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Effects of feeding sainfoin proanthocyanidins to lactating ewes on intake, milk production and plasma metabolites

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ABSTRACT

There is increasing interest in using sainfoin (*Onobrychis viciifolia*) to feed sheep, but it contains proanthocyanidins (**PACs**), and the associated effects of PAC on sheep production are not well-known. The aim of the study was to assess the effect of the presence of PAC from sainfoin, through the inclusion of polyethylene glycol (**PEG**), on the intake and productive parameters of local ewes bearing one male lamb. For the experiment, 20 ewes and their newborn male lambs were placed in individual indoor cages. All ewes were fed *ad libitum* fresh sainfoin plus 200 g/d barley. Twice daily, half of the ewes were orally dosed with only water (Sainfoin Group; $n = 10$), and the other half were orally dosed with 100 g/d PEG 4000 per ewe (Sainfoin + PEG Group; $n = 10$). Sucking lambs were permanently housed with their dams until they reached 10–12 kg BW. The intake of sainfoin was recorded daily, and its chemical composition was analysed. Weekly, the BW, body condition score (**BCS**), milk yields and individual milk and blood samples were recorded. At the beginning and end of the experiment, faecal samples were collected from ewes and analysed for the anthelmintic role of PAC. The chemical composition, polyphenol content and antioxidant capacity of the diet and milk were analysed. The presence of PAC did not affect the intake, BW, BCS or milk yield of the dams ($P > 0.05$); however, all parameters were affected by the week of lactation ($P < 0.05$). Milk components were affected by the week of lactation ($P < 0.001$), but only the polyphenol and urea contents were reduced in the presence of PAC ($P < 0.01$). Similarly, the presence of PAC decreased the plasma urea concentration ($P < 0.01$) without effect on the rest of metabolites, polyphenols and antioxidant activity ($P > 0.05$). The presence of PAC had no effect on parasitism ($P > 0.05$). In conclusion, the presence of PAC had no relevant effects on milk production, although it affected protein metabolism, as indicated by the urea contents in milk and plasma.

Implications

There is increasing interest in using sainfoin (*Onobrychis viciifolia*), a legume with medium content of proanthocyanidins; however, the associated effects of proanthocyanidins are not well-known. The aim of the study was to evaluate the effect of proanthocyanidins from fresh sainfoin on eweś performance during lactation. The effect of proanthocyanidins was not relevant on the intake, milk yield, milk composition and plasma metabolites, except for urea, which was reduced in milk and plasma. Diet constituted of 90% of sainfoin, regardless of the presence of proanthocyanidins, allows a good performance of local ewes rearing one lamb.

Introduction

In Mediterranean regions, meat from suckling lambs is traditionally consumed and well valued. Suckling lambs are fed exclusively maternal milk from birth to slaughter at 10–12 kg of BW, allowing for a cost-effective system. The suckling period required to reach the target weight is short, which gives an advantage to the Mediterranean autochthonous breeds characterised by a low genetic improvement, well adapted to the environment and able to produce meat lamb with low nutritional requirements. The flocks of these ewes are usually housed around parturition, and ewes are fed hay plus cereal grains or concentrate until weaning to guarantee adequate growth of the lambs. Uniform young lambs are obtained, with highly appreciated sensorial characteristics of

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the meat. However, in recent years, there has been increasing interest in reintroducing fresh forage and reducing the concentrate in the dams' diet, especially encouraging the use of leguminous forages because of their ability to fix nitrogen in soil. Sainfoin (*Onobrychis viciifolia*) is a Mediterranean forage legume with restorative effects, reducing the need for nitrogen fertilisers, the erosion, leaching and eutrophication of the soil. It also has high nutritional value due to its high protein content and high coefficient of digestibility for ruminants, but presents a medium content of proanthocyanidins (**PACs**), or condensed tannins, varying between 10 and 90 g/kg DM.

The intake of PAC can affect the productive performance and the quality of animal products (meat and milk), depending their effects on the concentration, structure and molecular weight, as well as on the diet and the physiological characteristics of the animals studied (Piluzza et al., 2014). When the proportion of PAC in the diet is high (>70 g/kg DM), negative effects on voluntary intake are observed (Hervás et al., 2003) due to their astringent power and the decrease in ruminal microbial activity (Piñeiro-Vázquez et al., 2015). However, when the diet has moderate PAC content (lower than 50 g/kg DM), positive effects are observed, such as a decrease in methane and ammonia production and reduced risk of bloat (Waghorn, 2008) and an increase in protein flow to the small intestine (Frutos et al., 2004). All these effects can involve changes in blood metabolites, decreasing glucose, urea, and increasing non-esterified fatty acids (**NEFAs**) (Kaneko et al., 2008). In addition, lower protein degradation can reduce the impact of intestinal nematodes and nematode larvae on the animal performance mainly by decreasing the availability of essential nutrients for their development (Waghorn, 1996). Nematodes cause mainly subclinical infections, producing important negative economic impacts on ruminant (Calvete et al., 2020). Anthelmintic resistance to products commonly used to control nematodes is becoming a growing problem, whereas natural additives with anthelmintic properties, such as PAC, could be used instead of synthetic products (Rivaroli et al., 2019).

