deactivates the effects of PAC. Ewes had fresh water and mineral blocks *ad libitum*. The experimental period started 2–3 postlambing days and lasted 28 days (divided into four lactation weeks), which corresponded to the time required for suckling lambs to reach the target slaughter weight (10–12 kg BW). During this process, the dam-lamb pair was allocated in the same pen and lambs had free access to suckling.

Measurements and sampling procedures

Composite samples per ewe and week were obtained from the daily offered fresh sainfoin and barley. Samples were freeze-dried (Genesis Freeze Dryer 25, Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, Spain), ground and sieved through a 0.2 mm screen (Rotary Mill, ZM200 Retsch, Haan, Germany) and stored in the dark until the FA content of forage was analysed. Weekly, before the morning meal distribution, BW was registered with an electronic balance (0.5 kg precision), and the body condition score was estimated by two trained technicians using a transformed scale from 0 to 5 with 0.25 intervals. Besides, to estimate milk production following the Doney et al. (1979) methodology, ewes were injected in the jugular vein with 5 IU oxytocin (Facilpart 10 UI/mL intravenous, SYVA, León, Spain) at 0800 and 1200 h and during this period, the lambs were separate from their dams. The individual milk samples were stored at −20 °C and freeze-dried to determine the FA profile.

Chemical analyses

Feedstuffs and milk FA were determined by gas chromatography with a flame ionisation detector. All the feedstuffs' analyses were run in duplicate. FA content was analysed following the methods described by Sukhija and Palmquist (1988) and Lee et al. (2012) after an optimisation process. Briefly, 0.5 g of feedstuff and 1 mL of the solution of internal standard C19:0 (methyl nonadecanoate N-19-M from Nu-Chek Prep, INC, Elysian, Minnesota, USA) in heptane were mixed. Afterwards, 4 mL of 0.5 M CH_3ONa/CH_3OH solution was added. The mixture was shaken in a vortex shaker (Heidolph reax top) and in a thermostatic bath with shaking (Wisd maxturdy 30) for 20 min at 70 °C before being left to cool. Four mL of the solution of acetyl chloride/CH_3OH (1:10, v:v) was added, and all the solution was shaken using the vortex for 30 s and put in the thermostatic bath for 1 h and 40 min at 70 °C. The sample was shaken in the vortex every 20 min. After being cooled, 2 mL of milli-Q water and 2 mL of heptane were added and shaken with the vortex and in the tube shaker (Heidolph multi reax) for 10 min at maximum speed and were centrifuged for 5 min at 3 500 rpm and 10 °C. The upper part (heptane) was collected and added to a 5 mL tube with anhydrous Na_2SO_4 and active carbon. The tube was shaken in the 5 mL Eppendorf shaker (Labbox vortex) for 10 min at ambient temperature and centrifuged at 3 500 rpm and 10 °C for 5 min. One mL of the supernatant was taken and poured into a suitable 2 mL vial for gas chromatography.

The method followed to analyse the FA content of milk was based on Kramer et al. (1997). Between 0.4 and 0.5 g of lyophilised milk and 1 mL of the solution of the internal standard C23:0 (methyl tricosanoate N-23-M from Nu-Chek Prep, INC, Elysian, Minnesota, USA) in heptane were mixed in a 15 mL polypropylene centrifuge tube. Then, 2 mL of heptane and 4 mL of the 0.5 M CH_3ONa/CH_3OH solution were added. The tube was shaken vigorously for 30 s in the vortex shaker and in a tube shaker for 45 min at 50 °C. After cooling, 4 mL of milli-Q water was added and vortex-shaken. The sample was centrifuged at 1000 rpm and 10 °C for 5 min to take the superior phase (heptane) and to add it to a 5 mL round-bottom tube with anhydrous Na_2SO_4. Afterwards, the tube was shaken for 30 s and centrifuged for 5 min at 1000 rpm and 10 °C. Approximately, 1 mL of the supernatant was recovered in a 2 mL vial for gas chromatography.

The technical specifications used for the FA determination of feedstuffs and milk are described in detail in Supplementary Tables S1 and S2, respectively. Briefly, a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, Massachusetts, USA) was used, equipped with a CP-8400 Autosampler (Bruker, Billerica, Massachusetts, USA), an SP-2560 column (100 m × 0.25 mm ID × 0.2 for feedstuffs samples and 200 m × 0.25 mm ID × 0.20 for milk samples, Saint Louis, Missouri, USA) and the Compass CDS software. FA identification was performed with the help of different certified reference materials GLC-(401, 463, 532, 538, 642, 643), C18:1 c11, C18:1 t11 from Nu-Chek Prep (INC, Elysian, Minnesota, USA) and relative retention times found in several sources (Kramer et al., 1997; Alves and Bessa, 2009; Bravo-Lamas et al., 2016). All the data related to the analytical parameters of feedstuffs and milk FA determination can be found in Supplementary Tables S3 and S4,

