TESIS DE LA UNIVERSIDAD DE ZARAGOZA
2024
83

Claudia Baila Bigne

# Use of sainfoin (Onobrychis viciifolia) in the diets of lactating dams and fattening lambs

Director/es
Lobón Ascaso, Sandra
Joy Torrens, Margarita



ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza


Universidad de Zaragoza
Servicio de Publicaciones

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

# USE OF SAINFOIN (ONOBRYCHIS VICIIFOLIA) IN THE DIETS OF LACTATING DAMS AND FATTENING LAMBS


Autor

Claudia Baila Bigne

Director/es

Lobón Ascaso, Sandra
Joy Torrens, Margarita

**UNIVERSIDAD DE ZARAGOZA**
**Escuela de Doctorado**

Programa de Doctorado en Producción Animal


2023

*PhD thesis*

# Use of sainfoin (*Onobrychis viciifolia*) in the diets of lactating dams and fattening lambs

**Clàudia Baila Bigné**

**Zaragoza, 2023**

**PhD supervisors:**

**Dra. Margalida Joy Torrens**

**Dra. Sandra Lobón Ascaso**

**CERTIFICACIÓN DE LAS DIRECTORAS DE TESIS**

***Margalida Joy Torrens*** y ***Sandra Lobón Ascaso***, Doctoras en Veterinaria e Investigadoras de la Unidad de Tecnología en Producción Animal del Centro de Investigación y Tecnología Agroalimentaria del Gobierno de Aragón (CITA),

***HACEN CONSTAR***

Que Dña. ***Clàudia Baila Bigné***, licenciada en Veterinaria y Máster en Nutrición Animal, ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada "Use of sainfoin (*Onobrychis viciifolia*) in the diets of lactating dams and fattening lambs", que se corresponde con el proyecto de Tesis aprobado por la comisión de Doctorado, y que cumple con los requisitos exigidos para optar al grado de Doctor por la Universidad de Zaragoza con mención de doctorado internacional, por lo que autorizan su presentación para que pueda ser juzgada por el Tribunal correspondiente.

Lo que suscribimos como directoras del trabajo en Zaragoza, a 30 de septiembre de 2023

Margalida Joy Torrens

Sandra Lobón Ascaso

# Index of contents

# Index of Tables

# Index of Figures

# Abbreviations

**ABTS** 2,2–azinobis–3–ethylbensothiazoline–6–sulfonic acid

**ADG** Average daily gain

**ADF** Acid detergent fibre

**AI** Atherogenicity index

**AIA** Acid insoluble ash

**BCFA** Branched–chain fatty acid

**BCS** Body condition score

**BH** Biohydrogenation

**BW** Body weight

**CAN** Canonical function

**CAP** Common Agricultural Policy

**CCW** Cold carcass weight

**CP** Crude protein

**CT** Condensed tannin

**DM** Dry matter

**DMI** Dry matter intake

**DOM** Digestibility of organic matter

**DP** Degree of polymerisation

**FA** Fatty acid

**GC-FID** Gas chromatography with flame ionisation detection

**H:h** Hyper-hypocholesterolemic index

**HCW** Hot carcass weight

**HT** Hydrolysable tannin

**IMF** Intramuscular fat

***LTL*** *longissimus thoracis et lumborum*

**MDA** Malondialdehyde

**mDP** Main degree of polymerisation

**MUFA** Monounsaturated fatty acid

**NDF** Neutral detergent fibre

**NEFA** Non–esterified fatty acid

**OCFA** Odd–chain fatty acid

**OM** Organic matter

**OMD** Organic matter digestibility

**PAC** Proanthocyanidin

**PC** Procyanidin

**PD** Prodelphinidin

**PEG** Polyethylene glycol

**PGI** Protected geographical indication

**PUFA** Polyunsaturated fatty acid

**RSD** Residual standard deviation

**SEM** Standard error of the mean

**SD** Standard deviation

**SE** Standard error

**SFA** Saturated fatty acid

**SOD** Superoxide dismutase

**SUM** Summation of the translated spectrum

**TI** Thrombogenicity index

**UPLC-DAD** Ultra performance liquid chromatography fluorescence and diode array detection

**VFA** Volatile fatty acid

# Summary

The inclusion of forage legumes in animal feed has been promoted in recent years to increase farm sustainability and self–sufficiency. These forages are characterised to present a good nutritional quality and to provide benefits to the soil, which makes them an attractive source to feed ruminants.

Sainfoin (*Onobrychis viciifolia*) is a legume with a medium content of proanthocyanidins (PAC), commonly known as condensed tannins, which have been studied for their potential good results when is fed to ruminants.

The aim of this doctoral thesis was to analyse the effects of feeding sainfoin to Aragonese sheep to produce suckling and finishing lambs. For this purpose, two different studies were carried out.

**Study 1** was conducted to assess the effects of sainfoin PAC in the diet of ewes during the rearing period of a suckling lamb. The experimental period lasted from birth until slaughter, when suckling lambs reached the target body weight (BW) of 10–12 kg. After lambing, twenty multiparous Rasa Aragonesa ewes and their male suckling lambs were randomly assigned to one of the two feeding treatments and each pair of dam–lamb were placed in individual indoor cages. All ewes were fed *ad libitum* fresh sainfoin plus 200 g/d of barley, however, half of the ewes were orally dosed twice a day, before each meal distribution, with only water (Sainfoin Group; *n* = 10) while the other half were orally dosed with 100 g/d of polyethylene glycol (PEG) per ewe (Sainfoin + PEG Group; *n* = 10) in order to block the effect of PAC. The suckling lambs were permanently with their dams with free access to suckling. The presence of PAC in dams' diet did not affect the dry matter intake, antioxidant status and parasitic load of ewes. Nevertheless, sainfoin PAC reduced blood and milk urea concentrations. No effect was found for milk yield and milk chemical composition but sainfoin PAC improved milk fatty acid (FA) profile, increasing C18:0, C18:2 n-6, C18:3 n-3 and total polyunsaturated FA (PUFA) n-6 and n-3 although milk C18:1 t11 and branched–chain fatty acid concentrations were reduced. The presence of PAC in the dams' diet did not affect growth, blood metabolites and carcass and meat quality of their suckling lambs, showing only an increase in the weight of the digestive compartments.

The main objective of **Study 2** was to analyse the effects of including dehydrated sainfoin in the finishing concentrates of light lambs. Twenty–six weaned male lambs of Rasa Aragonesa (14.0 ± 0.49 kg BW) were randomly distributed into one of the three diets (isoenergetic and isoproteic), with different levels of dehydrated sainfoin inclusion: 0% sainfoin (0SF; *n*=9), 20% sainfoin (20SF; *n*=9) or 40% sainfoin (40SF; *n*=8). The lambs were individually penned indoors with free access to concentrates, water, and minerals. The trial lasted 40 days until the lambs reached the target slaughter weight of 25 kg. Increasing amounts of sainfoin gradually decreased the estimated apparent digestibility of the diets which was reflected in a higher dry matter intake of 40SF lambs compared to their counterparts, while no differences were found between the other two groups. However, despite the higher intake of 40SF no effect was found on slaughter and carcass weights. Ruminal fermentation parameters were slightly affected by sainfoin inclusion, showing greater acetic proportions, regardless the level of inclusion. In contrast, noticeable effects were obtained on ruminal biohydrogenation (BH). Both 20% and 40% sainfoin concentrates increased C18:3 n-3 and decreased C18:1 t10 ruminal concentrations, suggesting a potentiation of t11 BH pathway due to sainfoin inclusion. Nevertheless, the highest C18:1 t11 and the lowest C18:2 n-6 proportions were only obtained when sainfoin was included at 40%, showing a stronger effect on t11 BH pathway promotion. Those changes in rumen due to sainfoin inclusion were not completely reflected on plasma fatty acid profile but increased PUFA n-3 and decreased PUFA n-6/PUFA n-3 ratio in meat of 20SF and 40SF lambs compared to 0SF. Again, these effects were greater with 40% sainfoin inclusion, as well as the increment of CLA c9,t11 and the reduction of C18:1 t10 in 40SF meat compared to their counterparts.

In view of the results obtained in both studies, it can be concluded that: i) the presence of sainfoin PAC in the diet of ewes allowed good productive results of ewes and improve milk FA profile, which was translated into good suckling lamb performance, without modifying the carcass and meat characteristics and ii) the inclusion of 20% or 40% dehydrated sainfoin in the lambs' finishing concentrate led to similar production results among groups while improved the fatty acid profile of the meat by modifying the BH pattern, especially when sainfoin was included at 40%. Therefore, sainfoin inclusion in the diet of dams rearing a suckling lamb or in the light lambs concentrate during the

fattening is an interesting option, allowing good performances in both ewes and lambs and improving PUFA n-3 concentrations in milk and meat. These results could contribute to reinforce the idea of including local products in animal feed, thus reducing imported sources.

# Resumen

Durante los últimos años se ha promovido el uso de leguminosas forrajeras en la alimentación animal con el fin de incrementar la sostenibilidad y autosuficiencia de las explotaciones. Estos forrajes se caracterizan por presentar una alta calidad nutricional y aportar beneficios al suelo, lo cual los convierte en un alimento adecuado para los rumiantes.

La esparceta (*Onobrychis viciiifolia*) es una leguminosa forrajera con un contenido moderado de proantocianidinas (PAC), comúnmente conocidas como taninos condensados, las cuales han sido estudiadas por sus posibles efectos beneficiosos cuando se incluyen en la dieta de los rumiantes.

El objetivo de la presente tesis doctoral fue analizar los efectos de la esparceta en la dieta de ovejas y corderos de raza Rasa Aragonesa para la producción de corderos lechales y ternascos. Para ello, se llevaron a cabo dos ensayos diferentes.

El **Ensayo 1** se realizó con el fin de estudiar los efectos producidos por la presencia de PAC de la esparceta en la dieta de ovejas durante el periodo de cría de un cordero lechal. El periodo experimental se extendió desde el nacimiento de los corderos hasta que éstos alcanzaron el peso de sacrificio del cordero lechal, fijado en unos 10–12 kg peso vivo. Tras el nacimiento, veinte ovejas de Raza Aragonesa y sus corderos machos fueron asignados aleatoriamente a uno de los dos tratamientos alimentarios y cada pareja de madre-cordero se estabuló en jaulas individuales. Todas las ovejas se alimentaron con esparceta fresca a libre disposición y 200 g/día de cebada. A la mitad de ellas se les administró agua dos veces al día, coincidiendo con la distribución de la ración, por vía oral con una jeringa (Grupo Esparceta; *n*= 10), mientras que a la otra mitad se les suministraba, en las mismas condiciones, una disolución de 100 g/oveja/día de polietilenglicol (PEG) (Grupo Esparceta + PEG; *n* = 10) con el fin de bloquear la acción de las PAC. Los corderos lechales estuvieron en todo momento con sus madres, por lo que tuvieron acceso continuo al amamantamiento. La presencia de PAC en la dieta no afectó a la ingestión de materia seca, a la capacidad antioxidante ni a la carga parasitaria de las ovejas. Sin embargo, la presencia de PAC redujo las concentraciones de urea en la sangre y la leche. El rendimiento lechero y la composición química de la leche no mostraron diferencias entre grupos, pero la presencia de PAC en la dieta mejoró el perfil de ácidos grasos (AG) de la leche, incrementando las concentraciones de C18:0, C18:2 n-6, C18:3

n-3 y ácidos grasos poliinsaturados (AGPI) totales n-6 y n-3, pese a que los porcentajes de C18:1 t11 y AG ramificados se redujeron. En cuanto a los efectos observados en los corderos lechales, la presencia de PAC en la dieta de las ovejas no afectó a su crecimiento, metabolitos sanguíneos ni a la calidad de la canal y de la carne, mostrando únicamente un mayor peso del retículo–rumen y del conjunto de los compartimentos que forman el estómago.

El objetivo del **Ensayo 2** fue evaluar los efectos de la inclusión de esparceta deshidratada en el pienso de cebo de corderos. Veintiséis corderos macho destetados de raza Rasa Aragonesa (14,0 ± 0,49 kg peso vivo) se distribuyeron de forma aleatoria en 3 grupos a los que se les asignaron 3 piensos (isoproteicos e isoenergéticos) que diferían en el nivel de inclusión de esparceta deshidratada: 0% esparceta (0ESP; *n*=9), 20% esparceta (20ESP; *n*=9) o 40% esparceta (40ESP; *n*=8). Los corderos se alojaron en jaulas individuales con pienso, agua y bloques minerales a libre disposición. La fase experimental se prolongó durante 40 días, coincidiendo con el tiempo requerido por los corderos para alcanzar el peso de sacrificio de 25 kg. El incremento del contenido de esparceta de los piensos redujo gradualmente la digestibilidad estimada de la materia orgánica, lo cual se reflejó en una mayor ingestión de materia seca por parte de los corderos 40ESP respecto a los otros dos grupos, mientras que no se observaron diferencias entre ambos grupos restantes. Sin embargo, las mayores ingestiones registradas en los corderos del grupo 40ESP no se reflejaron en mayores pesos al sacrificio o de la canal de estos animales. Los parámetros de fermentación ruminal se vieron poco afectados por la inclusión de esparceta en el pienso, mostrando únicamente un incremento de las proporciones de acético ante la presencia de esparceta en la dieta, independientemente del nivel de inclusión de ésta. En cambio, se obtuvieron efectos notables sobre la biohidrogenación (BH) ruminal. Tanto la inclusión de un 20% como la de un 40% de esparceta en el pienso aumentaron las concentraciones de C18:3 n-3 y disminuyeron las de C18:1 t10 en el rumen, lo que sugiere una potenciación de la ruta t11 de BH ruminal debido a la presencia de esparceta en la dieta. Sin embargo, los mayores porcentajes de C18:1 t11 y menores de C18:2 n-6 se obtuvieron cuando la esparceta se incluyó en un 40% en el pienso, mostrando un efecto mayor sobre la ruta t11 de BH. Estos cambios observados en el rumen como consecuencia de la presencia

de esparceta en el pienso no se reflejaron en su totalidad en el perfil de AG del plasma, aunque sí que lograron incrementar el porcentaje de AGPI n-3 y reducir la ratio AGPI n-6/AGPI n-3 de la carne de la carne de los corderos de los grupos 20ESP y 40ESP. Sin embargo, del mismo modo que en el rumen, estos efectos observados en la carne fueron mayores ante la inclusión de un 40% de esparceta en el pienso, logrando además mayores concentraciones de ácido linoleico conjugado c9,t11 y menores de C18:1 t10 en la carne de este grupo en comparación con la carne de los otros dos grupos.

A la vista de los resultados obtenidos a partir de ambos estudios, se puede concluir que: i) la presencia de PAC de esparceta en la dieta de ovejas durante la fase de cría de un cordero lechal logró buenos rendimientos de las ovejas y mejoró su perfil de AG de la leche, lo cual se tradujo en buenos crecimientos de los corderos sin modificar las características de su canal y su carne y ii) la inclusión de un 20% o un 40% de esparceta deshidratada en el pienso de cebo de corderos logró unos resultados productivos adecuados a la vez que mejoró el perfil de AG de la carne por medio de la modificación de los patrones de BH causados por la presencia de esparceta en la dieta, especialmente notables con la inclusión de 40% de esparceta.

Ante estas conclusiones, puede afirmarse que la inclusión de esparceta tanto en la dieta de ovejas durante la fase de cría de un cordero lechal como en los piensos de cebo de corderos es una opción viable y prometedora, ya que logra buenos resultados productivos de las ovejas y de los corderos en ambos sistemas de producción y permite mejorar las concentraciones de AGPI n-3 tanto de la leche como de la carne. Además, estos resultados podrían ayudar a reforzar la idea que aboga por promover el uso de recursos locales en la alimentación animal, permitiendo disminuir la dependencia de alimentos importados.

# Introduction

Small ruminant production has a long tradition in Spain and plays an important role in the agricultural sector, especially in marginal or less favoured areas, where sheep meat production is more prevalent. Spain is the second largest producer of goat and sheep meat in Europe and within Spain is the fifth in economic importance, accounting for around 7% of the total livestock production (MAPA, 2021). Aragón is the third Autonomous Community with the largest census, 12% of the reproductive sheep. Generally, adult flock maximize the use of natural resources or residues of crop, like stubbles. However, ewes are stalled indoor around lambing and during lactation, thus supplementation must be higher at this time, with high quality forages and/or concentrates. In addition, in areas and/or times of the year with low pasture availability, supplementation becomes even more important. Unlike ewes, lambs do not graze normally at any time and they are stalled permanently with their dams during the lactation period until weaning (Ripoll-Bosch et al., 2014). Thereafter, they are fattened with cereal–based concentrates indoors, until reaching the target slaughter weight necessary to achieve a carcass weight of 8–12.5 kg (approximately 17–26 kg of live weight), which is usually reached with less than 90 days of life (Sañudo et al., 1998). This production system is the most frequent of Aragonese lamb meat, producing homogenous carcasses, pale pink meat, and white fat (Sañudo et al., 2007). Approximately 46% of the lambs sold in Aragón are classified with the label of Protected Geographical Indication (PGI), being the label "Ternasco de Aragón" the most important of the five existing in the sheep sector in Spain. According to this PGI provided by the Regulation (EC) Nº 1107/96, the lambs marketed under this label must belong to one of the 5 Aragonese sheep breeds (Rasa Aragonesa, Ojinegra de Teruel, Roya Bilbilitana, Maellana, and Ansotana) and fed–indoor with cereal–concentrates.

Although the main sheep product in Aragón is the light lamb abovementioned, in the last decade some farmers have shifted towards suckling lamb production (Sanz et al., 2008). The rearing of these lambs is characterised by exclusive suckling feeding. Therefore, the lambs remain with their dam until they reach a live weight of about 10–12 kg (5.5–7 kg carcass weight) at approximately 35–45 days of life. It is a profitable type of meat production for farmers, since the cost of production is low and the time required is short. The meat obtained from these animals is highly appreciated for being very juicy

and tender, which is reflected in its price, 9.44€/kg on average in 2021 (MAPA, 2021), being the best paid lamb format in Spain.

Therefore, it can be stated that adult sheep system is characterised for being extensive, whereas fattening lamb production is a completely intensive system. This intensive system is facing some socio–economic challenges which are pushing towards some changes in production models. On one hand, global economic instability is forcing farmers to advocate for a system entailing local resources that provide them greater self–sufficiency. On the other hand, the self–sufficient systems have a lower environmental impact, making them a good alternative to the growing concern about the contribution of livestock farming to climate change (IPCC, 2022). Finally, there is also an increasing demand for healthier products by consumers, which is one of the most important current goals in animal production (Parodi, 2016; Willett et al., 2019).

The use of local forage legumes has been recently promoted in animal feeding as it responds to many of those challenges. The high protein content of local forage legumes could compete with some imported protein sources for animal feed (Moorby and Fraser, 2021), reducing the dependence on foreign markets. Besides, forage legumes have a high protein/carbohydrate ratio, contrarily to graminacae, making them an excellent choice to be combined with grass species in ruminant diets. In addition, some beneficial properties of forage legumes have been shown to decrease the detrimental environmental impact. The symbiotic relationships existing between this plant family with several species of soil bacteria capable of fixing atmospheric nitrogen (Hasanuzzaman et al., 2020) lead to a natural fertilisation of the soil that decreases erosion and nitrate leaching, thus reducing the need for industrial fertilisers and increasing soil enrichment when used in rotational cropping crops (Ledgard and Steele, 1992; Rochon et al., 2004; Chapagain and Riseman, 2015). For this reason, European policies encourage the cultivation of legumes through subsidies granted by the Common Agricultural Policy (CAP). Also, ruminant production systems based on legumes have shown lower $N_2O$ emissions per hectare and methane ($CH_4$) per kg of dry matter (DM) intake or per kg of milk or meat produced when compared to grasses (Rochette and Janzen, 2005; Eggleston et al., 2006; Waghorn and Clark, 2006).