The effects of PAC have been studied by comparing legumes with and without PAC, such as sainfoin and alfalfa (Lobón et al., 2017a). However, forages usually also differ in their chemical composition, and it is not possible to unravel whether the differences are due to the presence of PAC, differences in the chemical composition or a combination of both. Blocking agents of PAC, such as polyethylene glycol (**PEG**), have been used in several studies as polymers able to bind and deactivate PAC over a wide range of pH values. Thus, the objective of the present study was to assess the effect of the PAC from sainfoin on the intake and productive parameters of Mediterranean local dams rearing lambs.

Material and methods

Animal management and experimental design

The experiment was conducted in the facilities of the Research Centre (41°3′ N, 0°47′ W and 216 m above sea level) in Zaragoza, Spain. In spring 2019, 2–3 days after lambing, 20 single bearing ewes of Rasa Aragonesa breed and their male lambs were distributed into one of the two treatments, according to ewe BW (61 ± 6.2 kg), body condition score (**BCS**; 3.3 ± 0.57), lambing date (April 6 ± 0.1d) and lamb BW at birth (4.1 ± 0.64 kg). Each pair of dam lamb was placed in an individual indoor cage (1.5 m × 1.4 m). The trial length was 28 days, comprising of 4 weeks. The mean temperature during this period was 14.9 °C, 15.2 °C, 15.1 °C, and 16.2 °C in weeks 1, 2, 3, and 4, and the precipitation was 1.2 mm, 11.4 mm, 5.2 mm, and 0.6 mm, respectively.

All ewes received *ad libitum* fresh sainfoin (*Onobrychis viciifolia* cv Reznos, vegetative/start flowering stage) plus 200 g of barley grain per day that was distributed in two meals (0900 and 1600 h). Just before each meal administration, half of the ewes were orally dosed with 100 mL of water (Sainfoin Group; n = 10), whereas the other half were orally dosed with a solution of PEG (50 g of PEG 4000/100 mL; Sainfoin + PEG Group; n = 10), in order to inactivate the effects of PAC. The sainfoin was cut three times per week and stored indoors to avoid mould and overheating. Water and mineral blocks were offered *ad libitum*. Lambs suckled their dams *ad libitum*, and when they reached the target BW of 10–12 kg, they were slaughtered.

Measurements and sampling procedures

The amounts of feed offered and refused were recorded daily to calculate the individual intakes. The sainfoin offered was adjusted according to the refusal to allow *ad libitum* intake at 15% of refusal of the previous day. Composite samples of the offers and refusals per ewe and week were obtained.

Weekly, before the morning meal distribution, the BW of ewes and lambs was registered with an electronic balance (0.5 and 0.1 kg precision in ewes and lambs, respectively), and the BCS of ewes was estimated by two trained technicians. Also weekly, blood samples were collected from the jugular vein of ewes into tubes containing heparin (Vaccuette, Spain) and immediately centrifuged (3 000*g* for 15 min at 4 °C), and the plasma was stored at −20 °C until the metabolite analyses were performed.

Additionally, once per week, milk production was calculated by the oxytocin technique (Doney et al., 1979). Ewes were injected with 5 IU oxytocin (Facilpart 10 UI/mL intravenous, SYVA, León, Spain) in the jugular vein and machine-milked with hand finishing at 0800 and 1200 h (interval of 4 h). The standard milk yield was calculated as follows: standard milk production (L/d) = milk production (L/d) × [(0.0071 × crude fat (g/L) + (0.0043 × CP (g/L)) + 0.2224], as had been described Joy et al., (2012). The extracted milk was divided into two individual milk samples that were stored at 4 °C. One individual sample was preserved by the addition of azidiol (sodium azide, PanReac, Barcelona, Spain) until the chemical analyses, and the second sample was freeze-dried for determination of the polyphenols and antioxidant activity.

At the beginning and end of the study, faecal samples from ewes were collected from the rectum and kept refrigerated until parasite determination.

Chemical analyses

The composite samples of the offers were dried in an oven at 60 °C for 48 h, and the other part was freeze-dried (Genesis Freeze Dryer 25, Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, Spain). All samples were ground and sieved through a 1 mm screen (Rotary Mill, ZM200 Retsch, Haan, Germany), and a small portion of these samples was sieved through a 0.2 mm screen to analyse the CP, PAC, total polyphenol content, delphinidin:cyanidin ratio and antioxidant activity (measured as 2,2-azinobis-3-ethylbenso thiazoline-6-sulfonic acid; **ABTS**) of the feedstuffs. All samples were stored in total darkness and at −80 °C until further analyses.