respectively. The quantification of each individual FA was performed following the Standard UNE-EN ISO 12966-4:2015 and expressed as g of FA per 100 g of total FA (equations described in Supplementary Material S1), while total FA content was expressed as mg of FA per g of sample (equations described in Supplementary Material S2) using C19:0 as the internal standard for feedstuff and C23:0 for the milk samples. After performing FA identification and quantification, they were grouped into major sums and the corresponding ratios were calculated. The total amount of branched-chain FA (**BCFA**) in milk was calculated as the sum of *iso*-BCFA (represented by iC13:0, iC14:0, iC15:0, iC16:0, iC17:0 and iC18:0) and *anteiso*-BCFA (represented by aC13:0, aC15:0 and aC17:0), as described in detail in Vlaeminck et al. (2006). The n-6:n-3 ratio was calculated as (C18:2n-6 + C20:4n-6)/(C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3) and the hypocholesterolaemic/hypercholesterolaemic ratio as [(C18:1 c9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3)/(C14:0 + C16:0)], according to Santos-Silva et al. (2002). The Atherogenic Index was calculated as [(C12:0 + (4 × C14:0) + C16:0)]/[ΣMUFA + PUFA n-6 + PUFA n-3] and the Thrombogenic Index as (C14:0 + C16:0 + C18:0)/[0.5 × Σ MUFA + 0.5 × PUFA n-6 + PUFA n-3) + (PUFA n-3/PUFA n-6] following Ulbricht and Southgate (1991). The enzymatic activity levels of Δ^9-desaturase and elongase were determined using the mathematical model described by Malau-Aduli et al. (1997) according to the following equations: Δ^9-desaturase C16 = C16:1 c9/(16:0 + C16:1 c9); Δ^9-desaturase C18 = 18:1 c9/(18:0 + 18:1 c9); elongase activity = [(C18: 0 + C18:1 c9)/(C16:0 + C16:1 c9 + C18: 0 + C18:1 c9)] × 100.

Statistical analyses

Data were analysed with the SAS statistical software (v.9.3; SAS Inst. Inc., Cary, NC, USA). The FA profile evolution of feedstuffs was analysed by a general linear model with lactation week as the fixed effect. The FA profile evolution of milk was analysed by an analysis of variance with a mixed model (*proc mixed*) in the presence of PAC (Sainfoin or Sainfoin + PEG), lactation week (1–4) and their interaction as the fixed effects and ewe as the random effect. Degrees of freedom were adjusted with the Kenward-Roger correction. The least-square means and their associated SE were obtained, and Tukey's correction was used for pairwise comparisons. The effects were considered significant at $P < 0.05$.

A combination of three analysis types was used to discriminate between both treatments for their 115 individual FA detected in milk. These analyses were stepwise discriminant analysis (*proc stepwise*), discriminant analysis (*proc discrim*), and canonical discriminant analysis (*proc candisc*). The canonical discriminant analysis is a dimension-reduction technique that is related to the principal component analysis and canonical correlations. All the linear combinations of the original interval variables are those in which canonical discriminant analysis derives are called canonical functions (**CAN**) and summarise between-group variation. The equation followed by this combination technique and its details are described in Conte et al. (2018). The effective separation between treatments was assessed by the corresponding Hotelling's T-square test.

Results

Evolution of BW, body condition score as well as the DM intake and milk yield had been presented previously (Baila et al., 2022a). Briefly, BW, body condition score, DM intake and milk yield were not affected by the presence of PAC, with average values of 57.6 ± 2.12 kg BW, 3.07 ± 0.175 of body condition score, 1.88 ± 0.328 kg DM intake/d and 1.16 ± 0.206 kg milk/d. In contrast, the week of lactation had a significant effect on all these parameters ($P < 0.05$). The BW and body condition score decreased sharply in the first-week postlambing ($P < 0.05$). The DM intake increased as the week of lactation advanced, from 1.48 kg/ewe/day in week 1 to 2.28 kg/ewe/day in week 4. The milk production increases through the weeks of study, peaking at week 2, which was significantly greater. The productive results of the suckling lambs are presented in Baila et al. (2022b), showing no differences in lambs' growth and carcass yield due to the presence of PAC in the dams' diet.

Sainfoin fatty acids

The most relevant FAs of the sainfoin offered for the four lactation weeks are shown in Table 1. The main FAs were C18:3n-3, C16:0 and C18:2n-6. Week affected the total FA content, four individual FA ($P < 0.05$; Table 1), total saturated fatty acids (**SFAs**), PUFA and the PUFA:SFA ratio ($P < 0.05$; Fig. 1). Total FA content decreased throughout the time advanced until a minimum was reached at weeks 3 and 4 ($P < 0.001$). Conversely, C16:0 and SFA contents were lower at week 1 than at week 3 ($P < 0.05$), and C18:0 was lower at week 1 compared to weeks 2 and 3. For C16:1 and PUFA ($P < 0.05$), contents were higher at week 1 than at weeks 3 and 4, while the highest C18:3n-3 and PUFA:SFA ratio value appeared at week 1 ($P < 0.01$).

Milk fatty acids

In all, 115 individual FAs were identified, but only the most representative are shown, of which the most abundant were C16:0, C18:0 and C18:1 c9. Table 2 shows the total FA and SFA contents of milk. The total FA content in milk was not affected by the presence of PAC or by week ($P > 0.05$; Table 2). The total SFA, C12:0, C13:0 and iC13:0 contents were affected by the interaction between PAC and lactation week ($P < 0.05$; Table 2), but when comparing within the same week, no differences were observed due to the presence of PAC. Milk from Sainfoin group decreased its SFA content from week 1 to week 2 ($P < 0.01$) and thereafter remained steady ($P > 0.05$), whereas milk from Sainfoin + PEG did not show any changes ($P > 0.05$). The presence of PAC decreased the contents of C16:0, C9:0, C15:0, iC15:0, iC17:0, aC15:0, aC17:0 and the total odd-chain FA (**OCFA**), BCFA, and *anteiso*-BCFA ($P < 0.05$), but increased C18:0 and iC18:0 ($P < 0.05$). Lactation week affected most of the SFA, OCFA and BCFA. The SFA from C6:0 to C10:0 presented a similar weekly pattern, with higher concentrations at weeks 1 and 4 ($P < 0.05$). The C16:0 obtained higher values at week 1 compared to week 3 ($P < 0.05$), and C18:0 at week 3 was higher than the rest of the weeks ($P < 0.05$). All the OCFA, BCFA, *iso*-BCFA and *anteiso*-BCFA sums increased from week 1 to week 2, remaining steady thereafter.