Besides of simultaneously achieving greater sustainability and self–sufficiency and improving animal welfare, the inclusion of locally produced forages has been commonly studied for improving the fatty acid (FA) profile of meat and milk (Buccioni et al., 2015; Huyen et al., 2020; Moorby and Fraser, 2021; Santos-Silva et al., 2023). Forages are rich in C18:3 n-3 (Glasser et al., 2013), becoming an important source of this beneficial FA that can be potentially deposited on milk or meat. In addition, the increase of forage inclusion promotes the biohydrogenation (BH) pathway producing C18:1 t11, instead of the formation of C18:1 t10 isomer, which is associated with concentrate–rich diets (Griinari et al., 1998), and may lead to improvements in the FA profile of ruminant–derived products. These interesting characteristics of local forage legumes have led to great interest in the study of numerous species, which are widely used in ruminant feed (e.g. lucerne, *Medicago sativa*; clover, *Trifolium spp*.; birdsfoot trefoil, *Lotus corniculatus*; sainfoin, *Onobrychis viciifolia*; or sulla, *Hedysarum coronarium*), having shown beneficial results for ruminant production systems on animal performance, product quality, health, and environmental sustainability (Waghorn et al., 1998; Rochon et al., 2004; Waghorn and Clark, 2006; Aufrère et al., 2008; Bonanno et al., 2011; Ripoll et al., 2012; Lobón et al., 2016; Johansen et al., 2018).

All these positive aspects of forage legumes are reflected in the importance of the cultivation of these species. The cultivation of forage legumes in Spain entails a total of 454,700 ha, outstanding alfalfa with 64,800 ha cultivated (57% of the total area of forage legume production in Spain) (MAPA, 2020). Aragón is a major producer of forage legumes, accounting for 37% of the total alfalfa surface in Spain (84,546 ha). However, most forage legumes are grown under irrigated conditions and, given that the current scenario of drought and degraded soils is expected to worsen in the Mediterranean (Lead, 2020), therefore there is a growing need to look for feed resources that yield well with less need for water and irrigation. In this context, sainfoin (*Onobrichis viciifolia*) is a suitable forage legume. Despite being a minority crop, the total area under sainfoin cultivation in Spain is 6,800 ha, the distribution and conditions of the harvested area of sainfoin perfectly reflect that it is a rustic crop, well adapted to cold and water scarcity. Nevertheless, sainfoin has aroused interest due to its moderate proanthocyanidins (PAC; commonly known as condensed tannins) content, which has been the focus of most

recent studies on sainfoin. These secondary compounds have shown beneficial effects for animal production, such as a decrease in methane and ammonia production, a decrease of bloat risk (Waghorn, 2008) an increase in protein flow to the small intestine (Frutos et al., 2004), a reduction of the impact of intestinal nematodes (Niezen et al., 1995), and a beneficial modification of ruminal BH capable of improving the FA profile of the final product (Frutos et al., 2020).

In this framework, it would be interesting to study the effects of including sainfoin in the diet to find out whether it leads to good productive results and whether it can improve the quality of the edible products of different sheep production systems

# Background

## SAINFOIN

Sainfoin (*Onobrichis viciifolia*) is a legume widely adapted to the warm–temperate and dryland of Mediterranean areas (Hayot Carbonero et al., 2011). The cultivation of forage legumes in Spain is usually carried out with irrigation, with alfalfa being the main species as shown in Figure 1. The total area under sainfoin cultivation in Spain is 6,800 ha, of which 97% correspond to dry cultivation (Figure 1). Aragón accounts for 33% of the total cultivated area and of the sainfoin harvested in Spain, being the second most important autonomous community after Castilla y León, with 44% of the cultivated area used for grazing and the remaining 56% is harvested (MAPA, 2020).

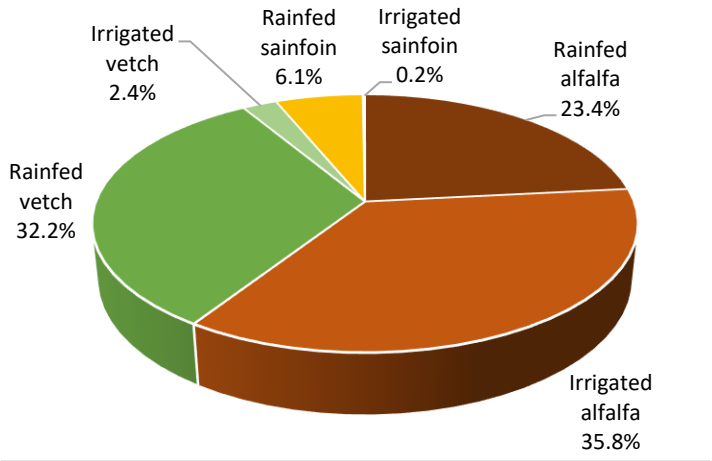


**Figure 1.** Percentage and type of crop of the main forage legumes in Spain (MAPA, 2020).

Although sainfoin dry matter (DM) yield is lower than that of alfalfa, when sainfoin is harvested at its optimum time, close to May, its yield is 4t DM/ha and it has less than 400 g neutral detergent fibre (NDF)/kg DM and an organic matter digestibility (ODM) of 650 g/kg (Borreani et al., 2003). As a legume, it is also characterised by their N fixation properties of approximately 130 and 160 kg/ha, as stated by Provorov and Tikhonovich (2003), which lead to a natural fertilisation of the soil, decreasing the dependence on N chemical fertilisers. It also shows good results in cold and high–altitude areas, like most legumes (Black et al., 2009). In addition, sainfoin is considered a pluriannual forage, with spring production accounting for two thirds of annual production (Delgado et al., 2008). The usual form of utilisation is a spring cut for hay, in full bloom, and a cutting or grazing of one or two regrowth (Cavallarin et al., 2005), being the quality of the late use notably

lower (Delgado et al., 2008). Therefore, sainfoin should be conserved to optimize its use, being more common in Spain to conserve it as hay (MAPA, 2020).

## TANNINS

Tannins are secondary compounds naturally occurring and widely distributed in the plant kingdom (Porter, 1992). Although they can be found in several plant tissues, including twigs, wood, bark, roots, seeds, and fruit (Porter, 1992), they must be found in foliage to be useful for animal nutrition (Waghorn, 2008). Gross (1999), following the definition of tannins that was formulated by Freudenberg in 1920, divided into two main groups: hydrolysable tannins (HT) and condensed tannins (CT), also known as proanthocyanidins (PAC); whose difference lies in their chemical structure. While HT consist of a carbohydrate core with hydroxyl groups esterified with phenolic acid (gallic or hexahydroxyohenic acids), PAC are polymers of flavonol units presenting usually higher molecular weight (Mueller-Harvey, 1999). Although this classification is still valid, many researchers point out that it is a rather poor view of the chemical structure of these compounds (Van Soest, 1994; Mueller-Harvey, 1999).

Tannins have been studied for more than 50 years and they were considered anti–nutritional or even toxic molecules (Reed, 1995). It was not until the beginning of the century that the first studies appeared to obtain desirable productive results associated with the consumption of tannin forages (Min et al., 2003). The effect of tannins on animals that ingest them is based on the fact that these compounds form insoluble complexes with various components of the ration. They show a special affinity for dietary proteins (Hagerman and Butler, 1981) to which they bind from phenolic hydroxyl groups (Naczk et al., 1996). By forming these complexes, they interfere in their metabolism, affecting the effect that certain nutrients have on the animals that ingest them. In addition, they also interact with other diet compounds such as cellulose, hemicellulose, and pectin (McSweeney et al., 2001), and even minerals, metal ions (Scalbert, 1991; McDonald et al., 1996) and ruminal microorganisms (Giner-Chavez, 1996). The chemical structure of tannins is highly variable and must be known, since the effect that these compounds will have on the animal performance depends on their chemical structure (*e.g.,* Mueller-Harvey, 1999; Molan et al., 2002; Kraus et al., 2003). For this reason,

several studies based on the chemical structure of tannins have been carried out in recent years. As sainfoin is characterised by its medium PAC content, this type of tannin is explained below in more detail.

**PROANTHOCYANIDINS (PAC) OR CONDENSED TANNINS**

Proanthocyanidins are a type of flavonoid, which are part of the secondary metabolites of plants widespread in dicotyledonous species and occur infrequently in graminacae (Waghorn, 2008). Their origin is not entirely clear, although it is thought to be an adaptive method of deterring herbivores from eating the PAC–containing plants (Feeny, 1976), as they can confer an astringent and undesirable taste (Jöbstl et al., 2004). However, although they do appear to repel insects (Salminen and Karonen, 2011), many ruminants have selected and preferred PAC–containing plants (Waghorn, 2008). Their concentration, type, and chemical structures, varies depending on the plant species and variety, vegetative stage, the method of forage preservation, climate, among others (Waghorn, 2008; Mueller-Harvey et al., 2019); thus, it is a very large and heterogeneous group of chemical compounds.

Proanthocyanidins are originated in the cell cytoplasm from phenylalanine and acetate and are subsequently stored in the vacuoles (Mueller-Harvey and McAllan, 1992; Zhao et al., 2010; Jonker and Yu, 2017). Proanthocyanidins are polymers from dimers to over 20 flavanol units with several flavan–3–ol structures within each polymer (Waghorn, 2008). Most of the PAC present in common forage species (*i.e.,* birdsfoot trefoil, big clover, sainfoin and lespedeza) are primarily composed of the four flavan–3–ol subunits, which differ in their chemical structure: catechin, epicatechin, gallocatechin and epigallocatechin (Figure 2). Catechin and epicatechin both have two hydroxyl groups on the so–called B-ring, but differ in their stereoisometry, with the former having the C3 hydroxy group in the *trans*-position and the latter in the *cis*-position. When these two polyenes are combined, they give rise to procyanidins (PC). In contrast, gallocatechin and epigallocatechin have 3 hydroxyl groups on the B-ring, the first having the C3 hydroxy group in the *trans*-position and the second in the *cis*-position and both give rise to prodelphinidin (PD) (Zeller, 2019).

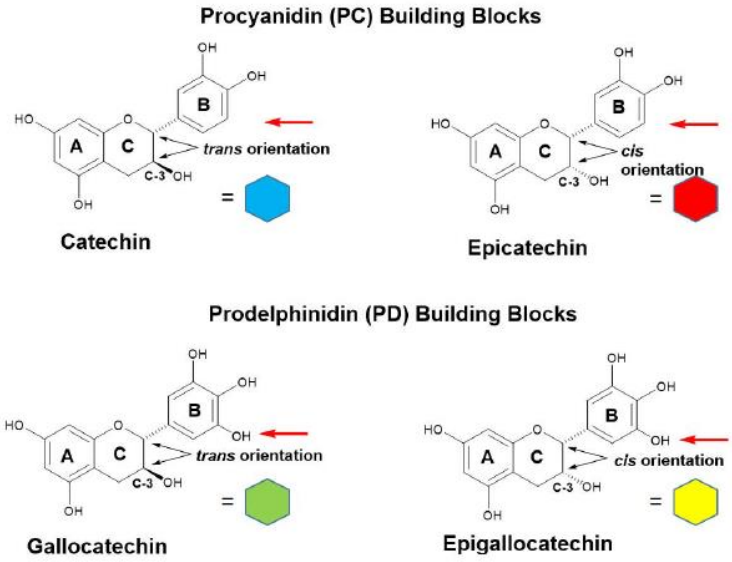


**Figure 2.** Flavan-3-ol subunits used as building blocks in production of proanthocyanidins in common forages, obtained from Zeller et al. (2019).

Therefore, the chemical structure must be taken into account when studying their effect on animals, being the PC/PD ratio one of the most important factors. This is because the PC and PD units have different structures that cause diverse effects on animals depending on the concentration of these units. Prodelphinidin units have more phenolic hydroxyl groups capable of forming hydrogen bonds with proteins and, therefore, has been shown to have a greater capacity to bind proteins than PC at similar molecular weight (Jones et al., 1976). Nevertheless, it has been established that the main degree of polymerisation (mDP) is a determining factor for PAC protein aggregation and precipitation (Zeller et al., 2015). A higher mDP, which is determined by the number of monomers forming the PAC, entails a greater surface area which increases the chances of them binding to compounds (Jones et al., 1976; Zeller et al., 2015). Other factors affecting PAC behaviour are *cis*/*trans* ratio and the type of linkages between flavonol monomers, called interflavan linkages (Zeller, 2019).

In addition to the complexity of the chemical structures of PAC and elucidating the effects they produce, it is important to understand the problems related to their detection and quantification methods. Differences in analytical methods may impair the comparability between studies in which tannins are used for feeding animals (Silanikove et al., 2006; Gravador et al., 2015). On one hand, PAC can be found free in the organism

(soluble PAC) or bound to compounds with which they form complexes, mostly bound to proteins (protein–bound PAC) or to fibre (fibre–bound PAC). Therefore, analysing only free PAC could lead to a serious quantification error. Determination of PAC concentration requires both unbound and bound PAC to be measured using purified PAC from the same species analysed as a standard (Waghorn, 2008) to better study and predict the effect of PAC inclusion in animal diets. Another aspect to consider is that there are several analytical methods, some more specific and recommended than others, to quantify the concentration of PAC, which makes the comparison between studies even more complex (Schofield et al., 2001; Waghorn, 2008; Mueller-Harvey et al., 2019).

Besides, PAC concentration in plants is highly variably and hardly predictable. It differs depending on several factors, as genetics, species, and variety (McMahon et al., 2000), although different contents can be found when analysing specimens of the same species (Hatew et al., 2015; Jonker and Yu, 2017). Other aspects affecting PAC concentration in plants are leaves/stems ratio and phenological stage of plants, showing a general decrease of PAC content associated with plant maturity (Wang et al., 2015). Forage conservation is also related to PAC content, as it can be reduced during forage processing due to the oxidation. The destruction of PAC during hay conservation is usually lower than those observed with silage due to partial rupture of plant cells, although the degree to which PAC are affected depends on temperature, ultraviolet light, and processing time of silage production process (Wang et al., 2015; Rufino-Moya et al., 2019a).

**Sainfoin PAC**

One of the reasons why sainfoin has been studied in the last years, is mainly due to its PAC content. The average PAC content of fresh sainfoin ranges between 10–90 g/kg DM (Mueller-Harvey et al., 2019). This variability depends on the variety, harvest time, location of the crop (Azuhnwi et al., 2013; Hatew et al., 2015), and phenological stage (Borreani et al., 2003). Although PAC are distributed in all parts of the sainfoin plant (Li et al., 2014), these secondary compounds are found in higher concentration in the leaves (McMahon et al., 2000) and, therefore, as proportion of leaves decreases with plan maturity, PAC concentration in fresh sainfoin decreases from vegetative to end–flowering stage (Rufino-Moya et al., 2019b). Most of the PAC of fresh sainfoin are found as

extractable PAC and no reduction in total PAC concentration was found when sainfoin was conserved as hay, although there was a slight decrease when it was kept as silage (Rufino-Moya et al., 2019a). An example of flavanol monomers and PAC structures of sainfoin are illustrated in Figure 3.

In addition to the variations found in PAC concentration, literature has demonstrated that sainfoin PAC exhibit highly structural variation (Gea et al., 2011; Novobilský et al., 2011; Azuhnwi et al., 2013; Malisch et al., 2015; Ramsay et al., 2015). A study of a large sainfoin germplasm collection from the EU 'HealthyHay' project found also great variability in PAC structure. Sainfoin samples from all the European territory displayed a wide range of differences: PAC contents varied from 0.57 to 2.80 g/100 g sainfoin and the mDP from 12 to 84 and, although PD and *cis*-flavanol units always predominate over PC and *trans*-flavanol units, the PD/PC ratio ranged from 52.7/47.3 to 94.8/5.2, and the proportion of *trans*-flavanol units from 12% to 34% (Stringano et al., 2012). All these types of variations could be the explanation for some contradictory effects or different responses of animals to the effects of sainfoin.

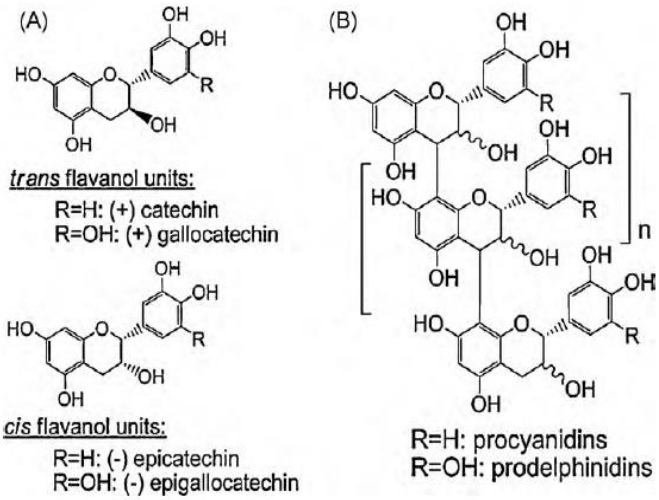


**Figure 3.** Sainfoin flavanol monomers (A) and PAC (B), obtained from Theodoridou et al. (2010).

**How PAC–containing forages inclusion in diets can affect animals?**

The variability of the effects that the intake of PAC can have on animals is very wide, as it depends on numerous factors, such as the dose and chemical structure of the tannins (Patra and Saxena, 2011), the type of diet (Vasta et al., 2009a), the animal

species (Robbins et al., 1991), and even the physiological or productive state of the animals (Piluzza et al., 2014), among others. A list of the effects of PAC on digestion in ruminants ordered according to whether they are observed more or less frequently can be found in the review of Waghorn (2008).

Two of what are possibly the most studied effects of PAC are their ability to reduce gastrointestinal parasites and gas formation and emission. The PAC have shown to reduce the impact of both nematode (Butter et al., 2000; Hoste et al., 2015; Rivaroli et al., 2019) and coccidian infections (Saratsis et al., 2016; Rivaroli et al., 2019) by decreasing the availability of essential nutrients, mainly protein, for the parasitism development (Niezen et al., 1995; Waghorn, 1996). This fact is of great importance because parasitic infections cause mainly subclinical infections, producing negative economic impacts on ruminant (Calvete et al., 2020). Furthermore, due to frequent anthelmintic treatments in livestock, resistance to chemicals commonly used nematodes control is becoming an increasing problem. For this reason, recent studies have focused on finding an optimal concentration and composition of PAC that can provide both nutritional and anti–parasitic effects (Cherry et al., 2014). Thus, natural additives with anthelmintic properties, such as PAC, could be used instead of synthetic products (Rivaroli et al., 2019).