All analyses of the feedstuffs were run in duplicate. The DM, CP, NDF exclusive of residual ash (**NDFom**), ADF exclusive of residual ash (**ADFom**), lignin determined by solubilisation of cellulose with sulphuric acid (lignin (sa)), content of PAC (obtained as the sum of extractable PAC, protein-bound PAC, and fibre-bound PAC) and total content of polyphenols of sainfoin and barley were obtained according to Rufino-Moya et al. (2019a). The determination of anthocyanidins (delphinidin and cyanidin) was carried out after hydrolysis by **UPLC-DAD** (High-Performance Liquid Chromatogra-

phy Fluorescence and Diode Array Detection, ACQUITY UPLC H-Class (Waters, Milford, Massachusetts, EE. UU)) following the procedure described in Assefa et al. (2019) with a Acquity UPLC BEH C18 (50 mm × 2.1 mm × 1.7 μm) column and a absorbance wavelength of 520 nm. The gross energy content was calculated through the combustion-specific heat obtained with a calorimetric bomb (Model Parr 1341 Plain Jacket Bomb Calorimeter, Parr Instrument Company, Illinois, USA). The antioxidant activity of the feedstuffs was determined using the Folin-Ciocalteu method as described in Makkar (2003). The concentration of ABTS was obtained according to Jiménez-Escrig et al. (2003). Results were read in a BioTek Epoch Spectrophotometric Microplate Reader (BioTek Instruments, Inc., Winooski, VT, EE. UU.) at 750 and 730 nm absorbance wavelength, respectively.

Milk protein, fat, lactose and urea contents and the number of somatic cells were determined in a Combifoss™ 7 (Foss, Hillerød, Denmark) device comprising a Fossomatic 7 DC somatic cell counter (based on the flow cytometry principle) and a MilkoScan™ 7 RM component. The milk content of polyphenols was determined with a Folin-Ciocalteu method according to Leal et al. (2019), and the antioxidant activity was estimated using ABTS, following the extraction method of Vázquez et al. (2015) and the determination described by Jiménez-Escrig et al. (2003) with a BioTek Epoch Spectrophotometric Microplate Reader (BioTek Instruments, Inc., Winooski, VT, EE. UU.) at 730 nm of wavelength.

Plasma concentrations of urea (kinetic method) and glucose (kinetic method) were analysed with an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). NEFAs (enzymatic method) were analysed using a commercial kit (Randox Laboratories Ltd., Crumlin Co., Antrim, UK). The antioxidant activity of plasma was studied based on polyphenols, superoxide dismutase (**SOD**), malondialdehyde (**MDA**) and ABTS. The plasmatic concentration of polyphenols was obtained by diluting 1:25 (plasma: Milli-Q water) and applying the method of Leal et al. (2019). The SOD was obtained using a colorimetric activity kit (Arbor Assays, DetectX, Michigan, USA), and the total MDA was determined as described in Bertolín et al. (2019). Finally, the method followed to determine ABTS was based on Jiménez-Escrig et al. (2003).

The concentrations of differentiable parasite forms in faeces were determined using a modification of the McMaster method. Faeces (2 g) were homogenised in 28 mL of zinc sulphate flotation solution (specific gravity 1.200) and filtered through double cotton gauze. The concentrations of parasite forms were estimated by screening two complete McMaster flotation chambers with a lower detection level of 15 parasite forms/g. In addition to their use in the coprological examinations, the faeces were used to produce bulk faecal cultures that were kept in an incubator at 23 °C for 13 days. On day 14, the infective larvae were harvested and identified (≈ 100 larvae per faecal sample) using identification keys for ruminant nematodes (Landau et al., 2002).

Statistical analyses

Data were analysed with SAS *statistical software* (v.9.3; SAS Inst. Inc., Cary, NC; EE.UU.) using the ewe as experimental unit. The intake, milk production and composition, BW, BCS and plasma metabolites of ewes were analysed through an analysis of variance with a mixed model (MIXED procedure) with the feeding treatment (Sainfoin or Sainfoin + PEG), week of lactation and their interaction as fixed effects and the ewe as the random effect. The degrees of freedom were adjusted with the Kenward-Roger correction. The least square means and their associated SEs were obtained, and Tukey's correction was used for pairwise comparisons. For the analysis of the number of faecal parasites, the egg counts were transformed into their logarithmic values to meet normality. The logarithm of the number of *Strongiloides* was analysed using the method of least squares, studying the sampling day and the effect of the treatment. Parasite presence ranges were then fixed and, due to being binomial characters, were analysed by a chi-squared procedure. The effects were considered significant at $P < 0.05$.

Results

The average chemical composition of the feedstuffs offered to dams during the experimental period is shown in Table 1, and the evolution of the most relevant parameters of the chemical composition and PAC of sainfoin during lactation is presented in Fig. 1. The CP decreased until week 3 ($P < 0.05$), whereas the NDFom and ADFom had an inverse evolution ($P < 0.05$). The gross energy was similar over time except during week 3, when a significant decrease was registered ($P < 0.05$). The content of polyphenols and PAC followed a similar pattern among them, being higher in week 1 and decreasing between weeks 2 and 3 ($P < 0.05$).