The effects of PAC and week on the milk monounsaturated fatty acid (**MUFA**) contents are presented in Table 3. The concentrations of total MUFA presented a higher value at week 1 compared to week 2 in Sainfoin milk ($P < 0.01$), while constant values were obtained in Sainfoin + PEG ($P > 0.05$). In contrast, no changes in total *trans*-MUFA were found in Sainfoin ewes, while Sainfoin + PEG group decreased its concentration from week 1 to week 2 ($P < 0.001$). The presence of PAC lowered the contents of C18:1 c12, C18:1 t10 and total *trans*-MUFA only in week 1 ($P < 0.05$), while C12:1 c9 content was lower in week 4 ($P < 0.05$). The presence of PAC generally reduced C15:1 c9, C16:1 c9 and C18:1 t11 ($P < 0.05$). For the effect of week, *cis*-MUFA (represented mainly by C18:1 c9) had a higher value at week 3 than weeks 1 and 4 ($P < 0.01$), whereas C18:1 t11 obtained a higher value at week 1 than at week 2 ($P < 0.01$).

Table 1
Evolution of the fatty acid (FA) composition of the fresh sainfoin offered to ewes.

	Fresh sainfoin						Barley	
	Week							
Fatty acids (FAs)	1	2	3	4	SE	*P*-value	Mean	SE
Total FA (mg/g DM)	37.4[a]	31.9[b]	28.8[c]	29.3[c]	0.49	<0.001	48.6	0.66
FA (g/100 g total FA)								
C12:0	0.42	0.49	0.47	0.56	0.037	0.15	0.03	0.002
C14:0	0.84	0.69	0.70	0.70	0.035	0.05	0.40	0.004
C15:0	0.16	0.23	0.20	0.17	0.023	0.19	0.07	0.002
C16:0	26.4[b]	28.2[ab]	28.9[a]	28.0[ab]	0.40	0.01	26.1	0.12
C16:1 c9	0.32[a]	0.16[ab]	0.10[b]	0.07[b]	0.034	0.004	0.08	0.004
C18:0	5.86[b]	7.61[ab]	8.51[a]	8.92[a]	0.566	0.02	7.0	0.11
C18:1 c9	0.95	1.36	1.39	1.40	0.117	0.07	12.4	0.16
C18:1 c11	0.60	0.48	0.41	0.36	0.099	0.40	0.55	0.012
C18:2n-6	14.3	14.0	14.4	13.7	0.42	0.60	49.1	0.18
C18:3n-3	50.2[a]	46.9[b]	44.8[b]	46.1[b]	0.69	0.003	4.31	0.169

In a feedstuff and parameter, the means with a different superscript differ at $P < 0.05$.

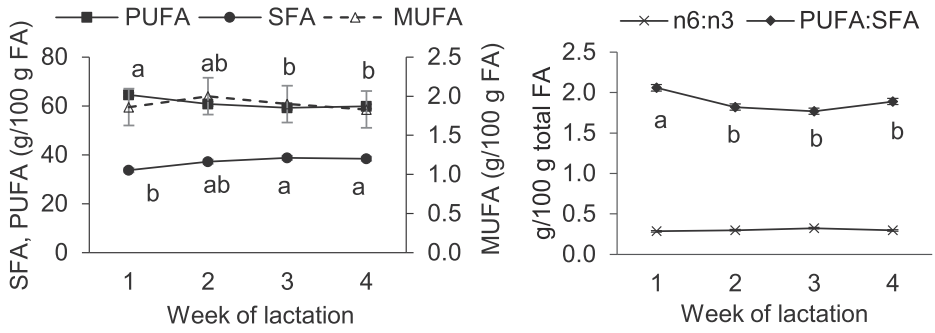

Fig. 1. Evolution of total SFA, MUFA, PUFA, and PUFA n-6:n-3 and PUFA:SFA ratios of sainfoin offered to ewes during the experimental period. Abbreviations: FAs = fatty acids; MUFAs = total monounsaturated fatty acids; PUFAs = total polyunsaturated fatty acids; SFAs = total saturated fatty acids; n-6:n-3 = total polyunsaturated fatty acids n-6: total polyunsaturated fatty acids n-3 ratio; PUFA:SFA = total polyunsaturated fatty acid: total saturated fatty acid ratio. [a,b]Values in a parameter with different superscripts differ significantly among weeks at $P < 0.05$. Vertical bars indicate the SE.

The main effects of sainfoin PAC and lactation week on milk PUFA are shown in Table 4. The interaction between PAC and lactation week affected total PUFA, total conjugated linoleic acid (**CLA**), CLA c9,t11, and CLA t9,c11 ($P < 0.05$; Table 4). As a result, the presence of PAC increased total PUFA at week 2 (6.50 vs 5.65 for Sainfoin vs Sainfoin + PEG; $P < 0.01$) and decreased CLA c9,t11 at weeks 1 (0.49 vs 0.73 for Sainfoin vs Sainfoin + PEG; $P < 0.001$) and 2 (0.47 vs 0.61 for Sainfoin vs Sainfoin + PEG; $P < 0.05$), whereas CLA t9,c11 (0.033 vs 0.071 for Sainfoin vs Sainfoin + PEG; $P < 0.01$) and total CLA (0.68 vs 1.04 for Sainfoin vs Sainfoin + PEG; $P < 0.001$) only decreased at week 1.