In the case of **sainfoin PAC**, their ability of to reduce gastrointestinal parasites is also one of the most studied aspects. The review of Hoste et al. (2015) about secondary compounds action to prevent and decrease the impact of digestive parasitism concluded that sainfoin has solid effect against gastrointestinal infections. However, sainfoin PAC concentration has been shown to affect the effectiveness of the response, suggesting a dose–response effect (Manolaraki, 2011; Novobilský et al., 2013). In addition, Manolaraki (2011) showed higher effectiveness of those sainfoin PAC with lower degree of polymerisation and lower molecular weight. The ability of sainfoin PAC to reduce parasitism has been shown to be useful in sheep fed sainfoin hay (Saratsis et al., 2012; Arroyo-López et al., 2014; Saratsis et al., 2016), hay with silage (Werne et al., 2013), pellets (Rivaroli et al., 2019; Komáromyová et al., 2022) and fresh sainfoin (Brunet et al., 2007) against several species (*e.g.*, *O. ostertagia, H. contortus, C. conrniculatus, Eimeria, T. columbriformis*).

The other important benefit provided by PAC is their effect on decreasing $N_2O$ and methane emissions, two potent greenhouse gases linked to livestock production. The cause of the decrease in the production of these gases by PAC is still under study. It seems that several factors are responsible, as the reduction of bacteria and protozoa population responsible of gas production or as the reduction of hydrogen production due to a general decrease of feed degradation in the rumen (Kingston-Smith et al., 2010; Patra and Saxena, 2011; Piluzza et al., 2014). In addition, the presence of PAC in fresh forages leads also to a lower bloat risk by reducing the stability of foam trapping the ruminal fermentation gases (Kingston-Smith et al., 2010; Mueller-Harvey et al., 2019). This feature can be interesting as is well–known that high fresh legumes forage intake can produce bloat in ruminants (*e.g*., alfalfa or clover).

Existing literature on the relationship between **sainfoin PAC** and rumen function has shown positive results. Lower ruminal fermentation and gas and ammonia productions were found for sainfoin hay compared to alfalfa hay in an *in vitro* assay (Toral et al., 2016). However, when sainfoin and alfalfa were evaluated in fresh, inexplicably, those differences were not found on gas and methane productions (Rufino-Moya et al., 2019b). Sainfoin pellet showed a reduction of urea N in blood and urine in dairy cows supplemented at 20% (Grosse Brinkhaus et al., 2016) and a reduction of methane production *in vitro* using 30% of supplementation (Niderkorn et al., 2020). However, it should be borne in mind that the results obtained depend on numerous factors. In relation to this, Hatew et al. (2015) showed that the PD/PC ratio of sainfoin is negatively related to methane production, which explains much of the variability in the results obtained with different sainfoin varieties.

Given the wide ranging of effects that PAC can have on animals, the factors on which they depend, and the large number of reviews on this topic, only the most relevant to this thesis are explained in depth below, along with the studies that have focused specifically on sainfoin.

**Effects of PAC on animal intake and growth**

As mentioned above, the capability of PAC to bind and form complexes with the nutrients of the ration is probably the most important effect produced by these

compounds. The interaction between PAC and protein begins in the mouth at the time animals ingest a PAC–containing feed, since PAC are able to bind with salivary and plant proteins during chewing (Mangan et al., 1976; Waghorn, 2008). The bond between these secondary compounds and salivary protein can produce an astringent sensation that can lead to a reduction in ration palatability and DM intake (Lamy et al., 2011). However, the formation of complexes with dietary protein is of particular interest. This binding renders the protein (and other nutrients) inaccessible to microorganisms (Mueller-Harvey and McAllan, 1992), decreasing their ruminal degradability and, therefore, increasing the small intestine reach (Wang et al., 1996). Once in the intestine, the protein is more efficiently absorbed and used as protein to meat, milk, or wool. Thus, dietary PAC can lead to a higher nutrient efficiency but, when the concentration of tannins in the diet is excessively high, these effects can negatively affect animal performance (Hervás et al., 2003; Waghorn, 2008). Therefore, there are several animal responses depending on the specific characteristics of the trials conducted, with studies showing an increase (Valderrábano et al., 2010; Giller et al., 2021), decrease (Girard et al., 2016), or no effect (Peng et al., 2016) of PAC on average daily gain (ADG) of lambs. This idea is reinforced by the recent meta–analysis of Álvarez-Rodríguez et al. (2020), who stated that results related to the effect of PAC on lambs' performance are not conclusive and pointing out that lambs' growth is more related to dietary fat and protein contents.

Concerning **sainfoin PAC** effects on animal performance, this forage has demonstrated promising results as ingredient in ruminal diets, showing a wide variety of effects on performance depending mainly on PAC concentration and the way that sainfoin is offered. *In vitro* assays showed a reduction of total volatile fatty acids (VFA) production with sainfoin hay compared to alfalfa hay (Toral et al., 2016), which could compromise animal performance, but greater organic matter degradability of sainfoin has also been reported (Rufino-Moya et al., 2019b). The lambs whose dams grazed sainfoin during lactation tended to obtain higher growth until weaning and greater slaughter weight after a fattening period compared to those whose dams' grazed alfalfa or were fed indoor with concentrate plus straw in Lobón et al. (2019a). Fattening lambs grazing alfalfa improved their growth when sainfoin pellets (42 g of PAC/kg of DM) were administrated at 36% of the diet (Rivaroli et al., 2019). However, growth and carcass

performance were reduced in lambs receiving sainfoin silage (104 g total PAC/kg DM) plus concentrate compared to their counterparts with alfalfa and red clover silages, despite similar DM intake among groups (Girard et al., 2016). These contradictory results are likely due to the different response of the animals depending on the concentration of sainfoin PAC in the diet.

**Effects of PAC and forage on ruminal biohydrogenation**

The final FA profile of ruminant products is given by preformed FA uptake and *de novo* synthesised FA. Preformed FA uptake includes all the FA that are absorbed in the digestive tract, which is a mixture of those with dietary origin, those that are formed in the rumen during the biohydrogenation (BH) process (Jenkins et al., 2008), and the FA provided by lipid mobilisation (Palmquist, 2006).

In the field of animal nutrition, one of the final aims is to improve the FA profile of animal products towards a healthier product for consumers (Parodi, 2016). Nowadays it is known that the ruminal BH process affects the fatty acid profile to be depot in animal tissues. It is therefore essential to know these ruminal processes in order to obtain the meat and milk quality demanded by consumers.

Ruminal BH is a complex process composed of multiple pathways that sequentially isomerise and hydrogenate the dietary FA. In the first place, when esterified FA reach the rumen, they are hydrolysed by lipases, releasing free unsaturated FA (Buccioni et al., 2012). From this moment on, free unsaturated FA are generally saturated through the ruminal population to reduce their toxicity (Jenkins et al., 2008), being the C18:0 the last product of the BH. Therefore, the final FA profile of ruminant–derived products will be more saturated FA as a consequence of ruminal BH (Scollan et al., 2017). Nevertheless, during the BH process there are intermediate stages forming intermediate FA, some of which are unique in ruminant products and beneficial for consumers health, such as some PUFA n-3, CLA c9,t11, and C18:1 t11 (Shingfield et al., 2008; Buccioni et al., 2015). Since ruminal BH is never produced to the same extent, the degree to which it is completed determines whether the final profile is more or less rich in PUFA,

intermediate FA or saturated FA, being the degree of ruminal BH extent of relevance for consumers health (Shingfield et al., 2008; Hennessy et al., 2011).

Therefore, due to the numerous factors on which ruminal BH depends, the improvement of the FA profile of ruminant products through the modification of ruminal BH entails a complex situation. However, some actions, such as the increase of forage inclusion in the diet or the presence of PAC, have been shown to be able to modify the natural course of those FA in the rumen reducing the ruminal saturation of dietary FA which leads to an increase in PUFA and some intermediate FA concentration in the rumen with bioactive and beneficial properties.

Effects of forage diets on ruminal biohydrogenation and FA profile of products

Increased dietary fibre intake affects ruminal fermentation patterns in several ways. Firstly, it promotes chewing and salivation, which increases the buffer capacity of the rumen (Blanco et al., 2014). This, together with a high development of the rumen papillae and their surface area, promoting the absorption of volatile fatty acids (VFA; the main source of energy in ruminants) and the rumen pH, which prevents problems related to ruminal keratinisation (Álvarez-Rodríguez et al., 2012). The occurrence of keratinisation has been linked to diets with a low forage/concentrate ratio, in which a large amount of concentrate produces an excess of VFA that cannot be absorbed by the papillae, reducing the rumen pH and causing damage to the rumen wall (Van Ackeren et al., 2009). Besides, the higher forage proportion in the diet can also lead to a longer retention time of the feed in the rumen and an improvement of environment for cellulolytic bacteria, which would lead to a greater extension of the BH and fermentation processes (Sackmann et al., 2003).

In addition, forages have a very beneficial FA profile, as they are mostly composed of PUFA, being C18:3 n-3 their major FA (Glasser et al., 2013). That makes the inclusion of forage in the diet a potential way to improve the FA profile of meat and milk. The increase of dietary PUFA from implies increased PUFA in ruminant products (Woods and Fearon, 2009; Álvarez-Rodríguez et al., 2022), despite the trend towards saturation due to the ruminal BH process.

The forage/concentrate ratio of the diet also affects the type of ruminal BH that takes place. Intensive systems, in which there is a high inclusion of concentrate in ruminant diets, lead to a shift in the ruminal BH pathway. There are two major C18:1 isomers produced during the BH process (Figure 4): C18:1 *trans*-11, which is associated with forage-rich diets, and C18:1 *trans*-10, related to concentrate–rich diets (Griinari et al., 1998; Bessa et al., 2005). The C18:1 *trans*-11 is considered the main BH pathway, however, when the concentrate is very high in the diet, this *trans*-11 pathway is replaced by the *trans*-10 BH pathway. This shift from the *trans*-11 pathway to the emergence of the *trans*-10 pathway is called ''*trans*-10 shifted''. Promoting the *trans*-11 pathway have been one of the main targets as is the way to enhance CLA c9,t11 production from C18:1 *trans*-11, both considered as beneficial FA with implications on human health (Palmquist et al., 2004). In contrast, the *trans*-10 BH pathway is considered non desirable as it has been linked to negative health (Aldai et al., 2013) and productive (Tricon et al., 2004) implications.

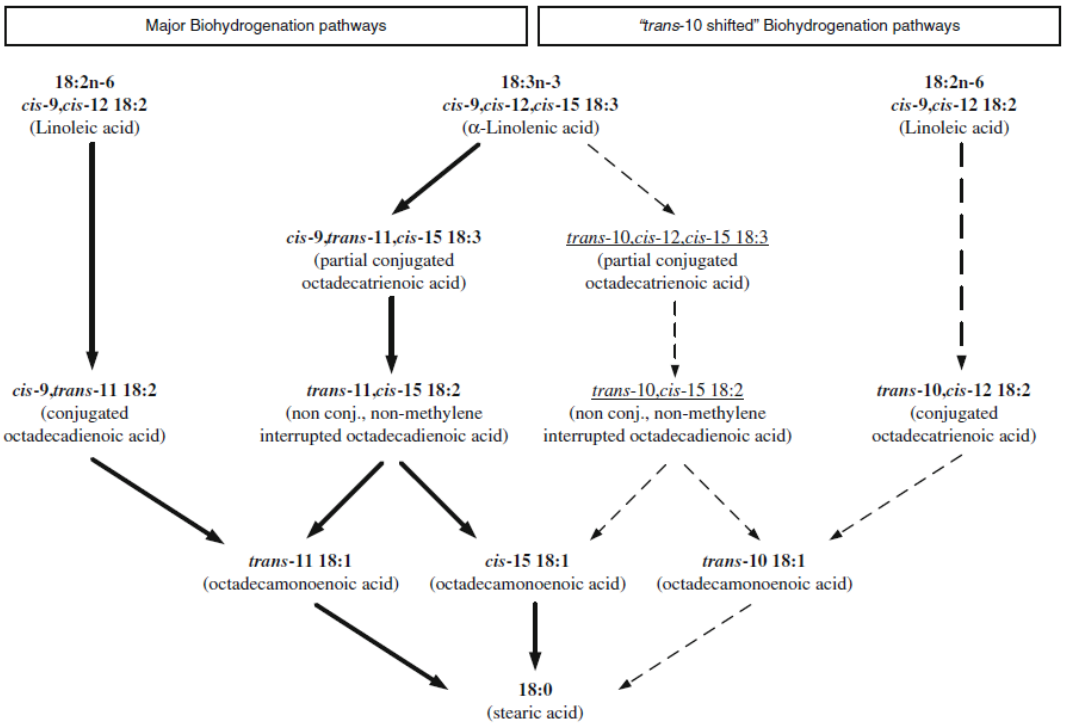


**Figure 4.** Main and ''*trans*-10 shifted'' ruminal biohydrogenation pathways of linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3), obtained from Alves and Bessa (2014).

Effects of PAC–containing diets on ruminal biohydrogenation and FA profile of products

Proanthocyanidins can influence the natural development of ruminal BH. The changes exerted by PAC on ruminal BH are due to a combined effect. In one side, PAC can modulate ruminal lipid metabolism by positively reducing the general extent of ruminal BH and, consequently, decreasing the FA saturation, or even affecting the activity of enzymes involved in the BH process, as $\Delta^9$-desaturase (Vasta et al., 2009a; Frutos et al., 2020). In the other side, PAC can modify the rumen microbial community (Costa et al., 2017; Vasta et al., 2019) due to changes in rumen conditions or directly interacting with bacterial cell membranes (Smith et al., 2005), affecting the BH reactions in which ruminal microorganisms are involved and, therefore, the synthesis of some FA produced by certain bacterial population (Vlaeminck et al., 2006). For example, ruminal cellulolytic bacteria are responsible for the synthesis of BCFA (Fievez et al., 2012), which have been considered healthier for consumers (Shingfield et al., 2008; Parodi, 2016).

However, there is no consensus on which step of the rumen BH is most affected by PAC. While some publications reported a specific effect of PAC reducing the first step of PUFA metabolism in the rumen (Campidonico et al., 2016; Alves et al., 2017), other studies pointed an effect on the last step of the BH process, when the beneficial C18:1 t11 (vaccenic acid) is saturated to C18:0 (stearic acid) (Vasta et al., 2009c; Rana et al., 2012). Depending on which step of the BH process the PAC is affecting, the final FA profile will present an enhanced concentration of one FA or others, as shown by the BH scheme (Figure 5) obtained from Frutos et al. (2020). Briefly, an inhibition of the initial step of BH would lead to a higher “preservation” of the dietary PUFA but would decrease the formation of some beneficial FA which are originated during the following steps. However, the inhibition of the next step, would decrease the dietary PUFA, which would be already transformed to different C18:3 and C18:2 isomers (known as BH intermediates). Lastly, the inhibition of the last step would favour the appearance of C18:1 isomers produced from C18:2 and C18:3 isomers, reducing the content of the latter. Again, the differences reported in the literature are possibly due to the concentration and structure of the PAC.

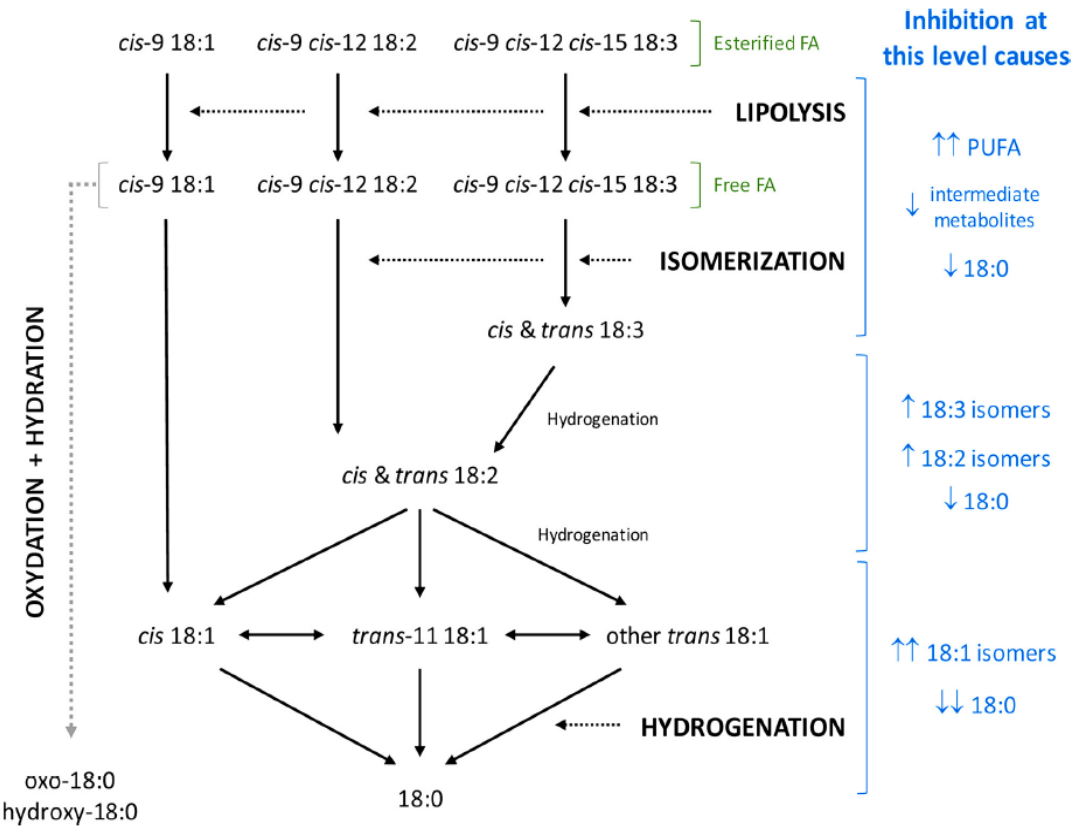


**Figure 5.** Schematic pathways of dietary unsaturated fatty acid metabolism in the rumen and the effects of their inhibition, obtained from Frutos et al. (2020).

The literature concerning the effects produced on ruminal BH by the presence of **sainfoin PAC** in the diet is scarce. An increase in C18:2 n-6, C18:1 c9, and total PUFA were found in *in vitro* assays using sainfoin hay compared to alfalfa hay (Toral et al., 2016), which were associated to a lower BH of dietary FA during the first steps of the process. Similarly, Campidonico et al. (2016) found greater concentrations of total PUFA and C18:3 n-3, leading to a lower MUFA formation in the subsequent steps of ruminal BH in lambs fed with sainfoin and red clover silages (as two PAC–containing legumes) compared to those receiving timothy (a grass without PAC). In the same study, lower total BCFA concentrations were also found and a reduced BCFA, suggesting changes in ruminal microbiota.

**Effects of PAC on product quality**

There are several parameters related to product quality that can be affected by the presence of PAC in animal diets, such as the FA profile, lipid oxidation, and colour

stability. Regarding the effect of PAC on the FA profile of meat and milk, Frutos et al. (2020) showed that, despite of the highly variable results, approximately in the 50% of the studies included in the review, PAC increased total PUFA and C18:1 t10 milk concentrations. However, most studies showed no effect on C18:1 c9, C18:1 t11, CLA c9,t11, and C18:0 concentrations in either meat or milk. In addition, the modulatory capacity of PAC on meat FA profile seemed to be less effective compared to changes observed in milk, with no significant changes due to PAC on most of the FA highlighted in the abovementioned review and only a slight increase in dietary PUFA, C18:3 n-3, and C18:2 n-6.