The DM intake (**DMI**) was not affected by the presence of PAC, but it increased with the week of lactation ($P < 0.05$; Table 2), except between week 2 and week 3 ($P > 0.05$). The BW and BCS of the ewes were only affected by the week of lactation ($P < 0.05$; Fig. 2). Both parameters decreased sharply between week 0 and week 1 of lactation ($P < 0.05$), and, thereafter, the BW increased slightly over lactation, only differing between week 1 and week 4 ($P < 0.05$), whereas the BCS remained unchanged.

The milk yield and composition are shown in Table 2. No interaction between the presence of PAC and week of lactation ($P > 0.05$) was observed, except for the milk urea content ($P < 0.05$), which increased throughout lactation in Sainfoin + PEG ewes, while it remained steady in Sainfoin ewes ($P < 0.05$; Fig. 3). The presence of PAC did not affect any milk parameters ($P > 0.05$) except polyphenols, which had higher content in the Sainfoin + PEG group ($P < 0.001$; Fig. 3). The week of lactation affected all milk parameters ($P < 0.05$ to <0.001). The milk yield increased from week 1 to week 2 ($P < 0.05$) and remained steady over the rest of lactation. The milk fat content decreased only from weeks 2 to 3 ($P < 0.05$), whereas protein diminished from weeks 1 to 2 ($P < 0.05$), remaining constant during the rest of lactation ($P > 0.05$). Lactose had an inverse evolution, with greater contents in week 3 and week 2 and the lowest content in week 1 ($P < 0.05$). The milk polyphenol concentrations were affected both by the presence of PAC ($P < 0.001$; Fig. 3) and by the week of lactation ($P < 0.01$), with the greatest content in the Sainfoin + PEG treatment in week 4. Regarding the antioxidant activity, ABTS decreased from weeks 1 to 2 ($P < 0.05$) and recovered its initial levels in week 3 of lactation ($P > 0.05$).

The concentrations of plasma metabolites throughout lactation are shown in Fig. 4. The concentration of urea was affected by the presence of PAC (23.7 vs 19.6 mg/dl for Sainfoin + PEG and Sainfoin ewes, respectively; $P < 0.05$) and the week of lactation ($P < 0.001$). The plasma urea decreased until week 2 and thereafter increased until week 4 ($P < 0.001$). The concentrations of glucose and NEFA were only affected by the week of lactation, showing an inverse evolution as glucose decreased ($P < 0.001$), while NEFA increased ($P < 0.05$) until week 3 of lactation.

Regarding the plasma polyphenols and the antioxidant activity (Fig. 5), the concentration of polyphenols was not affected by the presence of PAC or by the week of lactation ($P > 0.05$), whereas the antioxidant activity, which was measured as SOD and MDA, was only affected by the week of lactation ($P < 0.01$). The SOD values decreased until week 3 and then increased sharply during week 4 ($P < 0.001$), while the MDA decreased until week 2 ($P < 0.05$) and remained steady thereafter. The antioxidant activity

Table 1
Chemical composition, mean and SE, of the feedstuffs offered to the ewes.

	Sainfoin		Barley	
Item	Mean	SE	Mean	SE
Moisture (g/kg DM)	787	2.3	88	11.7
Ash (g/kg DM)	129	1.9	25	8.2
CP (g/kg DM)	116	0.7	95	2.9
NDFom (g/kg DM)	369	1.6	250	6.9
ADFom (g/kg DM)	264	1.3	87	5.5
Crude fat (g/kg DM)	–	–	20.8	1.14
Gross energy (MJ/kg DM)	17.2	0.04	18.2	0.18
Polyphenols (g/kg DM)	62.7	3.08	6.2	4.62
Proanthocyanidins (PACs)[1]				
Total	38.8	6.39	2.09	0.73
Extractable	34.0	6.10	1.58	0.717
Protein bound	3.18	0.587	0.40	0.044
Fibre bound	1.67	0.375	0.11	0.015
Delphinidin:cyanidin ratio	80:20	-	27:43	-
Antioxidant capacity, ABTS	137	33.7	82	7.1

ABTS = 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid, μmol eq. [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX)]/g DM.
[1] g eq.PAC sainfoin/kg DM.