Regarding the rest of PUFA, the presence of PAC produced an overall increase in total PUFA n-6, C18:2n-6, total PUFA n-3, C18:3n-3 and C20:5n-3 ($P < 0.05$) and a reduction in CLA c7,c9 ($P < 0.05$). Concerning the lactation week effect, both PUFA n-6 (represented mainly by C18:2n-6 and C20:4n-6) and PUFA n-3 (represented mostly by C18:3n-3) decreased throughout lactation ($P < 0.05$), although C18:3n-3 increased again at week 4 ($P < 0.05$).

The main milk FA ratios and enzymes are shown in Table 5. The n-6:n-3 and PUFA:SFA ratios and Δ^9-desaturase C18 were affected by the interaction between the presence of PAC and week of lactation ($P < 0.05$). The presence of PAC lowered the n-6:n-3 ratio (1.15 vs 1.57 for Sainfoin vs Sainfoin + PEG; $P < 0.001$) and Δ^9-desaturase C18 (58.1 vs 64.7 for Sainfoin vs Sainfoin + PEG; $P < 0.01$) at week 1, but increased the PUFA:SFA ratio at week 2 (0.101 vs 0.085 for Sainfoin vs Sainfoin + PEG; $P < 0.05$), with no other differences between treatments during the study ($P > 0.05$). Lactation week affected all the FA ratios ($P < 0.05$), except for the thrombogenic index ($P > 0.05$).

Multivariate analysis of milk fatty acids

The stepwise discriminant analysis process selected 46 FA of the 115 identified as being the most discriminant, to which the canonical discriminant analysis was applied. In all cases, the CAN was able to discriminate between the two treatments (*P*-value Hotelling's *t*-test < 0.001) with the selection of several FAs, as shown in Fig. 2. The FA forming the CAN varied according to week. It was possible to separate both treatments with C18:3n-3 content at week 1; with C18:1 c12 and C18:2n-6 at week 2; with iC17:0, C18:1 c9, C18:3 c9,t11,t15 and C20:5n-3 at week 3; with C14:0 8-Me, C20:5n-3 and C22:5n-6 at week 4.

Discussion

The results observed can be attributed to the presence of PAC, as the DM intake, performance and milk yield were similar in both treatments (Baila et al., 2022a).

Sainfoin fatty acids

In accordance with previous analyses of the FA content of different forages (Glasser et al., 2013), particularly of sainfoin (Rufino-Moya et al., 2022), the main FAs were C18:3n-3, C16:0 and C18:2n-6. Changes in plant maturity may have affected the sainfoin FA evolution during the trial. The maturation process decreases the proportion of leaves rich in C18:3n-3 (Glasser et al., 2013) with the most abundant FA in sainfoin, which explains the drop in the total FA content herein observed with advancing

Table 2
Effects of the presence of proanthocyanidins (PAC) and lactation week (Week) on total, saturated, odd- and branched-chain fatty acids of ewes' milk.