It should be borne in mind that the change in the FA profile of the product may entail other changes. Thus, although the effect of PAC on increasing PUFA concentration in animal products is a health desirable goal (Parodi, 2016; Toral et al., 2018), PUFA are more easily oxidised due to their chemical structure (Morrissey et al., 1998), which can compromise products stability, self–life, and quality. Lipid oxidation is an important process, as it can be also negatively reflected in several product attributes such as colour, texture, taste and aroma, leading to rancidity and off–flavours (Amaral et al., 2018). However, those effects can be counteracted by PAC, which has been shown to have antioxidant capacity (Hagerman et al., 1998; Soobrattee et al., 2005) so they can help to prevent lipid oxidation. The origin of this properties has been attributed to their free radical scavenging, chelation of redox active metals, and inactivation of haemoproteins by reducing highly prooxidant ferric states (Iglesias et al., 2012). This improvement of antioxidant activity of animal consuming PAC has great importance as it can increase the self–life of the final product and has been proved in meat (Vasta and Luciano, 2011; Valenti et al., 2019), milk (Santos et al., 2014; Delgadillo-Puga et al., 2019), and plasma and liver (López-Andrés et al., 2013).

Also related to iron metabolism, the inclusion of PAC in the diet of ruminants has been linked to a delay in metmyoglobin formation leading to an increase in colour stability (Priolo et al., 2002a; Luciano et al., 2011; Lobón et al., 2017a), which could also contribute to a greater self–life of the edible products and an improvement on consumer choice and acceptance. Several studies recorded lighter meat colour in lambs fed with PAC–containing diets compared to control diets or given the same diet plus polyethylene

glycol (PEG; as PAC blocking agent) (Priolo et al., 2000; Priolo et al., 2002a; Priolo et al., 2005; Lobón et al., 2017a), which is desirable in the Spanish lamb market as consumers prefer pale meat to dark meat (Sañudo et al., 1996). However, when Álvarez-Rodríguez et al. (2020) conducted a meta–analysis including 41 articles it was stated that feeding PAC diets to lambs was not clearly linked to an improvement in lamb meat colour, so this is not a generalised effect.

Finally, it has been discovered that forages rich in PAC can reduce skatole and indole ruminal formation (Priolo et al., 2009; Girard et al., 2016; Rivaroli et al., 2019) by decreasing protein degradation and the activity of microbes involved in their synthesis (Schreurs et al., 2008). This is a desirable effect on meat quality because the deposition of these compounds in lambs' fat confers a sheep meat' odour that is not well accepted by consumers.

The studies concerning the effect of **sainfoin PAC** in ruminant diets on product quality are mainly focused on the improvement of the FA profile of edible products. Grosse Brinkhaus et al. (2016) recorded higher C18:3 n-3 and lower C22:5 n-3 and C22:6 n-3 in milk from dairy cows receiving 20% of sainfoin pellets compared to cows fed with the same amount of birdsfoot trefoil, while dairy ewes fed with sainfoin silage showed reduced n-6/n-3 ratio in milk (Pascual et al., 2019). On the other hand, several studies had obtained a healthier FA profile in lambs' meat due to the presence of sainfoin in diet, showing greater concentrations of PUFA and PUFA n-3 and lower of SFA and n-6/n-3 ratio (Girard et al., 2016; Campidonico et al., 2016; Lobón et al., 2019b).

Other interesting results related to sainfoin PAC intake and meat quality are the decrease in lipid oxidation (Lobón et al., 2017a) and the increase in α-tocopherol content (Rufino-Moya et al., 2020) found in meat from lambs whose dams grazed sainfoin during lactation. Besides, lower skatole and indole levels of sainfoin–fed lambs were found in perirenal fat (Girard et al., 2016) and in perirenal and dorsal fat (Rivaroli et al., 2019) compared to alfalfa–fed lambs. These results confirm the theory affirming that higher ruminal indoles formation occurs in non–containing–PAC legumes, as alfalfa or white clover, compared to the PAC–containing, due to the greater protein degradability (Schreurs et al., 2008). This finding could help to improve consumers acceptance of meat

from lambs fed with high percentage of forage in the diet, as high content of indole in animal product can involve changes in taste, which may be rejected by consumers.

# Objectives

The main objective of the present thesis was to evaluate the effect of feeding sainfoin, a legume forage with medium content of proanthocyanidins, on two lamb production systems. To achieve the main objective, two studies were carried out to assess:

1. The effect of the proanthocyanidins of fresh sainfoin fed to lactating ewes, using polyethylene glycol as blocking agent, during the rearing period of a suckling lamb on:
    1.1. Body weight and body condition score, dry matter intake, metabolic and antioxidant blood status, and digestive parasitism of the dams.
    1.2. Yield, chemical, and fatty acid composition of dams' milk.
    1.3. Growth, metabolic status, digestive development, carcass traits, and meat quality of the suckling lambs.
2. The effect of increasing proportion of dehydrated sainfoin inclusion in the pelleted concentrate (0%, 20%, and 40% of sainfoin) of light lambs during the fattening period on:
    2.1. Growth, dry matter intake, metabolic and antioxidant blood status, estimated total digestibility, and carcass traits and quality of finishing lambs.
    2.2. Ruminal fermentation and biohydrogenation and fatty acids of plasma and meat.

**Objective 1** is addressed in Manuscripts I, II, and III, resulting from the completion of Study 1. **Objective 2** is addressed in Chapters IV and V originated from Study 2.

# Material and Methods

The thesis comprised two different studies that were carried out at the facilities of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) (41°3' N, 0°47' W and 216 m above sea level) in Zaragoza, Spain, with the objective of studying the effect of sainfoin inclusion in the diet of i) dams rearing their suckling lambs and ii) finishing lambs.

## EXPERIMENTAL STUDIES

### STUDY 1

In Spring 2019, 2–3 days after lambing, twenty single multiparous Rasa Aragonesa ewes of CITA's flock and their twenty male lambs were distributed randomly in two homogeneous groups 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). During the rearing period, the ewes were fed fresh sainfoin (*Onobrychis viciifolia* cv Reznos) *ad libitum* from vegetative to early flowering stage plus 200 g of barley daily divided into two meals (09:00 and 16:00 h). The sainfoin was grown in adjacent fields owned by CITA and it was cut three times per week and stored indoors to prevent mould and overheating. 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* and lambs had free access to suckling. The experimental period started 2–3 post–lambing 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).

#### Controls and sampling procedures

##### Feeding and feed samples

The amount of fresh sainfoin offered was daily adjusted to each ewe based on previous refusal, with at least 15% refusal allowing *ad libitum* intake. The amounts of feed offered and refused were recorded daily to calculate the individual intakes. Composite samples of the offers and refusals per ewe and week were obtained.

The feedstuff samples (offer and refuse) were divided in two subsamples: One sample were dehydrated in the oven (AFA150/5400, Dycometal, Barcelona, Spain) for 48 h to determine the daily individual dry matter (DM), and the second sample was frozen, kept at -20 °C until they were freeze–dried (Genesis Freeze Dryer 25, Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, Spain). All dried 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 crude protein (CP) content. On the other side, the freeze–dried samples were sieved through a 0.2 mm screen and used for the analyses of the secondary compounds (fatty acid, ABTS (2,2-azinobis-3-ethylbensothiazoline-6-sulfonic acid), PAC–fractions, delphinidin/cyanidin ratio and polyphenols) of sainfoin offers and refusals. For barley, only one sample was taken weekly for complete chemical analysis as for sainfoin samples.

Milk samples

Once a week, at 8:00 h before feeding, 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 08:00 and 12:00 h. During this interval of 4h, the lambs were separated from their dams and put in a different pen without access to milk. 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 (Genesis Freeze Dryer 25, Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, Spain) to analyse the polyphenols, the antioxidant activity (through ABTS determination), and milk FA profile.

The standard milk yield was calculated as follows:

Standard milk yield (l/d) = milk production (l/d) × [(0.0071 × crude fat (g/l) + (0.0043 × CP (g/l)) + 0.2224], as had been described by Bocquier et al. (1993).

Body weight, body condition score and blood samples

Weekly, at 8:00h 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 following the method of Russel et al. (1969). After, blood samples of ewes were collected from the jugular vein of ewes into 10 ml tubes containing heparin (Vaccuette, Spain) and immediately centrifuged (3000 *g* for 15 min at 4 °C), then, the plasma was stored at -20 °C until the metabolites analyses (urea, ABTS, SOD, MDA, and polyphenols) were performed.

Faecal samples

At the beginning and end of the study, faecal samples (approximately 50 g) from ewes were collected from the rectum, kept in sterile jars, and refrigerated for parasite determination (Strongiloides (*Teladorsagia spp*.)).

Lamb slaughtering procedures

When the lambs reached the target weight (10–12 kg BW), they were slaughtered in CITA's experimental slaughterhouse. At this time, their respective dams left the experiment, returning with the rest of the flock.

The lambs were slaughtered without prior fasting at the experimental slaughterhouse of CITA, placed next to the building where the animals were allocated. After registering their weight, the lambs were stunned by a captive bolt pistol and exsanguinated in the experimental abattoir following standard commercial procedures and according to Council Regulation (EC) Nº 1099/2009. Blood samples were taken from the jugular vein into 10 ml heparin tubes (Vaccuette, Madrid, Spain) and processed as had been explained above.

The contents of the digestive tract corresponding to the sections of reticulum–rumen, omasum–abomasum, and duodenum–jejunum were extracted and weighed. Then, the empty digestive compartments were cleaned and weighed. Hot carcass weight (HCW) was recorded without head and offal. After 24 h chilling at 4 °C in total darkness, the cold carcass weight (CCW) was recorded. Then, the dressing percentage was calculated as $\left(\frac{HCW}{Slaughter\ weight} \times 100\right)$ and the carcass shrinkage was recorded as $\left(\frac{HCW-CCW}{HCW} \times 100\right)$.

The carcasses were classified for the fatness degree of carcass, following the Community Scale for Classification of Carcasses of Ovine Animals and of Light Lambs (EEC 461/93) with a scale of 1 (low), 2 (slight), 3 (average), and 4 (high). Instrumental colour of caudal subcutaneous fat was measured on tail root using a Minolta CM-2006d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan), registering lightness (L*), redness (a*), and yellowness (b*), which were used to calculate hue angle ($h_{ab}$) and chroma ($C^*_{ab}$). The absolute value of the summation of the translated spectrum (SUM) was calculated following the method described in Prache and Theriez (1999) as:

$$SUM = \left[\left(\frac{TR_{450}}{2}\right) + TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + \left(\frac{TR_{510}}{2}\right)\right] \times 10$$

where TRi was the reflectance value at i nm.

After that, the carcasses were carefully split longitudinally into the two half carcasses and the *longissimus thoracis et lumborum* (*LTL*) muscles of both sides were collected. Perirenal fat deposits were extracted and weighed (BP 8100, Sartorius Stedim Biotech, Germany).

The *LTL* muscles from 4$^{th}$ to 6$^{th}$ lumbar vertebrae of the left side were used to measure the pH with a pH-meter equipped with a Crison 507 penetrating electrode (Crison Instruments, S.A., Barcelona, Spain) and to estimate the chemical composition by NIRs (FoodScanTM2, Foss Analytics, Hilleroed, Denmark). From 6$^{th}$ to 13$^{th}$ thoracic vertebrae from both sides were sliced into 2.5 cm–hick samples, the left ones were assigned to days 0, 2, and 7 of display and the right ones for days of display 5 and 9. The slices were placed in trays as is shown in Figure 6, wrapped with oxygen permeable polyvinyl chloride film, and kept in darkness at 4 °C until being measured for colour and haem pigment estimations. The 0-d samples were allowed to bloom in darkness at 4 °C for 1 h before being measured for colour and haem pigments. *LTL* colour was measured as had been explained above, while the relative contents of metmyoglobin (MMb) and


**Figure 6.** *LTL* muscles section of suckling lambs placed in trays for colour measurements.

oxymyoglobin ($MbO_2$) were estimated using the (K/S at 572 nm)/(K/S at 525 nm) and (K/S at 610 nm)/(K/S at 525 nm) ratios, where K is the absorption coefficient and S is the scattering coefficient, as described in Ripoll et al. (2013). After the measurements, the meat was freeze–dried, vacuum–stored (Sammic V-421 TI, Sammic S.L., Azpeitia, Spain), and kept in total darkness at -80 °C until the analysis of polyphenols and ABTS assay.

A schematic representation of the sampling and analytical procedures carried out in Study 1 is shown in Figure 7.

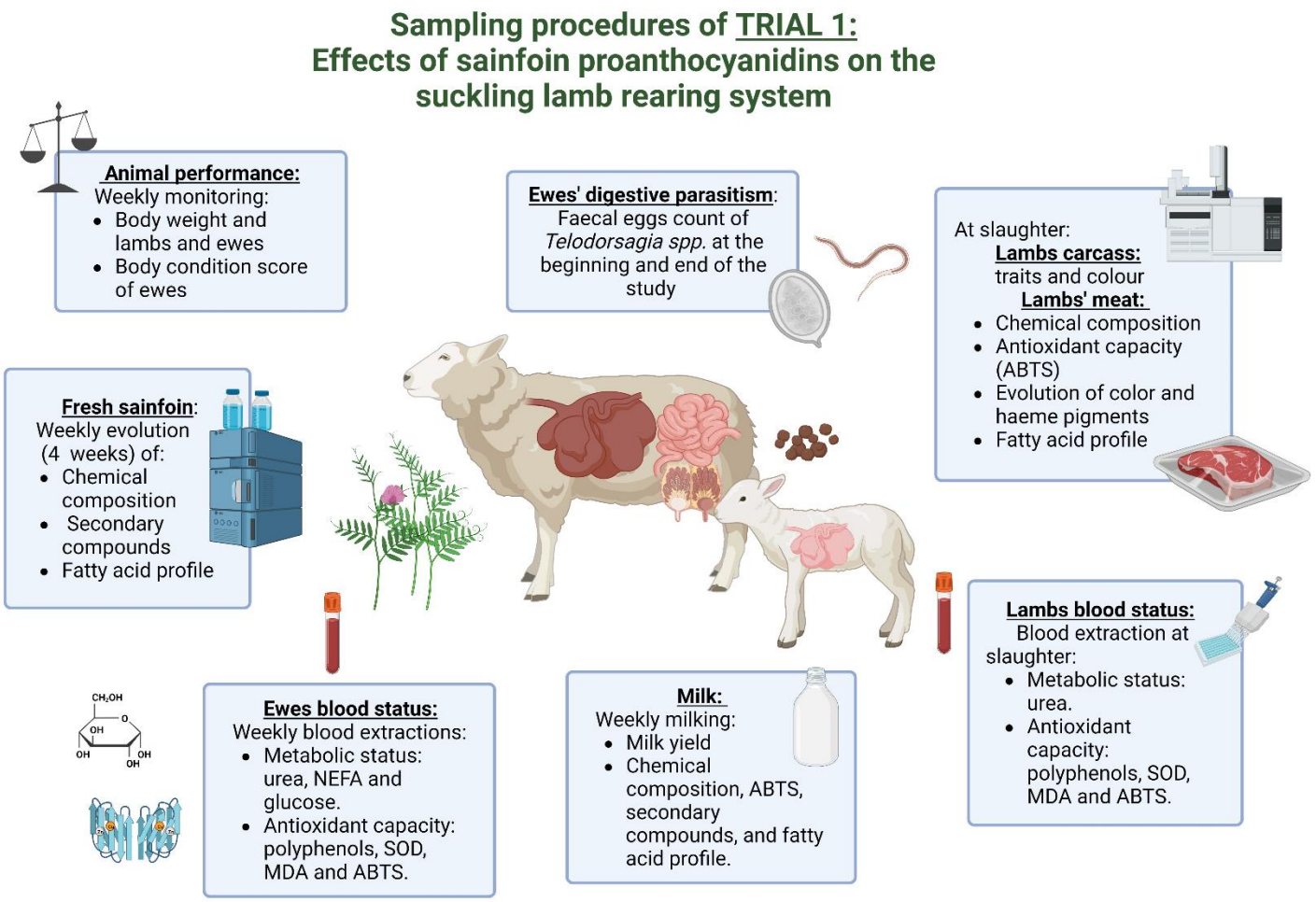


**Figure 7.** Graphical representation of the sampling and analytical procedures carried out in Study 1.

**STUDY 2**

In autumn 2020, after weaning, thirty male lambs of the Rasa Aragonesa breed of the CITA's flock raised by their dams with free access to suckling and initiation concentrate were randomly distributed in three groups. Groups were then consolidated according to weaning age (30.0 ± 1.99 d) and BW (14.0 ± 0.49 kg) to achieve a homogenous condition among groups as shown in Table 1.

**Table 1.** Average (mean ± standard deviation) age and BW at weaning of the lambs selected to be part of the groups assigned to the 3 diets.

| | Diet [1] | | |
|---|---|---|---|
| | 0SF | 20SF | 40SF |
| *n* | 10 | 10 | 10 |
| Age at weaning, days | 29 ± 1.4 | 30 ± 2.3 | 30 ± 2.3 |
| BW at weaning, kg | 13.9 ± 1.48 | 13.9 ± 1.39 | 14.1 ± 1.33 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate.

The lambs were individually placed in indoor pens (1.5 m × 1.4 m) and fed with the experimental concentrates containing 0% (0SF), 20% (20SF) or 40% (40SF) dehydrated sainfoin. The sainfoin included in the concentrates was left over after the end of Study 1, so it was cut in late spring, at the flowering stage. After a few days of natural drying, it was dehydrated with hot air and pelleted. The chemical composition of the sainfoin pellets used are shown in Table 2.

Afterwards, the ingredients of the concentrates (Table 3), which were formulated to be isoproteic and isoenergetic, were mixed with the sainfoin ground pellets and pelleted (3.5–mm diameter) to avoid selection. During all the experimental period, the lambs had free access to concentrates, water, and minerals. The study length comprised 40 days (5 weeks), which was the time required for the lambs to achieve the slaughter weight of approximately 25 kg BW. A total of 4 lambs (1 lamb belonging to the 0SF treatment, 2 to the 20SF treatment, and 1 to the 40SF treatment) were removed from the study during the experiment for reasons unrelated to the feeding treatment. Therefore, the

final number of lambs per treatment was *n*=9, *n*=8, and *n*=9 for the 0SF, 20SF, and 40SF, respectively.

**Table 2**. Chemical composition, proanthocyanidins (PAC) and their fractions, and fatty acid contents of sainfoin pellets.

| | mean ± standard deviation |
|---|---|
| Chemical composition | |
| Crude protein, g/kg DM | 121 ± 3.8 |
| Ash, g/kg DM | 117 ± 8.6 |
| Neutral detergent fibre, g/kg DM | 429 ± 3.0 |
| Acid detergent fibre, g/kg DM | 292 ± 4.5 |
| Lignin, g/kg DM | 53.7 ± 0.42 |
| Gross energy, MJ/kg DM | 18.11 ± 0.018 |
| Proanthocyanidins (PAC) [1] | |
| Total PAC | 17.4 ± 1.80 |
| Extractable PAC | 10.1 ± 1.83 |
| Protein–bound PAC | 4.81 ± 0.444 |
| Fibre–bound PAC | 2.45 ± 0.343 |
| Fatty acids (FA), g/100 g total FA | |
| C12:0 | 0.33 ± 0.024 |
| C14:0 | 1.08 ± 0.080 |
| C15:0 | 0.44 ± 0.040 |
| C16:0 | 27.5 ± 0.47 |
| C16:1 c9 | 0.95 ± 0.042 |
| C18:0 | 8.72 ± 0.021 |
| C18:1 c9 | 5.47 ± 0.166 |
| C18:1 c11 | 0.60 ± 0.015 |
| C18:2 n-6 | 16.3 ± 0.020 |
| C18:3 n-3 | 38.6 ± 0.54 |

[1] g eq. sainfoin PAC/kg DM

**Table 3.** Ingredients and chemical and fatty acid (FA) composition (mean ± standard deviation) of the experimental diets.

| | Diets [1] | | |
|---|---|---|---|
| | 0SF | 20SF | 40SF |
| Ingredients, g/kg DM | | | |
| Barley | 310 | 252 | 50 |
| Corn | 250 | 189 | 250 |
| Wheat | 50 | 50 | 102 |
| Gluten feed | 60 | 60 | 130 |
| Soybean meal 47% | 173 | 138 | 159 |
| Bran | 25 | 81 | 0 |
| Palm oil | 10 | 10 | 15 |
| Calcium carbonate | 15 | 13 | 4 |
| Sodium chloride | 5 | 5 | 5 |
| Premix vitamin 0.2% | 2 | 2 | 2 |
| Sainfoin pellet | 0 | 200 | 400 |
| Straw | 100 | 0 | 0 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate.