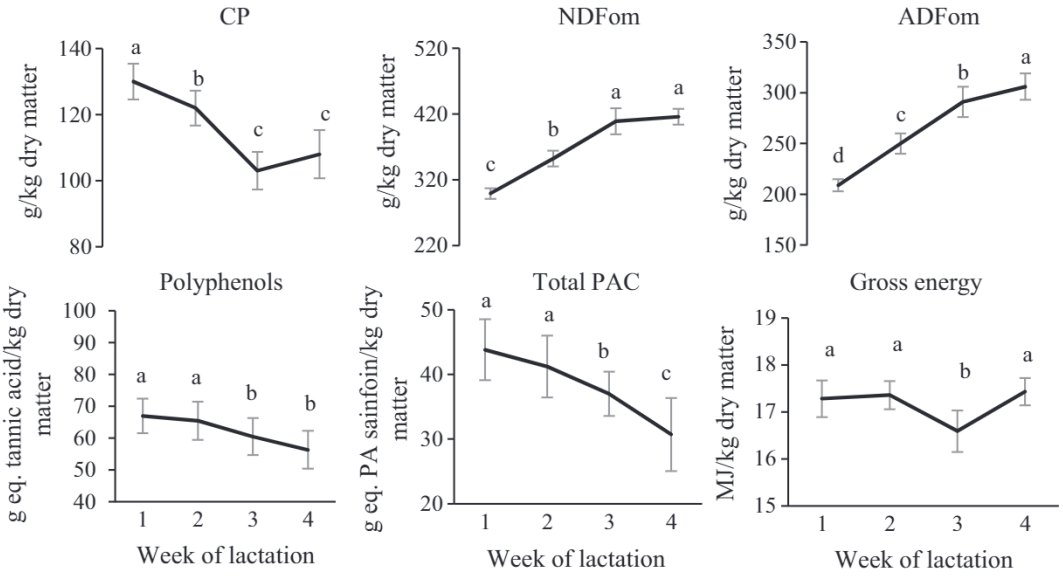

Fig. 1. Contents of CP, NDF exclusive of residual ash (NDFom), ADF exclusive of residual ash (ADFom), polyphenols, total proanthocyanidins (PACs) and gross energy of fresh sainfoin for ewes during lactation. Means with different letters differ at $P < 0.05$ among weeks.

Table 2
Effects of the presence of proanthocyanidins (PACs) and the week of lactation on the performance, milk yield and chemical composition of milk of ewes fed sainfoin.

	PAC		Week of lactation (W)					*P*-values		
Items	Sainfoin	Sainfoin + PEG	1	2	3	4	RSD	PAC	W	PACxW
DM intake (g/d)	1 845	1 914	1 484^{c}	1 913^{b}	1 836^{b}	2 283^{a}	356.4	0.45	<0.001	0.15
Milk yield (L/d)	1.23	1.27	0.94^{b}	1.52^{a}	1.28^{a}	1.26^{ab}	0.310	0.77	<0.001	0.25
Standard milk yield (kg/d)	1.02	1.27	0.96^{b}	1.42^{a}	1.03^{b}	1.21^{ab}	0.298	0.60	<0.001	0.32
Milk composition										
Fat (%)	6.27	6.81	7.08^{a}	6.74^{a}	5.20^{b}	7.16^{a}	0.869	0.38	<0.001	0.99
Protein (%)	5.00	5.04	5.50^{a}	4.90^{b}	4.78^{b}	4.91^{b}	0.198	0.82	<0.001	0.76
Lactose (%)	5.30	5.25	5.09^{b}	5.30^{a}	5.45^{a}	5.25^{ab}	0.159	0.61	<0.001	0.41
Somatic cells[1]	172	213	298^{a}	222^{ab}	113^{b}	137^{ab}	183.7	0.50	0.05	0.63
Urea, mg/L[*]	275^{b}	338^{a}	274^{b}	312^{a}	311^{a}	330^{a}	30.2	0.006	<0.001	0.002
Polyphenols[2]	42.3^{b}	51.8^{a}	44.2^{b}	43.4^{b}	48.6^{ab}	52.0^{a}	6.07	<0.001	0.01	0.34
ABTS	0.66	0.67	0.72^{a}	0.61^{b}	0.66^{ab}	0.68^{ab}	0.091	0.66	0.005	0.35

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).
ABTS = 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid (total antioxidant capacity) measured as μmol eq. [TROLOX]/g fresh milk.
Within a parameter and main effect, means with different superscript letters differ at $P < 0.05$.
[1] 1 000 cells/mL milk.
[2] μg eq. [gallic acid]/g fresh sample.
[*] Interaction is presented in Fig. 3.

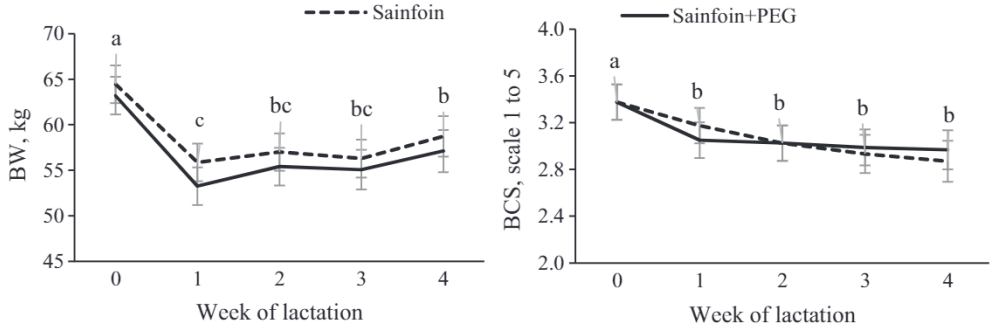

Fig. 2. Effects of the presence of proanthocyanidins (PACs) and the week of lactation on the evolution of BW and body condition score (BCS) of sainfoin-fed ewes. Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG). Means with different letters differ at $P < 0.05$ among weeks.