	PAC		Week					*P*-values		
Fatty acids	Sainfoin	Sainfoin + PEG	1	2	3	4	RSD	PAC	Week	PAC × Week
Total FA, (mg/g DM)	628	662	622	646	667	645	58.4	0.19	0.34	0.87
FA (g/100 g FA)										
SFA	65.7	65.9	67.2	65.3	63.4	67.2	2.35	0.85	<0.001	0.02
C4:0	2.12	1.99	2.04^{ab}	1.97^{b}	1.91^{b}	2.27^{a}	0.231	0.14	0.02	0.16
C6:0	2.07	2.05	2.14^{a}	1.88^{b}	1.98^{b}	2.25^{a}	0.150	0.81	<0.001	0.05
C8:0	2.17	2.17	2.33^{a}	2.00^{b}	2.05^{b}	2.30^{a}	0.195	0.99	<0.001	0.05
C10:0	5.81	6.02	6.76^{a}	5.62^{b}	5.07^{b}	6.22^{a}	0.642	0.63	<0.001	0.05
C12:0	3.34	3.48	4.18	3.32	2.74	3.40	0.377	0.62	<0.001	0.04
C14:0	7.99	8.40	8.67	8.07	7.19	8.86	1.064	0.38	0.06	0.16
C16:0	22.4^{y}	23.5^{x}	23.6^{a}	23.0^{ab}	22.2^{b}	23.0^{ab}	0.83	0.01	0.002	0.27
C18:0	13.5^{x}	11.7^{y}	11.4^{c}	12.8^{b}	13.8^{a}	12.5^{b}	0.83	0.004	<0.001	0.05
OCFA	2.95^{y}	3.35^{x}	3.00^{b}	3.22^{a}	3.20^{a}	3.18^{ab}	0.202	<0.001	<0.001	0.07
C5:0	0.27	0.31	0.25	0.27	0.32	0.33	0.075	0.12	0.05	0.56
C7:0	0.12	0.14	0.14^{a}	0.12^{b}	0.13^{ab}	0.13^{ab}	0.024	0.19	0.02	0.56
C9:0	0.067^{y}	0.094^{x}	0.10^{a}	0.063^{b}	0.068^{b}	0.087^{ab}	0.0197	0.047	<0.001	0.06
C11:0	0.12	0.14	0.15^{a}	0.12^{b}	0.11^{b}	0.15^{a}	0.019	0.23	<0.001	0.27
C13:0	0.069	0.088	0.098	0.073	0.062	0.079	0.0143	0.09	<0.001	0.03
C15:0	0.97^{y}	1.20^{x}	1.08	1.09	1.02	1.16	0.107	0.01	0.06	0.22
C17:0	1.36	1.37	1.18^{b}	1.50^{a}	1.51^{a}	1.26^{b}	0.094	0.74	<0.001	0.17
BCFA	2.09^{y}	2.37^{x}	2.02^{b}	2.30^{a}	2.24^{a}	2.35^{a}	0.161	0.004	<0.001	0.83
iso-BCFA	1.21	1.31	1.14^{b}	1.32^{a}	1.27^{a}	1.30^{a}	0.092	0.06	<0.001	0.79
iC13:0	0.022	0.024	0.023	0.020	0.023	0.025	0.0055	0.37	0.14	0.004
iC14:0	0.14	0.13	0.11^{b}	0.15^{a}	0.14^{a}	0.16^{a}	0.024	0.49	<0.001	0.56
iC15:0	0.20^{y}	0.24^{x}	0.16^{c}	0.23^{ab}	0.22^{b}	0.26^{a}	0.028	0.009	<0.001	0.35
iC16:0	0.31	0.34	0.32	0.34	0.31	0.34	0.036	0.18	0.09	0.98
iC17:0	0.51^{y}	0.57^{x}	0.49^{b}	0.56^{a}	0.58^{a}	0.53^{ab}	0.038	<0.001	<0.001	0.97
iC18:0	0.025^{x}	0.019^{x}	0.024	0.023	0.022	0.020	0.0035	0.003	0.10	0.78
anteiso-BCFA	0.88^{y}	1.03^{x}	0.88^{b}	0.98^{a}	0.96^{a}	1.00^{a}	0.092	<0.001	0.002	0.15
aC13:0	0.025	0.028	0.020^{c}	0.023^{bc}	0.026^{b}	0.036^{a}	0.0061	0.24	<0.001	0.36
aC15:0	0.38^{y}	0.48^{x}	0.39^{b}	0.43^{ab}	0.41^{b}	0.50^{a}	0.063	0.012	0.008	0.58
aC17:0	0.47^{y}	0.54^{x}	0.47^{b}	0.53^{a}	0.53^{a}	0.49^{ab}	0.035	0.002	<0.001	0.14

Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG).
Abbreviations: PAC = proanthocyanidins; FAs = fatty acids; SFA = sum of individual saturated fatty acids from C4:0 to C18:0; OCFA = sum of individual odd-chain fatty acids; BCFA = sum of *iso*- and *anteiso*- branched-chain fatty acids; *iso*-BCFA = sum of individual *iso*-branched-chain fatty acids; *anteiso*-BCFA = sum of individual *anteiso*-branched-chain fatty acids.
[x,y] Values within a row with different superscripts differ significantly between PAC treatments at $P < 0.05$.
[a–c] Values within a row with different superscripts differ significantly among weeks at $P < 0.05$.

Table 3
Effects of the presence of proanthocyanidins (PAC) and lactation week (Week) on monounsaturated fatty acids of ewes' milk.

	PAC		Week					*P*-values		
FA (g/100 g FA)	Sainfoin	Sainfoin + PEG	1	2	3	4	RSD	PAC	Week	PAC × Week
MUFA	27.8	28.2	25.9	28.6	30.7	26.8	2.21	0.77	<0.001	0.04
cis-MUFA	24.8	24.7	22.3^{c}	25.5^{ab}	27.5^{a}	23.6^{bc}	2.21	0.93	<0.001	0.08
C12:1 c9	0.029	0.037	0.034	0.030	0.029	0.039	0.0060	0.03	0.005	0.02
C14:1 c9	0.073	0.092	0.097^{a}	0.076^{b}	0.070^{b}	0.089^{ab}	0.0224	0.09	0.009	0.20
C15:1 c9	0.005^{y}	0.010^{x}	0.006	0.008	0.008	0.009	0.0036	0.008	0.08	0.39
C16:1 c9	0.60^{y}	0.68^{x}	0.58^{b}	0.65^{ab}	0.68^{a}	0.66^{ab}	0.075	0.009	0.006	0.07
C17:1 c9	0.40	0.43	0.34^{c}	0.44^{ab}	0.49^{a}	0.39^{bc}	0.055	0.27	<0.001	0.31
C18:1 c9	21.1	20.6	18.2^{c}	21.7^{ab}	23.7^{a}	19.9^{bc}	2.04	0.70	<0.001	0.15
C18:1 c11	0.68	0.69	0.59^{b}	0.75^{a}	0.77^{a}	0.63^{b}	0.068	0.71	<0.001	0.12
C18:1 c12	0.21	0.24	0.23	0.24	0.23	0.19	0.027	0.006	0.01	0.01
trans-MUFA	3.07	3.53	3.66	3.13	3.20	3.22	0.271	0.009	<0.001	0.01
C18:1 t10	0.24	0.30	0.33	0.26	0.25	0.25	0.044	0.01	<0.001	0.008
C18:1 t11	1.49^{y}	1.78^{x}	1.77^{a}	1.52^{b}	1.60^{ab}	1.64^{ab}	0.197	0.04	0.005	0.15

Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG).
Abbreviations: PAC = proanthocyanidins; FAs = fatty acids; MUFA = sum of *cis*- and *trans*- monounsaturated fatty acids; *cis*-MUFA = sum of individual *cis*-monounsaturated fatty acids; *trans*-MUFA = sum of individual *trans*-monounsaturated fatty acids.
[x,y] Values within a row with different superscripts differ significantly between PAC treatments at $P < 0.05$.
[a–c] Values within a row with different superscripts differ significantly among weeks at $P < 0.05$.

plant maturity. In addition, the decrease in PUFA and the increase in C18:0 and SFA concentrations with forage maturity agree with the results reported by Glasser et al. (2013).