**Controls and sampling procedures**

Feeding and feed samples

Concentrates were offered *ad libitum,* adjusting daily the amount offered to DM intake of the previous day, allowing a 15% of refusal. The amounts of concentrate offered and refused were recorded daily to calculate the individual intakes. Composite samples of the offers and refusals per lamb and week were obtained. The management and procedure of samples were the same that had been describe in Study 1.

Body weight and blood samples

The record of BW and blood samples were the same explained in Study 1, except that the lambs were bleeding fortnightly. Once the plasma was obtained, it was stored at -20 °C until the metabolite analyses of non–esterified fatty acid (NEFA), glucose, urea, ABTS, MDA, and polyphenols were performed.

Faecal samples

Faecal samples were collected using rectal stimulation before meal distribution the last 3 days of weeks 2, 4, and 6, obtaining a composite sample per week of collection.

The samples were kept in sterile jars, freeze–dried (Genesis Freeze Dryer 25, Hucoa Erlöss, S.A./Thermo Fisher Scientific, Madrid, Spain), and kept at -20 °C until the determination of digestibility following the acid–insoluble ashes (AIA) analysis.

Lambs slaughtering procedures

After 40 days of fattening, the lambs were slaughtered in the experimental abattoir of CITA Research Centre. Due to handling and staff issues at the slaughterhouse, the animals were slaughtered on two consecutive days. The slaughtering procedures were the same as for the suckling. Blood samples were taken per lamb form the jugular vein into 10 ml tubes for fatty acid analyses. The total ruminal contents of each lamb were extracted, and their weights were recorded (RX-60M, Accurex, Schofield, WI, USA; 10 g precision). Approximately 100 ml were stored unfiltered until it was freeze-dried (Lyobeta 25, Azbil Telstar, Japan) and kept at -20 °C until the fatty acid analysis was performed. After, approximately another 100 ml of the ruminal content were filtered through a double cheesecloth and stored in sterile jars. Immediately, the pH of the liquid was measured using a micropH 2002 pH-meter (Crison Instruments S.A., Barcelona, Spain). The rest of this sample was used to determine ruminal ammonia and volatile fatty acids (VFA). For ammonia analysis, 2.5 ml of ruminal liquid were mixed with 0.1 N HCl in a 1/1 ratio (v/v) and stored at -20 °C, and 0.5 ml of liquid was mixed with 0.5 ml of deproteinizing solution and 1 ml of distilled water and stored at -20 °C for subsequent analysis of VFA. Afterwards, the rumen was thoroughly cleaned and the colour was measured on the inner side (in contact with the ruminal papillae) of the ventral sac using a Minolta CM-2006d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan). The methodology followed for carcass traits (weight, degree of fatness, and colour of carcass and caudal subcutaneous fat) and the recording of perirenal fat weight was the same as previously explained for suckling lambs.

A schematic representation of the sampling and analytical procedures carried out in Study 2 is shown in Figure 8.

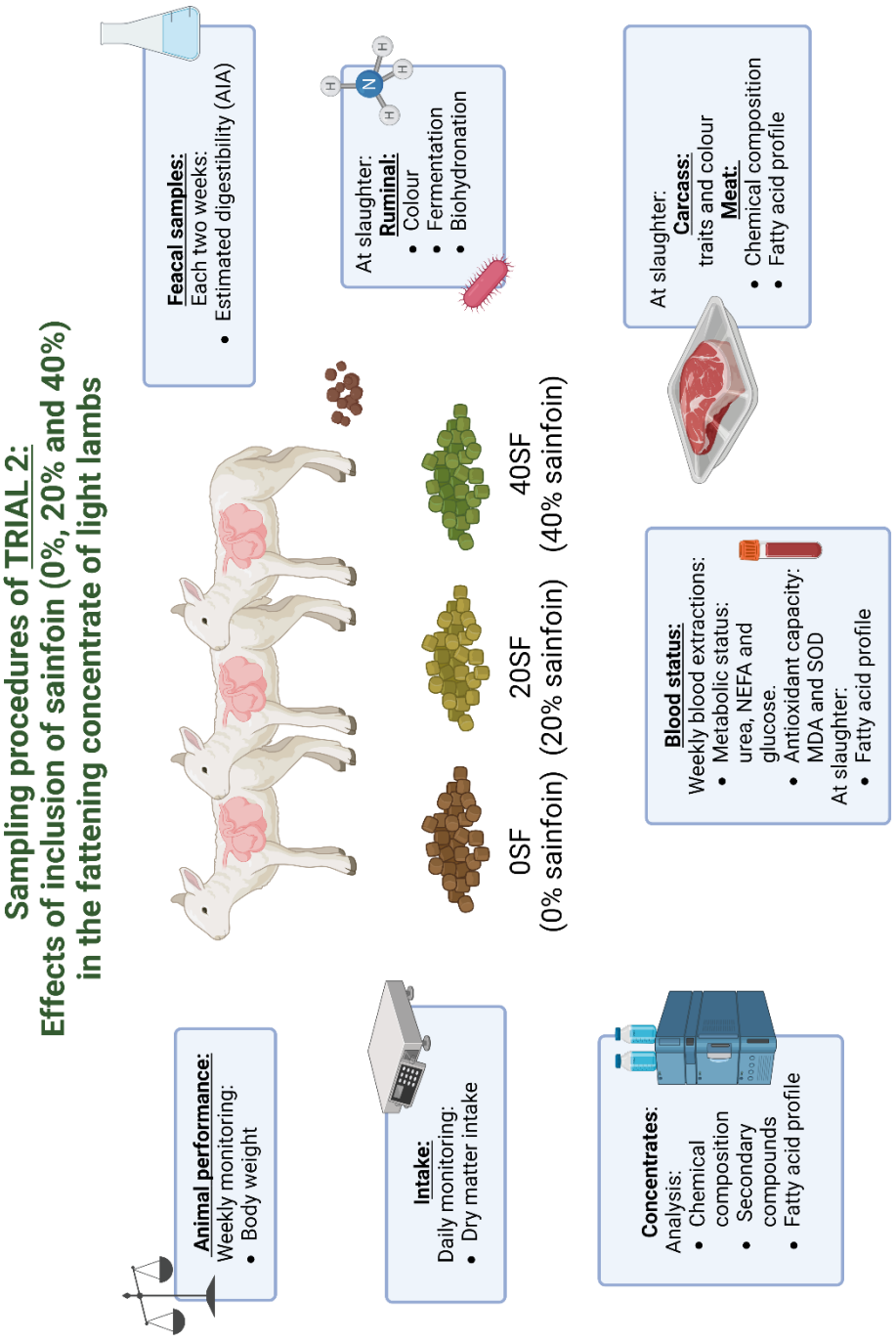


**Figure 8.** Graphical representation of the sampling and analytical procedures carried out in Study 2.

## CHEMICAL ANALYSES

### Feedstuffs

All analyses of the offered and refusal feedstuffs were run in duplicate. The dry matter (DM) (index nº 934.01) and ash (index nº 942.05) contents of the feedstuffs were determined in oven–dried samples 3 h at 550 °C (Heron 10-PR/400, Hobersal, Barcelona, Spain), according to the AOAC methods (AOAC, 2000). The content of CP (Nitrogen × 6.25) was determined following the Dumas Procedure (index nº 968.06) using a nitrogen analyser (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain) (AOAC, 2000). The contents of neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin of the feedstuffs were analysed following the sequential procedure of Van Soest et al. (1991) and using the Ankom 200/220 fibre analyser (Ankom Technology Corporation, Fairport, NY, USA). The NDF was assayed with a heat–stable amylase. The lignin was analysed in the ADF residues by solubilisation of cellulose with sulphuric acid. All values of NDF and ADF were corrected for ash–free content. The total polyphenols were extracted in freeze–dried samples following the method described by Makkar (2003) and quantified according to the method of Julkunen-Tiitto (1985) using the tannic acid as standard (Sigma Aldrich, Saint Louis, MO, USA). Both polyphenol contents of samples and standard calibration were measured with a Heλios β spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) at 725 nm, and expressed as tannic acid equivalents. The extractable PAC, protein–bound PAC, and fibre–bound PAC were extracted in freeze–dried samples and fractioned as described in Terrill et al. (1992) and quantified by the colorimetric HCl-butanol method described in Grabber et al. (2013). The standard used for the quantification of the samples was extracted and purified from sainfoin using the procedure described by Wolfe et al. (2008). Finally, samples and standard calibration were measured with the Heλios β spectrophotometer (Artisan Technology Group, IL, USA) at 550 nm and PAC concentrations were expressed as sainfoin PAC equivalents. The determination of anthocyanidins (delphinidin and cyanidin) proportions was carried out after hydrolysis by UPLC-DAD (High-Performance Liquid Chromatography Fluorescence and Diode Array Detection, ACQUITY UPLC HClass (Waters, Milford, MA, USA) following the procedure described in Assefa et al. (2019)

with a Acquity UPLC BEH C18 (50 mm × 2.1 mm × 1.7 µm) column (Waters, Milford, MA, USA) and an 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, IL, USA). For the net energy calculations, the values of each ingredient set by FEDNA (2019) were used together with an estimation for sainfoin pellets based on the calculation of Mertens (1983). The antioxidant activity of the feedstuffs was determined using the Folin–Ciocalteu method as described in Makkar (2003) and 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, USA) at 750 and 730 nm absorbance wavelength, respectively.

The ether extract of barley and concentrates was determined with an XT10 Ankom extractor (Ankom Technology Corporation, Fairport, New York, USA) following the Ankom Procedure (AOCS, 2005). The total starch of the concentrates was measured with the commercial kit K-TSTA-100A (Neogen Corporation, Lansing, Michigan, USA) following the amyloglucosidase/α-amylase method.

The total carotenoid content of the concentrates was analysed as described in Blanco et al. (2019). Briefly, carotenoids were determined from 50 mg samples according to the methodology proposed by Chauveau-Duriot et al. (2010).The extraction was performed with the procedures described by Fu et al. (2011) without saponification as it degrades part of the carotenoids. Thus, the samples were subjected to 3 subsequent extractions with 3 ml of methanol-acetone-petroleum ether solution (1-1-1, 0.01% butylated hydroxytoluene; BHT) until a white precipitate was obtained. Then, 1 ml of the supernatant was evaporated in a vacuum evaporator (Christ RVC 2-25, Germany) to a dry residue, to which 1 ml of acetonitrile-dichloromethane-methanol (75-10-15) was added and transferred to a 2 ml screw–capped glass vial for automatic sampling using 5 µl for UPLC determination.

The content of acid insoluble ashes (AIA) of the concentrates was analysed following the method of Shrivastava and Talapatra (1962). For that, 8 g of grounded through a 1 mm screen and freeze–died samples of concentrates were boiled with a mixture of 75 ml of 3N HCl and 25 ml of distilled water. After being filtered and incinerated at 550 °C for 3 h (Heron 10-PR/400, Hobersal, Barcelona, Spain), the AIA content of concentrates was obtained by weighing.

Fatty acids of feedstuffs were run in duplicate and determined by gas chromatography with a flame ionisation detector. The 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, MN, 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.

For the FA determination of feedstuffs, a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, MA, USA) was used, equipped with a CP-8400 Autosampler (Bruker, Billerica, MA, USA), an SP-2560 column (100 m × 0.25 mm ID × 0.20; Sigma Aldrich, Saint Louis, MO, USA), and the Compass CDS software. The 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, MN, USA) and relative retention times found in several sources (Kramer et al., 1997; Alves and Bessa, 2009;

Bravo-Lamas et al., 2016). 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, while total FA content was expressed as mg of FA per g of sample using C19:0 as the internal standard for feedstuff. After performing FA identification and quantification, they were grouped into major sums and the corresponding ratios were calculated.

**Plasma**

Plasma concentrations of urea, glucose, and creatinine (kinetic methods) were analysed with an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). Non esterified fatty acids (enzymatic method) were analysed using a commercial kit (Randox Laboratories Ltd., Crumlin Co., Antrim, UK). The plasmatic concentration of polyphenols was obtained by diluting 1:25 (plasma: milli-Q water) following the method of Leal et al. (2019). The superoxide dismutase (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).

Plasma FA were analysed as explained for feedstuffs from 2 ml of plasma but using a a SP-2560 capillary column (200 m × 0.25 mm ID × 0.20; Sigma Aldrich, Saint Louis, MO, USA). The quantification was performed as described in ISO 12966-4:2015 and expressed as g of FA per 100 g of total FA, while total FA content was 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.

**Milk**

The chemical analyses of milk were carried out for Study 1. Milk protein, fat, lactose and urea contents and the number of somatic cells were determined in a CombifossTM 7 (Foss, Hillerød, Denmark) device comprising a Fossomatic 7 DC somatic cell counter (based on the flow cytometry principle) and a MilkoScanTM 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, USA) at 730 nm of wavelength.

The methodology followed to analyse the FA content of milk was based on Kramer et al. (1997). The method used was the same as explained for FA feedstuffs determination but with some adaptations to milk matrix. Briefly, 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, MN, 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 1,000 rpm and 10 °C for 5 min to take the supernatant (heptane) and to add it to a 5 ml round-bottom tube with anhydrous $Na_2SO_4$. After, the tube was shaken for 30 s and centrifuged for 5 min at 1,000 rpm and 10 °C. Approximately, 1 ml of the supernatant was recovered in a 2 ml vial for gas chromatography.

For FA determination of milk, a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, MA, USA) was used, equipped with a CP-8400 Autosampler (Bruker, Billerica, MA, USA), an SP-2560 column (200 m × 0.25 mm ID × 0.20; Sigma Aldrich, Saint Louis, MO, USA) and the Compass CDS software. FA identification was performed as explained for plasma FA. 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, while total FA content was expressed as mg of FA per g of sample using C23:0 as the internal standard. After performing FA identification and quantification, they were grouped into major sums and the corresponding ratios were calculated as described in each manuscript.

**Faeces**

The concentrations of differentiable parasite forms in faeces were determined for Study 1, 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, 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).

The estimated organic matter digestibility (OMD) obtained through the analyses of AIA method based on Shrivastava and Talapatra (1962) were carried in Study 2. Thus, to determine the AIA content, 2 g grounded through a 1 mm screen and freeze–died faeces samples were analysed following the same method as feedstuffs. After, the AIA content of faeces a were obtained by weighing. The total digestive tract estimated OM digestibility was calculated using the nutrient–to–marker ratio in the diet (explained in the section corresponding to the chemical analysis of feedstuffs) and faeces, as follows:

Estimated organic matter digestibility (% OMD) = 100 − [100 × ($AIA_{diet}$/$AIA_{faeces}$) × ($OM_{faeces}$/$OM_{diet}$)]

where $OM_{faeces}$ and $OM_{diet}$ are the OM (%) in faeces and diet, respectively, and $AIA_{faeces}$ and $AIA_{diet}$ are the concentrations (%) of AIA in faeces and diet, respectively.

**Meat (*LTL* muscle)**

The chemical composition of meat was measured in suckling lambs' meat and finishing lambs' meat in Studies 1 and 2, respectively. The DM and intramuscular fat (IMF) were estimated by NIRs (FoodScanTM2, Foss Analytics, Hilleroed, Denmark). In Study 1, the polyphenols and ABTS contents of freeze–dried meat were determined according to Leal et al. (2019) and Vázquez et al. (2015), respectively.

The determination of cholesterol in the meat of finishing lambs of Study 2 was performed following the method of Bertolín et al. (2019) using an Acquity UPLC H-Class liquid chromatograph (Waters, Mildford, KA, USA) with a silica–based bonded phase column (Acquity UPLC HSS T3, 150 mm × 2.1 mm × 1.8 µm, Waters), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ Detector, Waters) and a fluorescence

detector (2475 Multi λ Fluorescence Detector, Waters) and controlled with a Empower 3 software (Waters). The absorbance of cholesterol was measured at 220 nm.

The FA determinations of meat samples of Study 2 were extracted according to Lee et al. (2012). Briefly, 500 mg of lyophilised meat were methylated as FA methyl esters using 4 ml of 0.5M $CH_3ONa/CH_3OH$ solution followed by 4 ml of the solution of acetyl chloride/$CH_3OH$ (1/10, v/v) and extracted in 3 ml of heptane. Afterwards, they were determined in a Bruker Scion 436-GC (Bruker, Billerica, MA, USA) gas chromatograph with a flame ionisation detector equipped with a CP-8400 autosampler and a SP-2560 capillary column (200 m × 0.25 mm ID × 0.20; Sigma Aldrich, Saint Louis, MO, USA). The FA identifications were performed as explained for milk FA.

**Ruminal digesta**

The chemical analyses of ruminal digesta were carried out in Study 2. The ruminal content of $NH_3$–N was analysed using the Berthelot reaction following the technique of Chaney and Marbach (1962) and using the ammonium chloride to as standard to elaborate the calibration curve (BioTek Instruments, Inc., Winooski, VT, USA) and setting the measurement at 625 nm, where ammonium chloride was used as standard. For ammonia–N determination, the samples and the standards were treated with phenol and sodium hypochlorite and incubated for 45 min at room temperature. Then, the samples were measured by the colorimetric method using an Epoch microplate spectrophotometer at 625 nm (BioTek Instruments, Inc., Winooski, VT, USA) and the ammonia concentrations were determined.

The concentrations of VFA were determined using the same GC described with a BR-SWax capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness; Bruker, Billerica, MA, USA) using helium as the carrier gas at a flow rate of 1 ml/min. The oven temperature programme was 100 °C, followed by a 6 °C/min increase to 160 °C. The injection volume was 1 µl at a split ratio of 1:50. The VFA were identified based on retention time comparisons

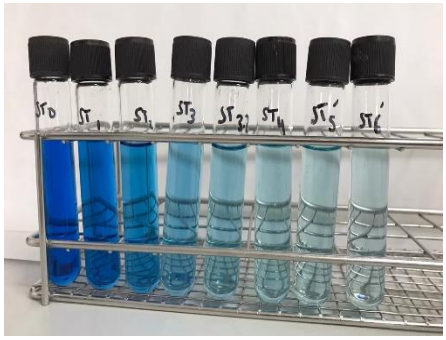

**Figure 9**. Calibration curve used to determine ruminal ammonia.

obtained through commercially available standards of acetic, propionic, iso–butyric, butyric, iso–valeric, valeric and 4–methyl–valeric acids of ≥ 99% purity (Sigma Aldrich, Saint Louis, MO, USA). The VFA contents were quantified with an external calibration curve using 4–methyl–valeric (Sigma Aldrich, Saint Louis, MO, USA) as internal standard as can be shown in Figure 9.