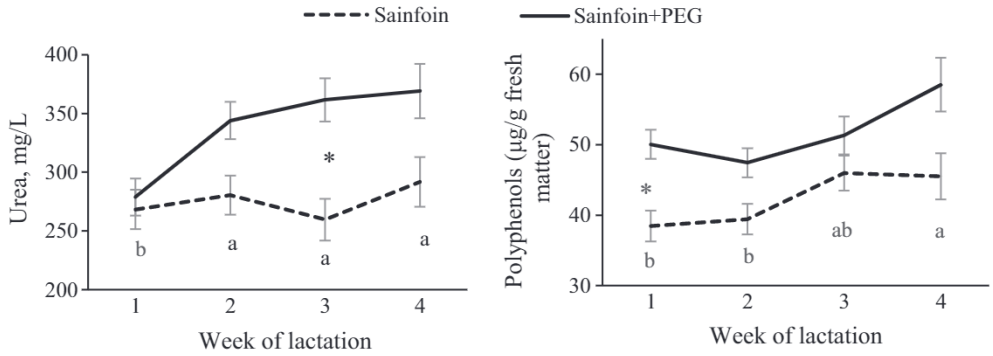

Fig. 3. Effects of proanthocyanidins (PACs) and the week of lactation on the milk urea and polyphenol concentration of sainfoin-fed ewes. Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG) Within a parameter and main effect, means with * differ at $P < 0.05$ Means with different letters differ at $P < 0.05$ among weeks.

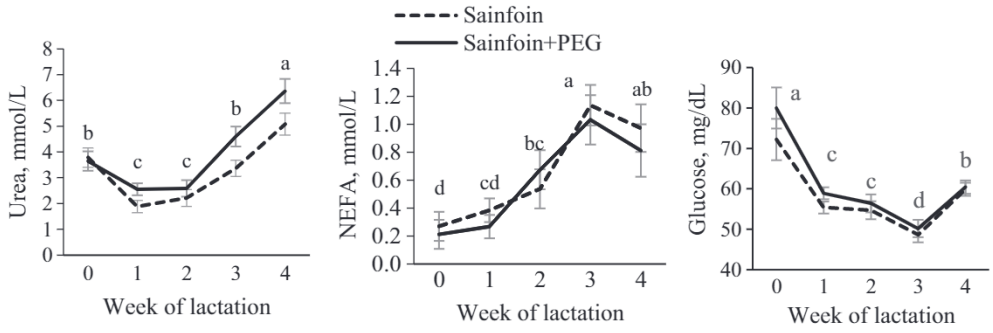

Fig. 4. Effects of proanthocyanidins (PACs) and the week of lactation on the urea, non-esterified fatty acid (NEFA) and glucose concentrations in the plasma of ewes fed sainfoin during lactation. Sainfoin: ewes fed *ad libitum* sainfoin + 200 g/d barley; Sainfoin + PEG: ewes fed *ad libitum* sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG). Means with different letters differ at $P < 0.05$ among weeks.

measured with ABTS tended to be affected by the presence of PAC, the week of lactation and their interaction ($P < 0.10$).

No differences in ewe parasitism were observed due to the presence of PAC, regardless of the moment of sampling (Table 3).

Discussion

The chemical composition of the fresh sainfoin was similar to what was reported in the vegetative state or at the start of flowering in sainfoin grown under similar conditions (Rufino-Moya et al., 2019a and 2019b), except for CP and PAC, which were slightly lower in the present study. As maturity progressed from vegetative stage to flowering, the CP, PAC and polyphenol contents decreased, and the NDFom, ADFom and lignin (sa) concentrations increased as a result of the reduction in the proportion of leaves to stems and the increase in lignified tissues. The evolution of the chemical composition of sainfoin was as expected, except for that observed on week 3, when the sainfoin was collected wet and, consequently, had lower CP and GE contents.

The increase in the sainfoin DMI as lactation advanced was expected and the presence of PAC did not affect the DMI in the current experiment, which might be related to the low-medium PAC content in the sainfoin fed. The source and chemical structure of fed PAC can explain the different effects on the DMI, when similar contents of PAC are included in the diet.

Regarding the effect of PAC on the BW and BCS of ewes, the literature is not conclusive. In agreement with the present study,