Milk fatty acids

Because both treatments had a sainfoin-based diet and there were no differences in either forage or concentrate DM intake (Baila et al., 2022a), it can be stated that FA intake was similar between treatments and, therefore, the effects observed in milk FA were directly linked with the presence of sainfoin PAC.

The milk FA profile is consistent with a diet composed mainly of forage (≈90% fresh sainfoin), where PUFA and CLA concentrations are high, and SFA concentrations and the n-6:n-3 ratio are low. Besides, C18:1 t11 isomer (vaccenic acid) content predominates over the C18:1 t10 isomer, which is also a characteristic of forage

Table 4
Effects of the presence of proanthocyanidins (PAC) and lactation week (Week) on polyunsaturated fatty acids of ewes' milk.

	PAC		Week					*P*-values		
FA (g/100 g total FA)	Sainfoin	Sainfoin + PEG	1	2	3	4	RSD	PAC	Week	PAC × Week
PUFA	6.50	5.93	6.81	6.07	5.98	5.99	0.340	0.005	<0.001	0.04
CLA	0.70	0.89	0.85	0.75	0.77	0.81	0.105	<0.001	0.03	0.04
CLA c9,t11	0.50	0.64	0.61	0.54	0.55	0.55	0.069	<0.001	0.02	0.02
CLA c7,c9	0.010^{y}	0.016^{x}	0.012	0.012	0.014	0.015	0.0041	0.006	0.29	0.26
CLA t9,c11	0.040	0.055	0.052	0.040	0.044	0.053	0.0155	0.06	0.08	0.01
PUFA n-6	2.33^{x}	1.99^{y}	2.47^{a}	2.24^{b}	2.07^{c}	1.86^{d}	0.130	<0.001	<0.001	0.11
C18:2n-6	2.06^{x}	1.72^{y}	2.14^{a}	1.96^{b}	1.83^{c}	1.64^{d}	0.124	<0.001	<0.001	0.11
C18:3n-6	0.046	0.044	0.046^{ab}	0.050^{a}	0.042^{b}	0.043^{ab}	0.0069	0.69	0.04	0.34
C20:2n-6	0.013	0.015	0.016	0.014	0.015	0.011	0.0049	0.45	0.13	0.06
C20:3n-6	0.029	0.034	0.038	0.031	0.027	0.031	0.0109	0.25	0.07	0.95
C20:4n-6	0.17	0.16	0.22^{a}	0.17^{b}	0.14^{c}	0.12^{d}	0.011	0.62	<0.001	0.14
C22:4n-6	0.008	0.009	0.008	0.009	0.009	0.007	0.0050	0.38	0.86	0.23
C22:5n-6	0.004	0.005	0.006	0.004	0.004	0.006	0.0050	0.49	0.53	0.39
PUFA n-3	2.11^{x}	1.52^{y}	1.90^{a}	1.70^{c}	1.73^{bc}	1.92^{ab}	0.148	<0.001	<0.001	0.11
C18:3n-3	1.74^{x}	1.21^{y}	1.51^{ab}	1.36^{c}	1.41^{bc}	1.62^{a}	0.138	<0.001	<0.001	0.16
C20:3n-3	0.020	0.019	0.021	0.019	0.019	0.021	0.0050	0.49	0.42	0.70
C20:5n-3	0.11^{x}	0.086^{y}	0.11	0.098	0.094	0.089	0.0173	0.02	0.15	0.51
C22:5n-3	0.17	0.14	0.18^{a}	0.16^{b}	0.14^{bc}	0.13^{c}	0.018	0.05	<0.001	0.89
C22:6n-3	0.075	0.062	0.090^{a}	0.073^{b}	0.060^{c}	0.051^{c}	0.0103	0.20	<0.001	0.24

Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG).
Abbreviations: PAC = proanthocyanidins; FAs = fatty acids; PUFA = sum of conjugated linoleic fatty acids and polyunsaturated fatty acids n-6 and n-3; CLA = sum of individual conjugated linoleic acid; PUFA n-6 = sum of individual polyunsaturated n-6 fatty acids; PUFA n-3 = sum of individual polyunsaturated n-3 fatty acids.
^{x,y} Values within a row with different superscripts differ significantly between PAC treatments at $P < 0.05$.
$^{a–d}$ Values within a row with different superscripts differ significantly among weeks at $P < 0.05$.

Table 5
Effects of the presence of proanthocyanidins (PAC) and lactation week (Week) on the ewes' milk fatty acid ratios and enzymes.