The analyses involving the FA determination of ruminal digesta were performed in the Laboratory of Animal Production and Nutrition of the Faculty of Veterinary Medicine of the University of Lisbon (Portugal) following the procedure described in Alves et al. (2017). Shortly, freeze–dried rumen contents (250 mg) were directly trans esterified according to Alves et al. (2018). The methyl nonadecanoate (C19:0) (internal standard) was used for quantification by GC with flame ionisation detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m × 0.25 mm, 0.20 μm film thickness, Supelco, Bellefonte, PA, USA) capillary column with the chromatographic conditions described in detail in Alves et al. (2018). The FA determinations were carried out by comparison with ruminal chromatograms of Alves et al. (2013) and Alves and Bessa (2014). The calculations corresponding to the estimate biohydrogenation extent of C18 dietary FA in rumen and the Completeness (%) of BH were calculated as explained in Alves et al. (2017).

**STATISTICAL ANALYSES**

All the statistical analyses were performed with SAS statistical software (v.9.3; SAS Inst. Inc., Cary, NC, USA). The animal (ewe and suckling and finishing lamb) was considered as experimental unit in all the analyses. The effects were considered significant at $P < 0.05$, and trends were discussed when $0.05 \leq P < 0.10$.

Variance analyses with a general linear model (*proc glm*) were carried out in Study 1 to analyse the plasmatic metabolites, carcass characteristics, and meat composition of the suckling lambs with the presence of PAC in dams' diet (Sainfoin or Sainfoin + PEG) as the fixed effect and the FA evolution of feedstuffs using the week of study (lactation week, 1-4) as the fixed effect. In Study 2 the variance analyses were used to study the data concerning the overall estimated OMD, the carcass traits, and the ruminal and

fermentation parameters of the finishing lambs using the concentrates (0SF, 20SF, and 40SF) as the fixed effect.

The analysis of variance with a mixed model (*proc mixed*) was carried out to study data with repeated measurements over time. In Study 1 was used for data corresponding to the intake, milk production, composition, and FA profile, BW, BCS and plasma metabolites of ewes with the experimental diets, week of lactation, and their interaction as fixed effects and the ewe as the random effect. The colour and haem pigments of meat measured in *LTL* muscle were also analysed with mixed models with feeding treatment, time of display (days 0, 2, 5, 7, and 9), and their interactions as fixed effects and the lamb as the random effect. In Study 2 the mixed model was used to analyse the intake, BW, weight gains, plasma parameters with the experimental diets (0SF, 20SF, and 40SF), week of fattening (0, 2, 4, and 6), and their interaction as fixed effects and the lamb as the random effect. For the FA profile of ruminal digesta, plasma, and meat the experimental diets were used as fixed effect and the group statement was included in the model to adjust the variance heterogeneity when significant. In all mixed analyses, the degrees of freedom were adjusted with the Kenward–Rodger correction, Tukey's correction was used for pairwise comparisons and the results were expressed as least square means with the standard error of the mean (SEM) or the residual standard deviation (RSD).

The number of faecal parasites were analysed in Study 1. For this purpose, 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 (start and end of study) and the effect of the dietary treatment. Parasite presence ranges were then fixed and, due to being binomial characters, were analysed by a Chi-squared procedure.

In addition, the 115 individual milk FA of Study 1 were analysed using a combination of three analysis types to discriminate between both treatments. These analyses were stepwise discriminant analysis (*proc stepwise*), discriminant analysis (*proc discrim*), and canonical discriminant analysis (*proc candisc*). The equation followed and its details are

described in detail in Conte et al. (2018). The effective separation between treatments was assessed by the corresponding Hotelling's T-square test.

# Results and Discussion

The **Results** of this doctoral thesis have been presented following a structure of 5 scientific manuscripts, which are listed below:

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

- **Manuscript II: "**Effect of sainfoin proanthocyanidins on milk fatty acids from ewes rearing suckling lambs*".* Baila, C., Joy, M., Bertolín, J. R., Blanco, M., Casasús, I., and Lobón, S. (2023). *Animal*, 17, 100862. doi:10.1016/j.animal.2023.100862

- **Manuscript III: "**Sainfoin in the dams' diet as a source of proanthocyanidins: effect on the growth, carcass, and meat quality of their suckling lambs*".* Baila, C., Lobón S., Blanco, M., Casasús, I., Ripoll, G., and Joy. M. (2022). *Animals*, 12, 408. doi:10.3390/ani12040408

- **Manuscript IV: "**Sainfoin can be included up to 40% in the finishing concentrate of lambs without affecting their performance, ruminal fermentation and carcass quality*".* Baila, C., M.Joy, Blanco, M., Casasús, I., Ripoll, G., and Lobón, S. Submitted to *Italian Journal of Animal Science*.

- **Manuscript V: "**Inclusion of sainfoin in the concentrate of finishing lambs: Fatty acid profile of rumen, plasma, and muscle*".* Baila, C., Joy. M., Bertolín, J. R., Alves, S. P., Bessa, R. J. B., Blanco, M., and Lobón, S. Under review in *Journal of Agricultural and Food Chemistry*.

The general **Discussion** focuses on the contribution of the five Manuscripts, integrating the different topics addressed by each Manuscript. Finally, the **Conclusions** section summarises the evidence found.

# Manuscript I

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

C. Baila, M. Joy, M. Blanco, I. Casasús, J. R. Bertolín, and S. Lobón

**Abstract**

There is increasing interest in using sainfoin (*Onobrychis viciifolia*) to feed sheep, but it contains proanthocyanidins (PAC), 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, 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.

**Keywords**

*Onobrychis viciifolia,* polyethylene glycol, milk quality, performance, nematodes.

**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 proanthocyanids from fresh sainfoin on ewes´ 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, allow 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 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 (PAC), or condensed tannins, varying between 10–90 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 (NEFA) (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., 2012). 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), BCS (3.3 ± 0.57), lambing date (April 6 ± 0.1 d) 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 week 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 (09:00 and 16:00 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 (3000 *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 08:00 and 12:00 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 × crude protein (g/l)) + 0.2224], as had been described in Bocquier et al. (1993). 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–ethylbensothiazoline–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 Chromatography Fluorescence and Diodo Array Detection, ACQUITY UPLC H–Class (Waters, Milford, MA, USA)) following the procedure described in Assefa et al. (2019) with a Acquity UPLC BEH C18 (50 mm × 2.1 mm × 1.7 µm) column (Waters, Milford, MA, USA) and an absorbance wavelength of 520 nm. The gross energy (GE) content was calculated through the combustion specific heat obtained with a calorimetric bomb (Model Parr 1341 Plain Jacket Bomb Calorimeter, Parr Instrument Company, IL, 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). NEFA (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 colourimetric activity kit (Arbor Assays, DetectX, MI, 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–Rodger correction. The least square means and their associated standard errors 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 4, and the evolution of the most relevant parameters of the chemical composition and PAC of sainfoin during lactation is presented in Figure 10. The CP decreased until week 3 ($P < 0.05$), whereas the NDFom and ADFom had an inverse evolution ($P < 0.05$). The gross energy (GE) 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 ($P < 0.05$).

**Table 4.** Chemical composition (mean ± standard error) 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 |
| Crude protein, 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 (PAC) [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 |

[1] g eq.PAC sainfoin/kg DM

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

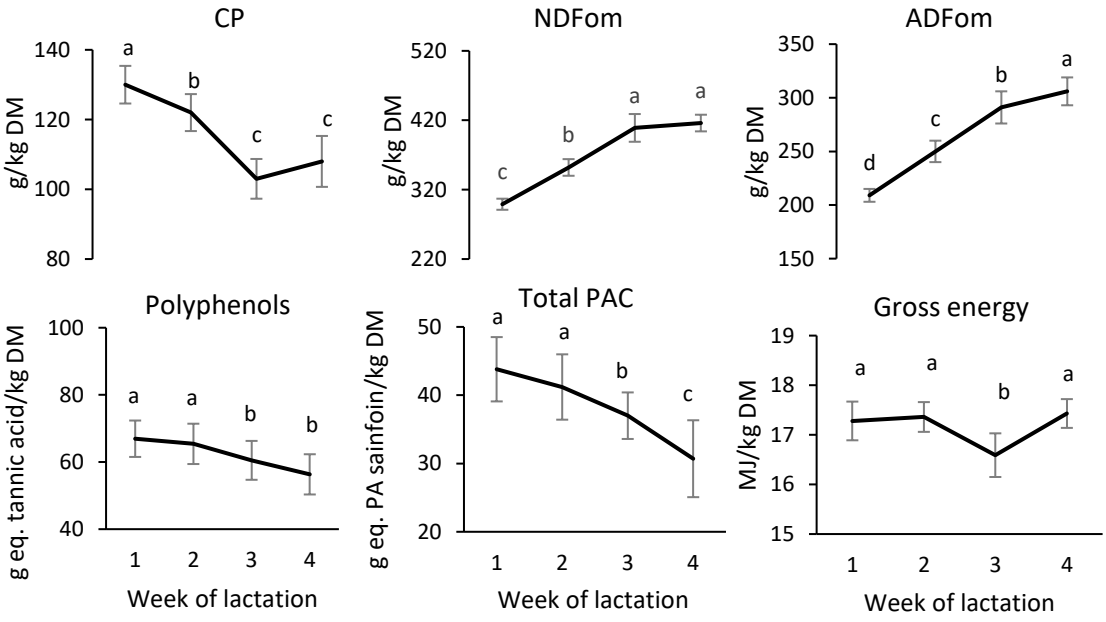


**Figure 10.** Contents of CP, NDF exclusive of residual ash (NDFom), ADF exclusive of residual ash (ADFom), polyphenols, total proanthocyanidins (PAC) and gross energy of fresh sainfoin during lactation.

Means with different a, b, c or d superscripts differ at $P < 0.05$ among weeks.

The dry matter intake (DMI) was not affected by the presence of PAC, but it increased with the week of lactation ($P < 0.05$; Table 5), 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 ($P < 0.05$; Figure 11). 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 5.

**Table 5.** Effects of the presence of proanthocyanidins (PAC) and the week of lactation on the performance, milk yield, and chemical composition of milk of sainfoin–fed ewes.

| | PAC | | Week of lactation (W) | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Sainfoin | Sainfoin +PEG | 1 | 2 | 3 | 4 | RSD | PAC | W | PAC x W |
| Dry matter 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

[1] 1000 cells/ml milk

[2] µg eq. [gallic acid]/g fresh sample

* interaction is presented in Figure 12

Within a parameter and main effect, means with different a, b or c superscripts differ at $P < 0.05$

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 ($P < 0.05$; Figure 12). The presence of PAC did not affect any milk parameters ($P > 0.05$) except polyphenols, which had higher content in the Sainfoin+PEG group ($P < 0.001$; Figure 12). 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 ($P < 0.001$; Figure 12) 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 ($P > 0.05$).

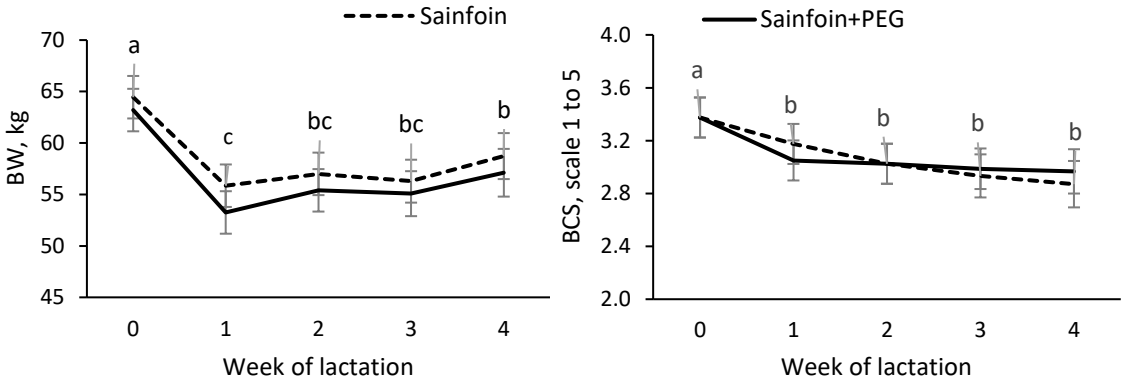


**Figure 11.** Effects of the presence of proanthocyanidins (PAC) and the week of lactation on the evolution of BW and BCS of sainfoin–fed ewes.

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 a, b or c superscripts differ at $P < 0.05$ among weeks.

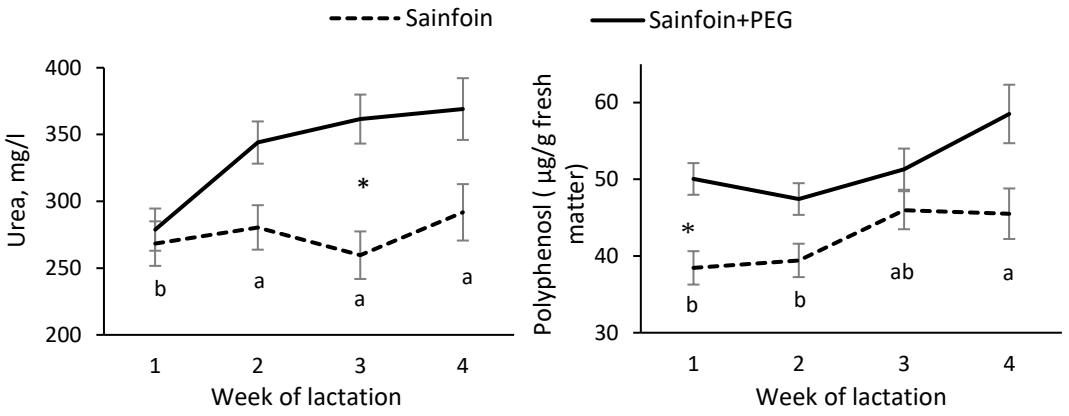


**Figure 12.** Effects of proanthocyanidins (PAC) and the week of lactation on the milk urea and polyphenol concentration of sainfoin–fed ewes.

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 a, b c or d superscripts differ at $P < 0.05$ among weeks.

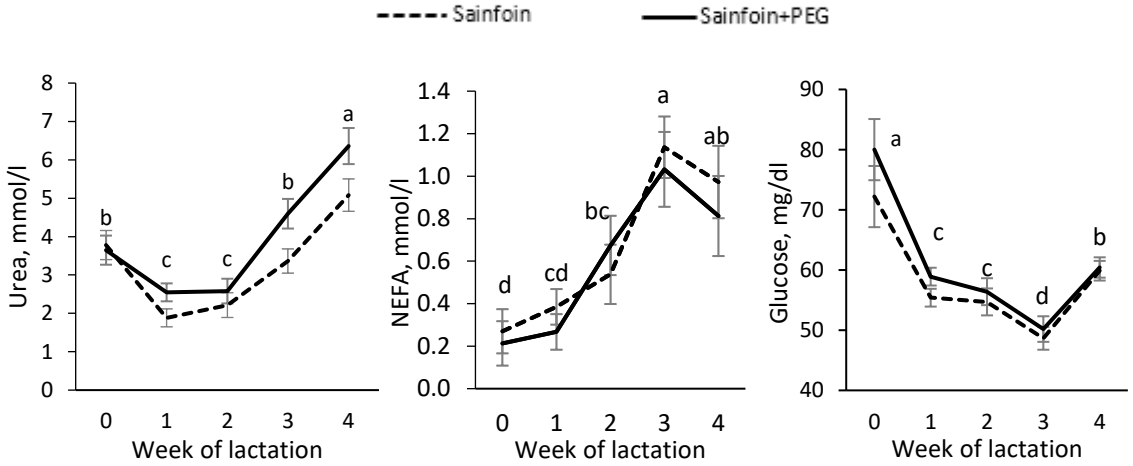


**Figure 13.** Effects of proanthocyanidins (PAC) and the week of lactation on the urea, non–esterified fatty acid (NEFA) and glucose concentrations in the plasma of ewes fed sainfoin during lactation.

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 a, b, c or d superscripts differ at $P < 0.05$ among weeks.

The concentrations of plasma metabolites throughout lactation are shown in Figure 13. 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 (Figure 14), 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).

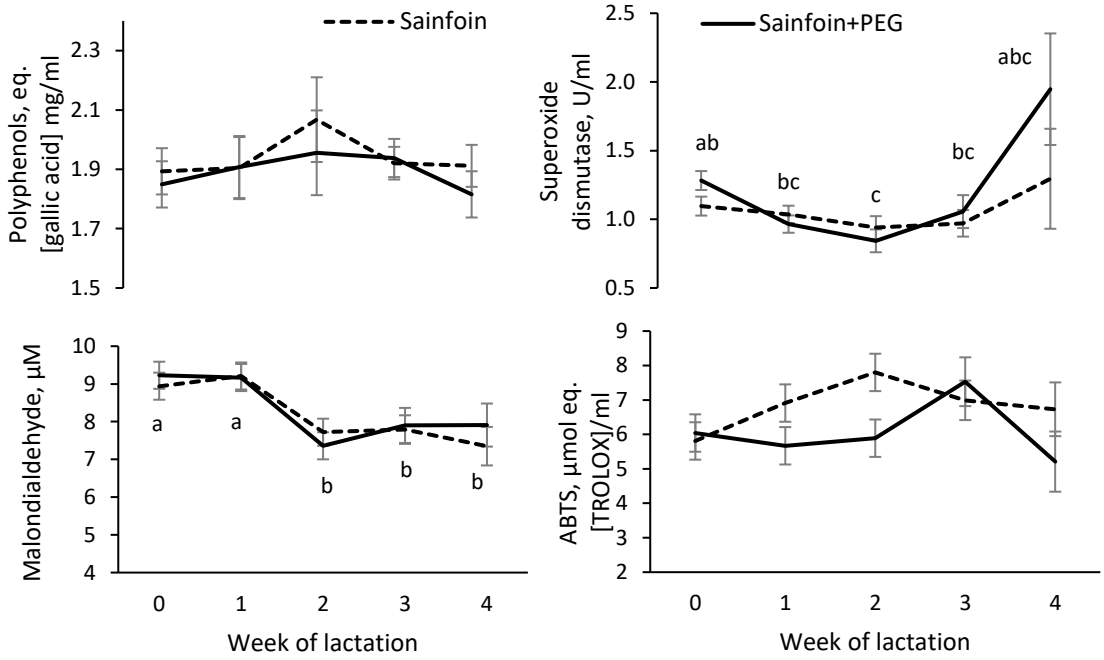


**Figure 14.** Effects of proanthocyanidins (PAC) and the week of lactation on the polyphenol concentration and antioxidant activity parameters (superoxide dismutase, malondialdehyde and 2,2‑azinobis‑(3‑ethylbensothiazoline)‑6‑sulfonic acid) in the plasma of ewes fed.

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 a, b, c or d superscripts differ at $P$ < 0.05 among weeks.

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 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 6).

**Table 6.** Effects of proanthocyanidins (PAC) 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 | | |
|---|---|---|---|---|---|---|
| | Sainfoin | Sainfoin +PEG | *P*-value | Sainfoin | Sainfoin +PEG | *P*-value |
| *n* | 8 | 8 | | 8 | 8 | |
| Log–transformed count, nº eggs/g | 45.7 | 14.45 | 0.31 | 33.1 | 16.6 | 0.59 |
| (log–values ± standard error) | (1.66±0.356) | (1.16±0.356) | | (1.52±0.356) | (1.22±0.356) | |
| Ewes excreting Strongiloides, % | | | | | | |
| >0 eggs/g feces | 87.5 | 62.5 | 0.25 | 75 | 62.5 | 0.59 |
| >10 eggs/g feces | 87.5 | 62.5 | 0.25 | 75 | 62.5 | 0.59 |
| >200 eggs/g feces | 12.5 | 25 | 0.52 | 12.5 | 25 | 0.52 |
| >500 eggs/g feces | 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)

**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; Rufino-Moya et al., 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, 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 Ben 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 post–lambing 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, increase milk yields (Provenza et al., 2000). In contrast, the higher milk urea content in Sainfoin+PEG ewes confirms the effectiveness of PEG in neutralizing the action of PAC, because PAC reduces 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 favor 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 abovementioned 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 with 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 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 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 increased 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 study, which could have led to an increased passage rate and a large amount of undegraded protein reaching to 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 (Aufrère 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.