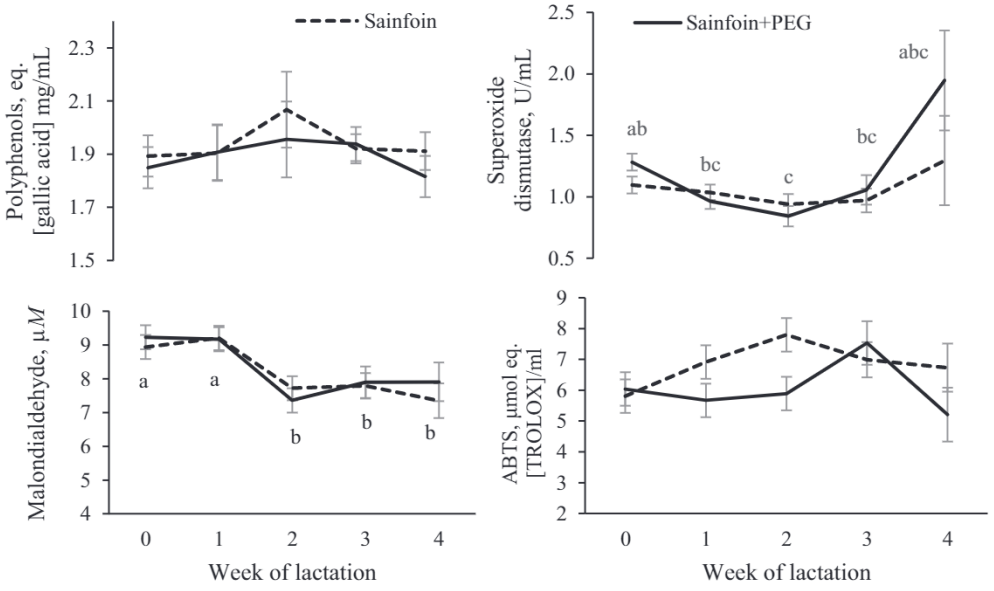

Fig. 5. Effects of proanthocyanidins (PACs) and the week of lactation on the polyphenol concentration and antioxidant activity parameters (superoxide dismutase, malondialdehyde and ABTS (2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid) in the plasma of ewes fed sainfoin during lactation. Sainfoin: 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). Means with different letters differ at $P < 0.05$ among weeks.

Table 3
Effects of proanthocyanidins (PACs) and the week of lactation on faecal Strongiloides (*Teladorsagia* spp.) parasitism of ewes at the start and end of lactation.

	Start of lactation			End of lactation		
Items	Sainfoin	Sainfoin+PEG	*P*-value	Sainfoin	Sainfoin+PEG	*P*-value
N	8	8		8	8	
Log-transformed count (n° eggs/g)	45.7	14.45	0.31	33.1	16.6	0.59
(log-values ± SE)	(1.66 ± 0.356)	(1.16 ± 0.356)		(1.52 ± 0.356)	(1.22 ± 0.356)	
Ewes excreting Strongiloides (%)						
>0 oocysts/g faeces	87.5	62.5	0.25	75	62.5	0.59
>10 oocysts/g faeces	87.5	62.5	0.25	75	62.5	0.59
>200 oocysts/g faeces	12.5	25	0.52	12.5	25	0.52
>500 oocysts/g faeces	0	12.5	0.31	0	0	1

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).

Lobón et al. (2017b) observed no effect on these parameters when quebracho was included at 100 g/kg DM (PAC = 8.1 g cyanidin equivalent/kg DM), whereas Salem et al. (1999) observed a negative effect when *Acacia cyanophylla* was fed *ad libitum* (PAC = 51 g catechin equivalent/kg DM). In the current study, the lack of an effect of PAC could be partially related to the low PAC intake, similar DMI and the short experimental period. Regardless of the treatment, ewes decreased their BW sharply during the first week postlambing as a consequence of uterine involution. Subsequently, the intake capacity increased, and ewes started gaining weight. The loss of BCS was coupled with the increasing demand for nutrients to support milk yield.

The absence of difference in the DMI between treatments could be the main cause of the lack of observed effects on the milk yield and composition. Similarly, Benchaar et al. (2008) did not find effect of quebracho on the DMI and milk production of cows eating 45 g PAC/kg DM. The lack of effect of the treatment on the milk fat and protein contents contradicts the theory that the inactivation of PAC by the inclusion of PEG increases the availability of macronutrients required for milk synthesis and, thus, increases milk yields (Provenza et al., 2000). In contrast, the higher milk urea content in Sainfoin + PEG ewes confirms the effectiveness of PEG in neutralising the action of PAC, because PAC reduces the ruminal degradability of the protein and thus decreases the urea content (Peng et al., 2016). This effect on protein is also consistent with one of the main characteristics of prodelphinidins, the main polymer of sainfoin PAC, which favour the interaction with protein and their metabolism (Jonker and Yu, 2017). The absence of an effect of PAC on somatic cell counts observed in the present study contrasts with the bactericidal capacity of PAC in the mammary gland (Nudda et al., 2020). The above-mentioned authors suggest that several tannin extracts inhibit the proliferation of the most important pathogens in the mammary gland, such as *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*. However, in the present study, the farm hygiene conditions were good, and no mastitis was registered in any treatment.

The greater polyphenol content in milk in Sainfoin + PEG treatment was unexpected. This result can be related to the high variability of the analytical method used, which is less precise in sheep milk than in goat or cow milk (Vázquez et al., 2015). Moreover, another possible explanation of this result can be related to some cross-reactivity between polyphenols and reducing substances detected with the analytical method (Sánchez-Rangel et al., 2013). The PEG contains a large number of oxygen atoms capable of forming hydrogen bonds with the phenolic and hydroxyl groups of tannins (Silanikove et al., 2001) inhibiting them over a wide range of pH. However, in this study, PEG could also have been linked to polyphenols with similar structures, avoiding their

degradation in rumen and promoting their posterior absorption, which could pass into the milk, and thus increasing the total content of polyphenols in this treatment. In addition, the mammary gland has a very complex metabolism and its functioning is not well understood in the presence of exogenous substances, such as PEG, that could cause the mammary gland to react differently. In addition, PAC polymers are not easily absorbed in the small intestine because of their large molecular weight (Scalbert and Williamson, 2000), which could have resulted in the lower polyphenol content in milk from sainfoin. Greater content of polyphenols in milk usually involves a higher antioxidant activity (Jordán et al., 2010). However, in the current study, no differences in antioxidant activity were found. Further studies regarding the degradation of polyphenols are required to discern the relationship between PAC, polyphenols and antioxidant activity.