	PAC		Week					*P*-values		
Items	Sainfoin	Sainfoin + PEG	1	2	3	4	RSD	PAC	Week	PAC × Week
PUFA n-6:n-3 ratio	1.11	1.33	1.36	1.35	1.22	0.97	0.139	0.002	<0.001	0.02
PUFA:SFA ratio	0.10	0.09	0.10	0.093	0.096	0.09	0.007	0.03	0.002	<0.01
C18:1 t10:t11 ratio	0.16	0.17	0.19^{a}	0.17^{ab}	0.16^{b}	0.16^{b}	0.027	0.47	0.005	0.30
Atherogenic index	1.82	1.98	2.10^{a}	1.84^{ab}	1.57^{b}	2.09^{a}	0.377	0.26	0.003	0.10
Thrombogenic index	1.99	2.15	2.13	2.09	1.92	2.13	0.212	0.10	0.06	0.21
h:H ratio	0.84	0.77	0.70^{c}	0.83^{ab}	0.94^{a}	0.76^{bc}	0.106	0.21	<0.001	0.17
Δ^{9}-desaturase C16 (%)	2.62	2.83	2.40^{b}	2.74^{a}	2.97^{a}	2.80^{a}	0.300	0.12	<0.001	0.10
Δ^{9}-desaturase C18 (%)	60.6	63.5	61.4	62.8	63.2	61.0	2.69	0.03	0.15	0.02
Elongase (%)	60.2	57.2	55.1^{c}	59.1^{b}	62.3^{a}	58.3^{b}	2.45	0.06	<0.001	0.30

Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG).
Abbreviations: PAC = proanthocyanidins; PUFAs = total polyunsaturated fatty acids; SFAs = total saturated fatty acids; h:H = hypocholesterolemic to hypercholesterolemic ratio.
$^{a–c}$ Values within a row with different superscripts differ significantly among weeks at $P < 0.05$.

diets (Griinari and Bauman, 1999). The former isomer is one of the most desirable from a human health point of view, because it is the precursor of CLA c9,t11 (rumenic acid), the main bioactive CLA whose origin lies only in ruminant-derived products (Palmquist, 2006).

Most of the even-chain SFA of 6–14 carbon atoms, and approximately half of the 4:0 and 16:0 found in milk, come from the *de novo* synthesis in mammary gland (Chilliard et al., 2000). Except for C16:0, no effect on this milk FA group was observed. Thus, it can be asserted that there was a minimum effect of PAC on FA mammary synthesis. An inhibitory effect of PAC on the growth and activity of ruminal microorganisms has also been reported (Min et al., 2003; Min et al., 2005). Accordingly, a reduction in OCFA and BCFA contents in milk was observed in the Sainfoin treatment in the present study because these FAs are synthesised by rumen cellulolytic bacteria (Vlaeminck et al., 2006). Similar results have been observed by Cabiddu et al. (2009) in ewes fed sulla (*Hedysarum coronarium*, ≤3% PAC), which suggest that PAC concentrations as low as those herein used may suffice to alter specific rumen bacterial populations.

The presence of PAC in diet can inhibit ruminal BH in several stages. As C18:0 is produced in the last step of this process, a drop in its concentrations due to any of these possible inhibitions would have been anticipated (Frutos et al., 2020). Thus, the higher milk C18:0 concentrations associated with sainfoin PAC were unexpected but have also been reported when dairy ewes were fed sulla (Addis et al., 2005). Frutos et al. (2020) recently showed wide variability for the effect of PAC on milk C18:0 content, with most studies reporting non-significant changes and less than 20% describe either an increase or decrease. In the present study, the Sainfoin group tended to have higher activity of elongase. However, since the contribution of this enzyme in catalysing the synthesis of C18:0 from C16:0 in milk fat appears to be very low (Palmquist, 2006), the differences in C18:0 between treatments should not be justified by the activity of this enzyme and the origin remains unclear. The literature also shows inconsistency between C18:0 contents in digesta, milk and meat, which implies that factors other than ruminal BH are involved, such as Δ^{9}-desaturase, which will be subsequently discussed.

The increase in dietary PUFA (C18:3n-3 and C18:2n-6) and the decrease in several C18:1 and C18:2 isomers in Sainfoin ewes' milk indicate that the inhibition in ruminal BH occurred in an initial stage, when dietary PUFAs are exposed to isomerisation and disappearance. The lower concentrations of OCFA, BCFA and

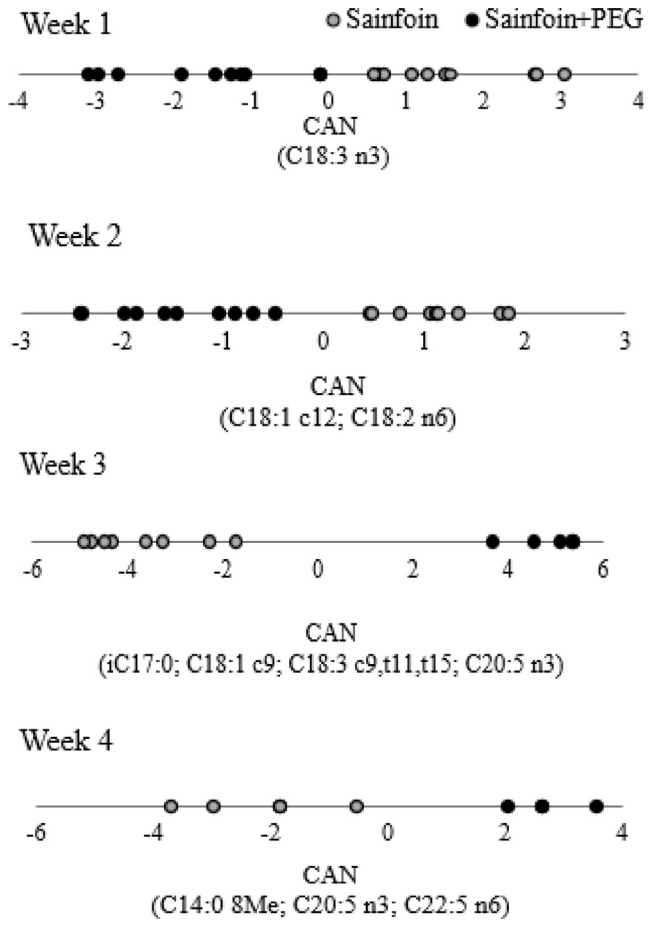

Fig. 2. Graph of the canonical function (CAN) of milk fatty acids for Sainfoin and Sainfoin + PEG ewes and for weeks 1, 2, 3, and 4. Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG). The position to left or right of the graph is related to the negative or positive canonical effects, respectively. Abbreviations: CAN = canonical function.