**Conclusions**

In conclusion, the effect of PAC from sainfoin had no effect on dry matter 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.

# Manuscript II

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

C. Baila, M. Joy, J. R. Bertolín, M. Blanco, I. Casasús, and S. Lobón

**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 post–lambing). 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 C18:3 n-3 decreased from week 1 to 2, C16:0 and C18:0 increased from week 1 to 3, and C18:2 n-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, C18:2 n-6, C18:3 n-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 FA, suggesting some changes in ruminal biohydrogenation.

**Keywords**

*Onobrychis viciifolia*, polyethylene glycol, forage, condensed tannins, lipid metabolism.

**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 C18:3 n-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 (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., 2017a) 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) has 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., 2019b). Within this framework, this study aims 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), BCS (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 (09:00 and 16:00 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 post–lambing days and lasted 28 days (divided into 4 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 BCS 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 08:00 and 12:00 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, MN, USA) in heptane were mixed. Afterwards, 4 ml of 0.5M $CH_3ONa/CH_3OH$ solution were 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) were 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, MN, USA) in heptane were mixed in a 15 ml polypropylene centrifuge tube. Then, 2 ml of heptane and 4 ml of the 0.5M $CH_3ONa/CH_3OH$ 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 were 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.

For the FA determination of feedstuffs and milk a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, MA, USA) was used, equipped with a CP-8400 Autosampler (Bruker, Billerica, MA, 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; Sigma Aldrich, Saint Louis, MO, 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, MN, USA) and relative retention times found in several sources (Kramer et al., 1997; Alves and Bessa, 2009; Bravo-Lamas et al., 2016). 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, while total FA content was expressed as mg of FA per g of sample 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 (C18:2 n-6 + C20:4 n-6)/(C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3) and

the hypocholesterolaemic/hypercholesterolaemic ratio as [(C18:1 c9 + C18:2 n-6 + C20:4 n-6 + C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-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 × ΣMUFA + 0.5 × PUFA n-6 + PUFA n-3) + (PUFA n-3/PUFA n-6] following Ulbricht and Southgate (1991). The enzymatic activity levels of $\Delta^9$-desaturase and elongase were determined using the mathematical model described by Malau-Aduli et al. (1997) according to the following equations: $\Delta^9$-desaturase C16 = C16:1 c9/(16:0 + C16:1 c9); $\Delta^9$-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 to 4) and their interaction as the fixed effects and ewe as the random effect. Degrees of freedom were adjusted with the Kenward–Rodger 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–groups 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, BCS, as well as the DM intake and milk yield had been presented previously (Baila et al., 2022a). Briefly, BW, BCS, 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 BCS, 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 BCS decreased sharply in the first week post–lambing ($P < 0.05$). 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. Regarding 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 FA of the sainfoin offered for the four lactation weeks are shown in Table 7. The main FA were C18:3 n-3, C16:0 and C18:2 n-6. Week affected the total FA content, four individual FA ($P < 0.05$; Table 7), total saturated fatty acids (SFA), PUFA and the PUFA/SFA ratio ($P < 0.05$; Figure 15). 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 C18:3 n-3 and PUFA/SFA ratio value appeared at week 1 ($P < 0.01$).

**Table 7.** Evolution of the fatty acid (FA) composition of the fresh sainfoin offered to ewes.

| | Fresh sainfoin | | | | | | Barley | |
|---|---|---|---|---|---|---|---|---|
| | Week | | | | | | | |
| Fatty acids (FA) | 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 |
| C18:2 n-6 | 14.3 | 14.0 | 14.4 | 13.7 | 0.42 | 0.60 | 49.1 | 0.18 |
| C18:3 n-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 $P < 0.05$

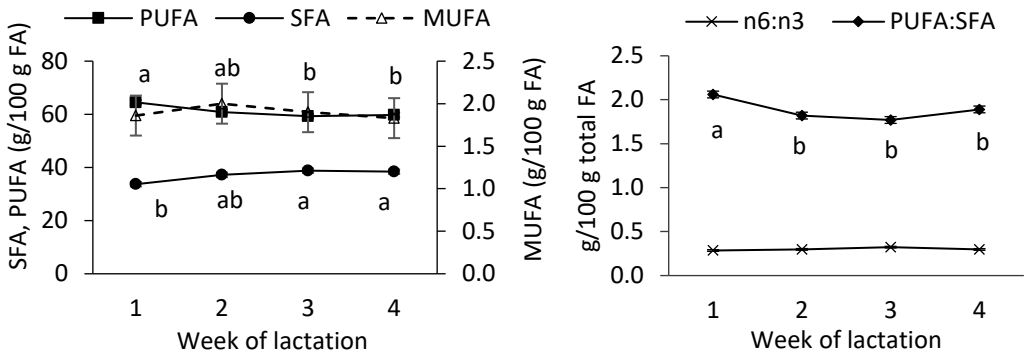


**Figure 15.** Evolution of total SFA, MUFA, PUFA, and PUFA n-6/n-3 and PUFA/SFA ratios of sainfoin offered to ewes during the experimental period.

Abbreviations: FA = fatty acids; MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids; SFA = 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 acids: total saturated fatty acids ratio

[a,b] Values in a parameter with different superscripts differ significantly among weeks at $P < 0.05$. Vertical bars indicate the SE.

### *Milk fatty acids*

In all, 115 individual FA were identified, but only the most representative are shown, of which the most abundant were C16:0, C18:0 and C18:1 c9. Table 8 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 8).

**Table 8.** Effects of the presence of proanthocyanidins (PAC) and lactation week (W) on total, saturated, odd- and branched-chain fatty acids (FA) of ewes' milk.

| | PAC | | Week | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sainfoin | Sainfoin +PEG | 1 | 2 | 3 | 4 | RSD | PAC | W | PAC x W |
| 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; FA = 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 $P < 0.05$.

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 8), 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 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 2, remaining steady thereafter.

The effects of PAC and week on the milk monounsaturated fatty acids (MUFA) contents are presented in Table 9. 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 2 ($P < 0.001$). The presence of PAC reduced 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 9.** Effects of the presence of proanthocyanidins (PAC) and lactation week (W) 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 | W | PAC x W |
| 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; FA = 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 $P < 0.05$.

The main effects of sainfoin PAC and lactation week on milk PUFA are shown in Table 10. 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 10). 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, C18:2 n-6, total PUFA n-3, C18:3 n-3 and C20:5 n-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 C18:2 n-6 and C20:4 n-6) and PUFA n-3 (represented mostly by C18:3 n-3) decreased throughout lactation ($P < 0.05$), although C18:3 n-3 increased again at week 4 ($P < 0.05$).

**Table 10.** Effects of the presence of proanthocyanidins (PAC) and lactation week (W) 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 | W | PAC x W |
| 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 |
| C18:2 n-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 |
| C18:3 n-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 |
| C20:2 n-6 | 0.013 | 0.015 | 0.016 | 0.014 | 0.015 | 0.011 | 0.0049 | 0.45 | 0.13 | 0.06 |
| C20:3 n-6 | 0.029 | 0.034 | 0.038 | 0.031 | 0.027 | 0.031 | 0.0109 | 0.25 | 0.07 | 0.95 |
| C20:4 n-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 |
| C22:4 n-6 | 0.008 | 0.009 | 0.008 | 0.009 | 0.009 | 0.007 | 0.0050 | 0.38 | 0.86 | 0.23 |
| C22:5 n-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 |
| C18:3 n-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 |
| C20:3 n-3 | 0.020 | 0.019 | 0.021 | 0.019 | 0.019 | 0.021 | 0.0050 | 0.49 | 0.42 | 0.70 |
| C20:5 n-3 | $0.11^{x}$ | $0.086^{y}$ | 0.11 | 0.098 | 0.094 | 0.089 | 0.0173 | 0.02 | 0.15 | 0.51 |
| C22:5 n-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 |
| C22:6 n-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; FA = 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 $P < 0.05$.

The main milk FA ratios and enzymes are shown in Table 11. The n-6/n-3 and PUFA:SFA ratios and $\Delta^9$-desaturase C18 were affected by the interaction between the presence of PAC and week of lactation ($P < 0.05$). The presence of PAC decreased the n-6/n-3 ratio (1.15 *vs.* 1.57 for Sainfoin *vs.* Sainfoin+PEG; $P < 0.001$) and $\Delta^9$-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 ($P > 0.05$).

**Table 11.** Effects of the presence of proanthocyanidins (PAC) and lactation week (W) on the ewes' milk fatty acid ratios and enzymes.

| | PAC | | Week | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Items | Sainfoin | Sainfoin +PEG | 1 | 2 | 3 | 4 | RSD | PAC | W | PAC x W |
| 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 |
| $\Delta^9$-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 |
| $\Delta^9$-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; PUFA = total polyunsaturated fatty acids; SFA = total saturated fatty acids; h/H = hypocholesterolemic to hypercholesterolemic ratio.
[a-c] Values within a row with different superscripts differ significantly among weeks at $P < 0.05$.

### *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 FA, as shown in Figure 16. The FA forming the CAN varied according to week. It was possible to separate both treatments with C18:3 n-3 content at week 1; with C18:1 c12 and C18:2 n-6 at week 2; with iC17:0, C18:1 c9, C18:3 c9,t11,t15 and C20:5 n-3 at week 3; with C14:0 8-Me, C20:5 n-3 and C22:5 n-6 at week 4.

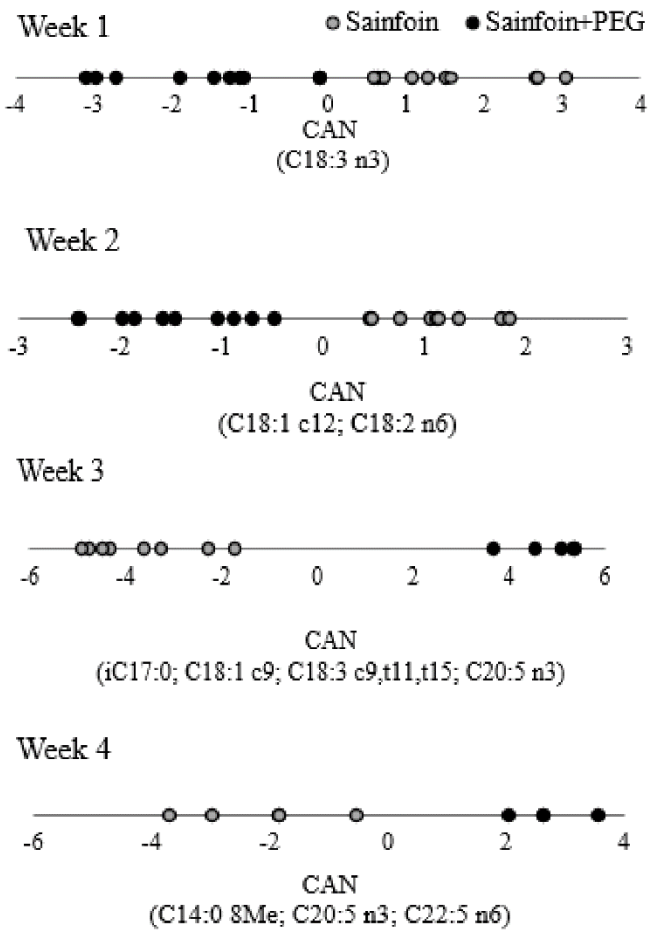


**Figure 16.** Graph of the canonical function (CAN) of milk fatty acids for Sainfoin and Sainfoin+PEG ewes and for weeks 1, 2, 3, and 4.

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

## 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 FA were C18:3 n-3, C16:0 and C18:2 n-6. Changes in plant maturity may have affected the sainfoin FA evolution during the study. The maturation process decreases the proportion of leaves rich in C18:3 n-3 (Glasser et al., 2013), the most abundant FA in sainfoin, which explains the drop in the total FA content herein observed with advancing 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 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 were observed in the Sainfoin treatment in the present study because these FA 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 $\Delta^9$-desaturase, which will be subsequently discussed.

The increase in dietary PUFA (C18:3 n-3 and C18:2 n-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 PUFA are exposed to isomerisation and disappearance. The lower concentrations of OCFA, BCFA and *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 (C18:3 n-3, C18:2 n-6, and C18:1 c9), and from some of the FA 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., 2009a), and even between–animal variability (Harnly et al., 2022).

The milk FA profile also depends on mammary gland activity, where $\Delta^9$-desaturase enzyme introduces a double bond at the $\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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 FA 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, $\Delta^9$-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 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.

# Manuscript III

## Sainfoin in the dams' diet as a source of proanthocyanidins: effect on the growth, carcass, and meat quality of their suckling lambs

C. Baila, S. Lobón, M. Blanco, I. Casasús, G. Ripoll, and M. Joy

**Simple Summary**

Several studies point out that the use of local forage legumes, such as sainfoin, can be appropriate for feeding sheep autochthonous breeds, with additional benefits also for the soil. Besides, sainfoin has a medium content of proanthocyanidins (PAC), also known as condensed tannins, the effects of which have been studied in fattening lambs but seldom on suckling lambs. The aim of the study was to evaluate the effect of PAC of sainfoin fed to dams on the productive traits, weight of the digestive organs, and on carcass and meat quality of their suckling lambs. The inclusion of PAC from sainfoin in the dam diet did not produce detrimental changes on the growth and carcass and meat characteristics of their suckling lambs. Therefore, sainfoin can be fed to ewes during lactation to produce suckling lambs, achieving good performances and meat quality.

**Abstract**

Sainfoin (*Onobrychis viciifolia*) is a forage legume with a medium content of proanthocyanidins (PAC), which may affect animal performance and product quality. The objective of the present study was to assess the effect of PAC from sainfoin fed to dams, using polyethylene glycol (PEG) as a blocking agent, on the performance and carcass and meat quality of their suckling male lambs. After lambing, twenty lactating dams were fed fresh sainfoin *ad libitum* plus 200 g per day of barley; ten were orally dosed with water (Sainfoin), and ten were dosed orally with a water dilution of 100 g PEG (Sainfoin + PEG). Their lambs (4.1 ± 0.64 kg at birth) suckled *ad libitum* until they reached the target slaughter weight of 10–12 kg. The presence of PAC in the dams' diet did not affect the growth, blood metabolites and carcass weight and fatness of the suckling lambs but decreased the lightness of caudal fat ($P < 0.05$) and increased the weight of the digestive compartments ($P < 0.05$). Regarding the meat characteristics, PAC only decreased polyphenols content ($P < 0.05$). In conclusion, the presence of PAC in the dams' diet had not significant effects on the performance and product quality of their suckling lambs.

**Keywords**

*Onobrychis viciifolia*, condensed tannins, performance, plasma metabolites, meat colour

**Introduction**

Nowadays, there is increasing social pressure for livestock production systems to minimize their negative environmental impacts, and to reduce the inclusion of feed components that compete for land use with human food crops (Mottet et al., 2017). In the last decades, the European Union has encouraged the use of local legumes for animal feeding in order to reduce the dependency on soybean meal, and to benefit from their positive environmental effects (Calabrò et al., 2009; Guyader et al., 2016). Among legume forages, sainfoin (*Onobrychis viciifolia*) has proven to be an excellent forage to be fed during lactation in ewes (Lobón et al., 2017a). Furthermore, consumers are increasingly aware of the importance of food quality on human health, which has increased the demand for products obtained from forage–fed animals as they are considered healthier than those obtained from concentrate–fed diets (Girard et al., 2016; Lobón et al., 2017a), as well as more respectful with animal welfare.

In the Mediterranean area, the traditional production of suckling lambs (slaughtered at 10–12 kg BW) is based on a system in which dams are fed diets mainly composed by straw, cereals and byproducts, and lambs are fed exclusively on their dams' milk. In this framework, the inclusion of high proportions of fresh forage in the diet of the ewes could be an interesting alternative. In fact, grazing sainfoin during lactation improved the meat quality of light lambs even after a finishing period on concentrates, when compared to lambs reared with dams grazing alfalfa (Lobón et al., 2019b). This could be due to a possible synergy between dietary proanthocyanidins (PAC) present in sainfoin and other antioxidant components in the muscle (Valenti et al., 2019) or the milk (Leparmarai et al., 2019).

Previous research concerning the effect of PAC on lamb performance is not conclusive, as some studies reported that weight increased (Valderrábano et al., 2010; Giller et al., 2021), decreased (Girard et al., 2016) or did not change (Peng et al., 2016). Regarding meat quality, there is also no consensus about the relation between PAC in lamb diets and the colour parameters and haem pigments contents (Luciano et al., 2009; Seoni et al., 2018). On the other hand, improvements in the antioxidant capacity of tissues due to the action of PAC have been observed (Soobrattee et al., 2005; López-Andrés et al., 2013). Therefore, this great variability of results may depend on molecular

weight, structure, and degree of polymerisation of PAC, as well as on the type of diet and animal studied (Piluzza et al., 2014).

Most of the studies regarding the inclusion of PAC have been carried out in fattening lambs with limited research on suckling lambs, the diet of which is based almost exclusively in milk. Therefore, to study the effect of PAC on the meat of suckling lambs, the source of PAC has to be included in their dam's diet, and results cannot be extrapolated from those obtained in fattening lambs. Hence, the aim was to evaluate the effect of PAC of fresh sainfoin fed to dams on productive parameters, weight of the digestive organs, and carcass and meat quality of suckling lambs.

## Material and methods

### *Experimental site*

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, were approved by The Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA).

### *Animal management and experimental design*

The experiment was conducted in the facilities of CITA in Zaragoza, Spain (41°3′ N, 0°47′ W and 216 m above sea level) in spring 2019, during 28 days. All the methodology carried out during the experiment has been explained in detail in a previous study (Baila et al., 2022a). Briefly, after lambing twenty multiparous Rasa Aragonesa ewes with their male lambs were assigned into two homogeneous groups according to ewe BW (61 ± 6.2 kg), BCS (3.3 ± 0.57), lambing date (April 6 ± 0.1 d) and lamb BW at birth (4.1 ± 0.64 kg). All dams were fed fresh sainfoin (*Onobrychis viciifolia* cv Reznos) [dry matter (DM): 213 g/kg; crude protein (CP): 116 g/kg DM; neutral detergent fibre (NDF): 369 g/kg DM; acid detergent fibre (ADF): 264 g/kg DM; total PAC: 38.8 g eq. PAC sainfoin/kg DM], water and mineral blocks ad libitum and 200 g/head/day of barley [DM: 912 g/kg; NDF: 250 g/kg DM; ADF: 87 g/kg DM; CP: 95 g/kg DM] distributed in two meals. Before each meal, ten ewes were drenched with 100 ml of water (Sainfoin), whereas ten ewes were orally

dosed with 100 ml of polyethylene glycol (PEG) solution (50 g of PEG 4000/100 ml; Sainfoin + PEG to inactivate the effects of PAC. Each pair of dam–lamb was placed in an individual pen (2.2 $m^2$). Lambs exclusively suckled their dams ad libitum until they reached the target slaughter weight of 10–12 kg BW.