The content of polyphenols in plasma was similar between treatments and among weeks, which contradicts the above-mentioned comments regarding milk. To explain these differences, a UPLC-MS/MS (High-Performance Liquid Chromatography with mass spectrometry) should be used to identify the metabolites that differed between milk and plasma, as well as between treatments. The antioxidant activity in plasma, measured as SOD or MDA, was not affected by treatment, whereas when it is measured as ABTS showed a tendency to have greater antioxidant activity in Sainfoin treatment. Peng et al. (2016) did not find any effect on either the MDA or SOD content in lamb serum when purple prairie clover was fed. The wide heterogeneity of the analytical methods used to measure the plasma antioxidant activity is partially responsible for the variability of results observed in the literature.

The urea concentration in plasma and milk increases as lactation progressed, but in different weeks. Blood urea increased in both treatments from week 2 onwards, whereas the milk urea concentration increased from week 1 onwards, in Sainfoin + PEG treatment only. This mismatch between blood and milk urea nitrogen contents can be attributed to a time lag between blood and milk sampling (blood was collected 2 days later) and to the low milk yield recorded in week 1. Another factor to take into account is the high DMI recorded in the present trial, which could have led to an increased passage rate and a large amount of undegraded protein reaching the intestine. In this case, the concentration of milk urea would be more related to the total amount of protein absorbed from the small intestine than to the amount of the urea coming from the protein degraded in the rumen. When the urea generated by ruminal protein degradation decreases, gluconeogenesis from amino acids was found to be more important than rumen ammonia concentration as a source of blood urea variation (Cannas et al., 1998). This would generate a "lag" or a lower correlation between the blood urea nitrogen and milk urea nitrogen. The reduction in the blood urea concentration in the presence of PAC is similar to results from Peng et al. (2016), due to the greater protein degradation. Similarly, an increase in blood urea nitrogen concentration in male lambs was observed when PAC from sulla was blocked by the addition of PEG (Stienezen et al., 1996). The decrease of blood urea nitrogen and milk urea nitrogen produced by PAC could translate into a lower urea production rate in the liver (Cannas et al., 1998). In turn, this could be reflected in a lower excretion of urinary N, desirable from an environmental point of view (Galles et al., 2011). However, the effects observed on blood urea nitrogen and milk urea nitrogen are related to the level of inclusion of sainfoin in the diet (Aufrere et al., 2008; Theodoridou et al., 2010).

The lack of differences in plasma glucose contents between treatments was caused by the similar DMI. Regarding the plasma metabolites, the evolution of NEFA was inverse to the dynamics of the blood glucose concentration. The poor quality of the sainfoin in week 3 (lower protein and energy content and higher fibre content) was reflected in the lowest glucose concentration and the highest concentration of NEFA, indicating that fat mobilisation was occurring at this time.

In relation to the effect of sainfoin PAC on parasitism, Rivaroli et al. (2019) reported a delay in the onset of both helminth and coccidian infections in lambs supplemented with sainfoin pellets. Other authors also showed an anthelmintic effect with different sources of PAC, such as quebracho (Villalba et al., 2010) and *Acacia cyanophilla* (Akkari et al., 2008). However, in the current study, there was no differences in parasitism in ewes due to the presence of PAC, which could be ascribed to: i) the fact that ewes were dewormed one month before lambing and were allocated into individual cages after lambing, such that the parasitic load was minimal; ii) the large variability of the data and the small number of observations; and iii) the short length of the present study (28 days) compared with others that reported a positive effect of the presence of PAC with longer study periods.

Conclusion

In conclusion, the effect of PAC from sainfoin had no effect on DM intake, milk production or parasitism, but reduced the milk and plasma urea contents, which could be a consequence of reduced protein degradation in the rumen. Further studies regarding the effect of this feeding management on carcass and meat quality of suckling lambs' commercial category should be done.

Ethics approval

The Animal Ethics Committee of the CITA approved the experimental procedures (CEEA, 2017-07), which were in compliance with the guidelines of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September on the protection of animals used for experimental purposes.

Data and model availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conceptualization, **M.J., S.L.** and **M.B.**; formal analysis, **C.B.** and **S.L.**; chemical analysis **J.R.B.**; Investigation, Writing-Original draft preparation, Reviewing and Editing **C.B., S.L., M.B., I.C., J.R.B.** and **M.J.**; project administration, **M.J.**; funding acquisition, **M.J.** All authors have read and agreed to the published version of the manuscript.

Declaration of interest

None.

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