trans-MUFA, which origin is strictly ruminal, obtained in Sainfoin milk confirmed the lower BH of this treatment. To support these pieces of evidence, the canonical analyses were able to discriminate treatments through dietary PUFA (C18:3n-3, C18:2n-6, and C18:1 c9), and from some of the FAs resulting from their ruminal BH (C18:1 c12 and C18:3 c9,t11,t15). Although the lesser disappearance of dietary PUFA is desirable for final product quality, some C18:1 and C18:2 isomers, especially rumenic acid (CLA c9, t11) and its precursor vaccenic acid (C18:1 t11), are also considered beneficial (Shingfield et al., 2006), and both decreased with PAC in the present study. The reduction in total CLA and CLA c9, t11 due to the presence of PAC was only significant at week 1. Our findings are similar to those obtained by Cabiddu et al. (2009), who observed that an average of 34 g eq. of sulla PAC/kg DM also inhibited BH in early stages and, on average, CLA c9,t11 and C18:1 t11 milk contents were 40% higher when condensed tannins were blocked by PEG. In the above-mentioned review, Frutos et al. (2020) indicated that condensed tannins did not affect milk CLA c9,t11 content in 65% of studies, while 11% and 24% showed a reduction or increase, respectively. This lack of uniformity in the results is because PAC can inhibit rumen BH in different stages, which results in the formation of this FA being greater or lesser. The level at which this inhibition occurs depends on several factors, such as PAC dose and chemical structure (Patra and Saxena, 2011), the interactions between the ingredients of diet (Vasta et al., 2009), and even between-animal variability (Harnly et al., 2022).

The milk FA profile also depends on mammary gland activity, where Δ^9-desaturase enzyme introduces a double bond at the Δ^9-position in a broad FA spectrum (Ntambi and Miyazaki, 2004), including C12:0, C15:0, C16:0, C17:0, C18:0, C18:1 t11, and C18:1 c7. In the present study, C12:1 c9, C15:1 c9, C16:1 c9, CLA c9,t11, and CLA c7,c9 contents were lower in the Sainfoin ewes' milk but no differences were observed in C17:1 c9. Only the C15:1 c9 and C17:1 c9 are exclusively from endogenous origin, while the rest can come also from a dietary origin. The lack of differences in the C17:1 c9 proves that the differences between treatments are not due solely to the effect of Δ^9-desaturase activity. Furthermore, the lack of effect on C18:1 c9 also does not clarify this issue, as it can come directly from dietary intake, lipid mobilisation, as well as being produced during the BH process. This mixed origin prevents us from discerning whether PAC action inhibits the BH of dietary C18:1 c9 in the first ruminal process stages or decreases the Δ^9-desaturase activity (Frutos et al., 2020). In the present study, concentrations of milk C18:1 c9 should not be related to lipid mobilisation since plasma concentrations of non-esterified FA did not differ between PAC treatments (Baila et al., 2022a).

Disregarding differences in Δ^9-desaturase activity between groups, the decrease of CLA c9,t11 in Sainfoin milk could be explained by the lower concentration of milk C18:1 t11 in this group, since it is estimated that almost the 50% of CLA c9,t11 secreted in ewe's milk comes from the endogenous production from C18:1 t11 (Chilliard et al., 2000).

In the present study, milk SFA and MUFA underwent inverse evolution, which can be explained by the BH process because SFA comes partially from ruminal MUFA saturation. No changes were obtained in PUFA concentration in the same treatment, even though SFA and MUFA are partly derived from the BH of dietary PUFA. This different evolution pattern can be linked with the direct passage of some FA from forage to milk. It is important to highlight that, when specific milk FAs were affected by the interaction between PAC and lactation week, this effect was significant only in early lactation. The fact that both treatments differed at week 1 (C18:1c12, total *trans*-MUFA, total CLA, n-6:n-3 ratio, Δ^9-desaturase C18), at week 2 (total PUFA and PUFA:SFA ratio), or at both weeks 1 and 2 (CLA c9,t11), supports previous evidence that the effects of PAC are time-related due to possible rumen microbiota adaptation (Cabiddu et al., 2009).

Conclusions

Milk FA contents did not mirror changes in sainfoin FA concentrations, which suggests that the milk FA profile is related mostly to changes in ruminal metabolism. The inhibition of rumen BH by sainfoin PAC seemed to occur in the early stages of this process, as shown by higher milk PUFA contents and a decrease in BH intermediates, such as MUFA. Our results support the beneficial effect of forage-based diets on the milk FA profile, which renders sainfoin an interesting option for the diets of lactating ewes.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100862.

Ethics approval

All the experimental procedures carried out in this trial were approved (CEEA, 2017-07) by the Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria de Aragón Research Centre in compliance with the guidelines of Directive

2010/63/EU of the European Parliament and Council, of 22 September, on the protection of animals used for experimental purposes.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of interests

None.

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