The detailed chemical composition of feedstuffs and milk has been reported in a previous study (Baila et al., 2022a). The dry matter intake (1879.5 ± 281.3 g DM/d) and the milk yield and chemical composition was similar between groups (milk yield: 1.25 l/d; crude fat: 6.54%, CP: 5.02%, lactose: 5.28%), except for the polyphenols (42.3 *vs.* 51.8 µg eq. [gallic acid]/g fresh sample, for Sainfoin and Sainfoin + PEG, respectively) and urea (275 *vs.* 338 mg/l, for Sainfoin and Sainfoin + PEG, respectively).

***Measurements and sampling procedures***

Lambs were weighed weekly at 8:00 h with an electronic scale (0.1 kg precision) to calculate the average daily gain (ADG). Blood samples were obtained the day of slaughter, from the jugular vein into heparin tubes (Vaccuette, Madrid, Spain). Samples were immediately centrifuged (3000 *g* for 15 min at 4 °C) and stored at −20 °C until the metabolites analyses were performed.

When lambs reached the target weight of 10–12 kg BW, they were stunned by a captive bolt pistol and exsanguinated in the experimental abattoir of the Research Centre, using standard commercial procedures and according to Council Regulation (EC) Nº 1099/2009. The contents of the digestive tract corresponding to the sections of reticulum–rumen, omasum–abomasum, and duodenum–jejunum were extracted and weighed. Then, the empty digestive compartments were weighed. Hot carcass weight (HCW) was recorded without head and offal. After 24 h chilling at 4 °C in total darkness, the cold carcass weight (CCW) was obtained. The dressing percentage was calculated as:

$$\frac{\text{HCW}}{\text{slaughter weight}} \times 100$$

and the carcass shrinkage was calculated as:

$$\left(\frac{\text{HCW} - \text{CCW}}{\text{HCW}}\right) \times 100$$

The fatness degree of the carcasses was determined following the Community Scale for Classification of Carcasses of Ovine Animals and of Light Lambs (EEC 461/93) and scored from 1 (1-, very low) to 4 (4+, very high) following the scale of 1 (low), 2 (slight), 3 (average), and 4 (high). Caudal subcutaneous fat colour was measured on tail root using a Minolta CM-2006d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan), registering lightness (L*), redness (a*), and yellowness (b*), which were used to calculate hue angle ($h_{ab}$), and chroma ($C^*_{ab}$). The absolute value of the summation of the translated spectrum (SUM) was calculated as:

$$SUM = \left[\left(\frac{TR_{450}}{2}\right) + TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + \left(\frac{TR_{510}}{2}\right)\right] \times 10$$

where $TR_i$ was the reflectance value at i nm. An extensive explanation of the baselines of the method is exposed in Prache and Theriez (1999).

After that, the carcass was carefully split longitudinally into the two half carcasses and the *longissimus thoracis et lumborum* (*LTL*) muscles of both sides were collected. Perirenal fat deposit was extracted and weighed.

***Meat quality***

The *LTL* muscles from 4$^{th}$–6th lumbar vertebrae of the left side were used to measure the pH with a pH-meter equipped with a Crison 507 penetrating electrode (Crison Instruments, S.A., Barcelona, Spain) and to estimate the chemical composition by NIRs (FoodScan$^{TM}$2, Foss Analytics, Hilleroed, Denmark). From 6th to 13th thoracic *vertebrae* from both sides were sliced into 2.5 cm–hick samples, the left ones were assigned to days 0, 2 and 7 of display and the right ones for days of display 5 and 9. The slices were placed in trays, wrapped with oxygen permeable polyvinyl chloride film, and kept in darkness at 4 °C until being measured for colour and haem pigment estimations. *LTL* colour was measured as had been explained above, while haem pigments were measured as described in Lobón et al. (2017). The 0-d samples were allowed to bloom also in darkness at 4 °C for 1 h before being measured. After that, meat was freeze–dried and vacuum–stored in total darkness at -80 °C until the analysis of polyphenols and a 2,2–azinobis–3–ethylbensothiazoline–6–sulfonic acid (ABTS) assay, which were determined according to Leal et al. (2019) and Vázquez et al. (2015), respectively.

### *Plasma analysis*

Plasma concentrations of creatinine and urea (kinetic methods) were analysed with an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). The methodology to determine antioxidant activity of plasma regarding to polyphenols concentration, superoxide dismutase (SOD) and ABTS and the method of the determination of lipid oxidation (measured as malondialdehyde; MDA) are described in Baila et al. (2022a).

### *Statistical analysis*

Data were analysed with the SAS (v.9.3; SAS Inc.: Cary, NC, USA) using the lamb as the experimental unit. The productive traits (animal performances and plasmatic metabolites), carcass characteristics and meat chemical composition of the suckling lambs were analysed through a variance analysis with a general linear model, with the presence of PAC as the fixed effect. Colour and haem pigments of *LTL* muscle were analysed with mixed models (MIXED procedure) with presence of PAC, time of display and their interactions as fixed effects and the lamb as the random effect. The degrees of freedom were adjusted with the Kenward–Rodger correction. Results were reported as least square means and their associated standard errors of the means, and the Tukey correction was applied for pairwise comparisons. The effects were considered significant at $P < 0.05$.

## Results

### *Lamb performance and plasma metabolites*

The productive traits of the suckling lambs are shown in Table 12. The presence of PAC in the dams' diet did not affect any productive trait studied, such as average daily gain, age, and weight at slaughter ($P > 0.05$). Regarding the plasma metabolites at slaughter, the presence of PAC in the dams' diet did not affect creatinine and urea plasmatic concentrations of the suckling lambs ($P > 0.05$). In the same line, the plasma polyphenols concentration and the antioxidant activity measured as SOD, ABTS and MDA were not affected by the presence of PAC in the dams' diet ($P > 0.05$).

**Table 12.** Effect of the presence of proanthocyanidins (PAC) in the dams' diet [1] on the performance, plasma metabolites and antioxidant (AO) status of their suckling lambs.

| Item | Sainfoin | Sainfoin + PEG | s.e.m [2] | *P*-Value |
|---|---|---|---|---|
| Birth weight, kg | 4.0 | 4.2 | 0.29 | 0.59 |
| Average daily gain, g/d | 272 | 283 | 21.0 | 0.63 |
| Slaughter age, d | 28.1 | 25.7 | 2.38 | 0.33 |
| Slaughter weight, kg | 11.6 | 11.1 | 0.33 | 0.14 |
| Plasma metabolites | | | | |
| Creatinine, µmol/l | 52.2 | 55.4 | 7.28 | 0.67 |
| Urea, mmol/l | 4.86 | 5.16 | 0.619 | 0.63 |
| Polyphenols, eq. [gallic acid] mg/ml | 1.62 | 1.70 | 1.160 | 0.49 |
| Antioxidant status | | | | |
| Superoxide dismutase (SOD), U/ml | 0.68 | 0.63 | 0.115 | 0.65 |
| Total AO capacity- ABTS [3] | 5.32 | 5.79 | 0.294 | 0.13 |
| Lipid oxidation, µM MDA [4] | 9.69 | 9.51 | 0.699 | 0.31 |

[1] Sainfoin: lambs whose dams were fed ad libitum sainfoin + 200 g/d barley; Sainfoin + PEG: lambs whose ewes were fed ad libitum sainfoin + 200 g/d barley + 100 g/d polyethylene glicol (PEG); [2] standard error of the mean; [3] 2,2–azinobis–(3–ethylbensothiazoline)–6–sulfonic acid, µmol eq. [TROLOX]/ml; [4] malondialdehyde.

***Digestive compartments and carcass traits***

The presence of PAC in the dams' diet significantly increased the weight of the content in the reticulum–rumen ($P < 0.05$) and, concomitantly, in the forestomach ($P < 0.01$) and increased the weight of the digestive compartments ($P < 0.05$), except for the omasum–abomasum (Table 13). Nevertheless, most of the carcass characteristics were not affected by the treatment (Table 13). The colour of subcutaneous fat of the suckling lambs was similar between groups, except for lightness, which was decreased with the presence of PAC in the dams' diet ($P < 0.01$).

**Table 13.** Effect of the presence of proanthocyanidins (PAC) in the dams' diet [1] on the weights of the digestive compartments, the carcass characteristics, and the colour of caudal fat deposits of their suckling lambs.

| Item | Sainfoin | Sainfoin+PEG | s.e.m [2] | *P-v*alue |
|---|---|---|---|---|
| Weight of digestive content, g fresh matter (FM) | | | | |
| Reticulum–rumen | 284 | 192 | 39.5 | 0.033 |
| Omasum–abomasum | 185 | 161 | 41.6 | 0.57 |
| Forestomach | 469 | 353 | 47.5 | 0.011 |
| Duodenum–jejunum | 69 | 46 | 18.4 | 0.23 |
| Weight of digestive compartments, g FM | | | | |
| Reticulum–rumen | 126 | 78 | 16.3 | 0.009 |
| Omasum–abomasum | 95 | 73 | 14.8 | 0.15 |
| Forestomach | 221 | 151 | 17.2 | 0.001 |
| Duodenum–jejunum | 171 | 132 | 13.8 | 0.011 |
| Carcass traits | | | | |
| Hot carcass weight, kg | 7.78 | 7.64 | 0.264 | 0.61 |
| Cold carcass weight, kg | 6.19 | 6.15 | 0.238 | 0.87 |
| Dressing percentage, % | 55.6 | 57.2 | 1.03 | 0.13 |
| Carcass shrinkage, % | 3.98 | 2.99 | 0.595 | 0.12 |
| Fatness score, 1–4 scale | 2.10 | 2.15 | 0.103 | 0.74 |
| Perirenal fat weight, g | 128 | 129 | 24.7 | 0.98 |
| Colour of caudal fat deposits | | | | |
| Lightness (L*) | 68.8 | 71.4 | 0.48 | 0.015 |
| Redness (a*) | 2.6 | 2.5 | 0.31 | 0.89 |
| Yellowness (b*) | 12.3 | 11.9 | 0.45 | 0.66 |
| Hue angle ($h_{ab}$) | 78.2 | 78.9 | 0.16 | 0.77 |
| Chroma ($C^*_{ab}$) | 12.6 | 12.2 | 0.49 | 0.71 |
| SUM [3] | 102 | 110 | 13.1 | 0.75 |

[1] Sainfoin: lambs whose dams were fed ad libitum sainfoin + 200 g/d barley; Sainfoin + PEG: lambs whose ewes were fed ad libitum sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG); [2] standard error of the mean; [3] estimator of carotenoids.

***Meat quality***

There were no differences between groups in the pH values of *LTL* at 24 h *post–mortem*, DM, CP, intramuscular fat, total collagen, and ash contents (Table 14). The polyphenol content of meat significantly decreased with the presence of PAC in the dams' diet ($P < 0.05$) but did not affect the total antioxidant capacity estimated by ABTS content.

**Table 14.** Effect of the presence of proanthocyanidins (PAC) in the dams' diet [1] on the pH, chemical composition, and total antioxidant (AO) capacity of the meat of their suckling lambs.

| Item | Sainfoin | Sainfoin + PEG | s.e.m [2] | *P*-Value |
|---|---|---|---|---|
| $pH_{24\ h}$ | 5.5 | 5.53 | 0.032 | 0.46 |
| Dry matter, % fresh matter (FM) | 21.0 | 21.0 | 0.07 | 0.85 |
| Crude protein, % FM | 21.0 | 21.3 | 0.28 | 0.17 |
| Intramuscular fat, % FM | 2.36 | 2.19 | 0.124 | 0.18 |
| Total collagen, % FM | 0.77 | 0.77 | 0.153 | 0.99 |
| Ash, % FM | 1.81 | 1.78 | 0.103 | 0.75 |
| Polyphenols, µg eq gallic acid/g FM | 71.0 | 81.5 | 4.17 | 0.021 |
| Total AO capacity–ABTS [3] | 0.44 | 0.45 | 0.035 | 0.76 |

[1] Sainfoin: lambs whose dams were fed ad libitum sainfoin + 200 g/d barley; Sainfoin + PEG: lambs whose ewes were fed ad libitum sainfoin + 200 g/d barley + 100 g/d polyethylene glycol (PEG); [2] standard error of the mean [3] 2,2–azinobis–(3–ethylbensothiazoline)–6–sulfonic acid, µmol eq. [TROLOX]/g FM.

No significant interactions were observed between the presence of PAC in the dams' diet and the time of display of meat. The *LTL* colour (Figure 17) was not modified by the presence of PAC on the dams' diet, but it was affected by the time of display ($P < 0.05$). All colour variables increased from day 0 to day 2 ($P < 0.05$), and thereafter L* and b* remained steady, whereas $C^*_{ab}$ and a* increased until day 5, remaining unchanged onwards ($P < 0.001$).

Similarly to the colour parameters, the presence of PAC in the dams' diet had no significant effect on haem pigments (Figure 18), but the day of display had a significant effect ($P < 0.05$). Metmyoglobin and oxymyoglobin showed a similar evolution over time, increasing until day 5 ($P < 0.001$), whereas deoxymyoglobin followed the inverse pattern, decreasing until 5 day ($P < 0.001$) and remaining steady thereafter.

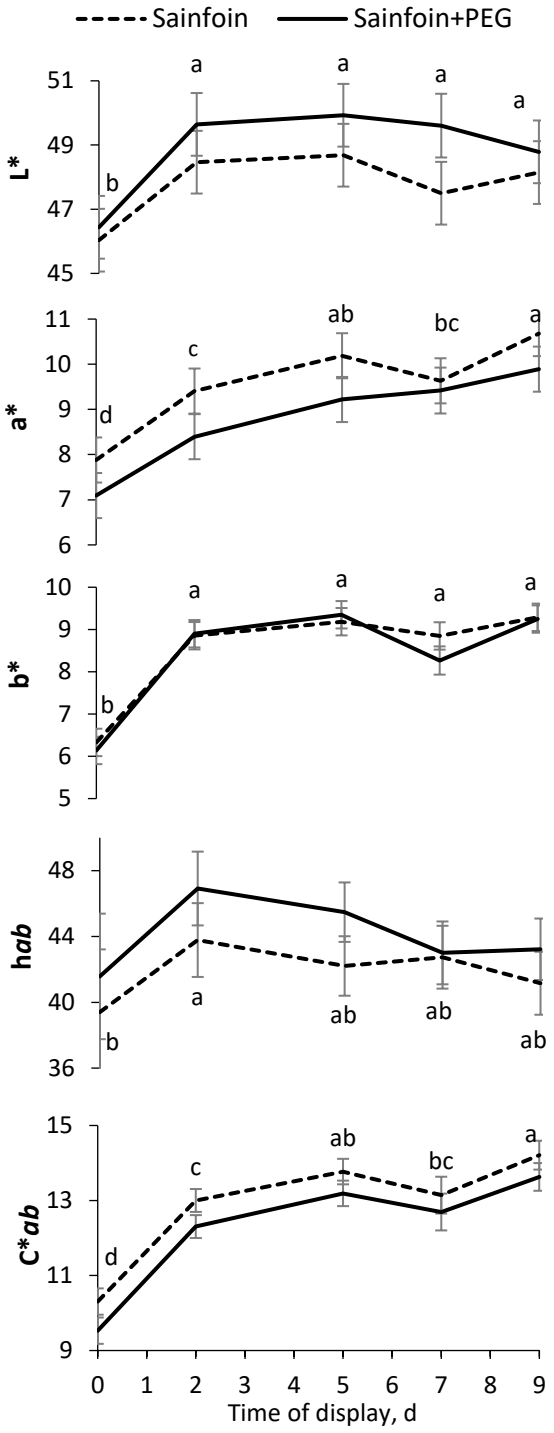


**Figure 17.** Evolution of instrumental colour [lightness (L*), redness (a*), yellowness (b*), hue angle ($h_{ab}$), and chroma ($C^*_{ab}$)] of meat of suckling lambs according to the presence of proanthocyanidins (PAC) in their dams' diet.

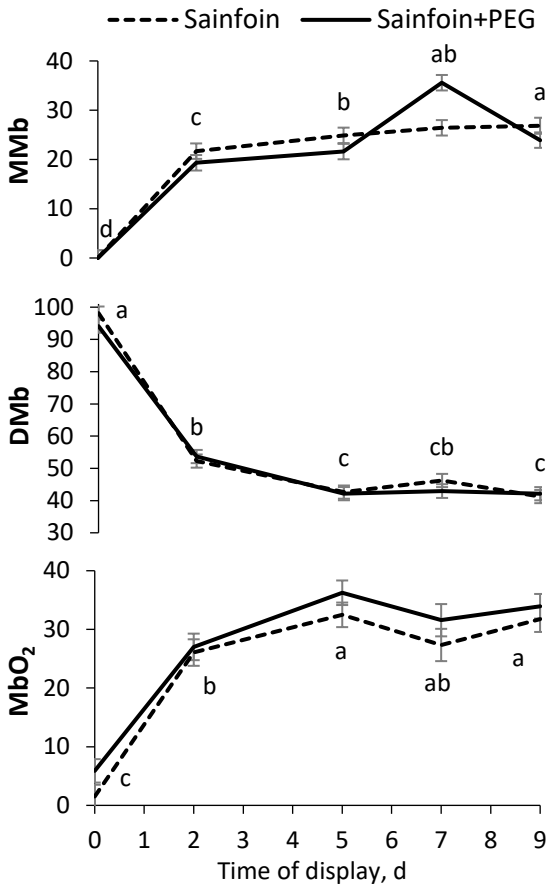


**Figure 18.** Evolution of haem pigments [metmyoglobin (MMb), deoxymyoglobin (DMb), and oxymyoglobin ($MbO_2$)] of meat of suckling lambs according to the presence of proanthocyanidins (PAC) in their dams' diet.

**Discussion**

The lack of effect of the presence of PAC from sainfoin in the diet of lactating ewes on the weight gains and carcass characteristics of their suckling lambs in the current study can be related to the similar milk production and quality of the dams, as previously shown. Milk yield and composition are the main factors responsible for suckling lamb growth (Gallardo et al., 2014), the protein intake being the most determinant (Gargouri et al., 2006). A previous study (Giller et al., 2021) reported a higher ADG in suckling lambs whose dams' received polyphenols of grape seed extract, but not when the supplementation was given directly to fattening lambs. Therefore, it is possible that polyphenols elicit a more pronounced effect during the suckling period compared to the post–weaning phase, as shown in lambs supplemented with grape pomace (Kafantaris et al., 2018). In a meta–analysis, it was reported that the performance of weaned lambs

was not modified when the concentrations of PAC in their diet ranged between 16 and 25 g PAC/kg DM (Javier Álvarez-Rodríguez et al., 2020).

The growth of the suckling lambs of this study was comparable to that observed when dams were fed concentrates indoors, and higher than those obtained with dams fed on pasture during lactation (Fusaro et al., 2020). Therefore, in this study, a diet based mainly on fresh sainfoin with only a 10% of supplementation was sufficient to achieve good performances in rearing a male lamb of this autochthonous breed. This alternative feeding management could be advisable to allow diversification of the production system and to increase system resilience in an unfavourable situation from a meteorological, social, and economic point of view (Ripoll-Bosch, 2013).

The similar plasma urea concentration at slaughter of lambs of both treatments was unexpected, because dams from the Sainfoin group had lower urea concentrations both in the plasma and the milk (Baila et al., 2022a). The Sainfoin + PEG suckling lambs ingested a greater quantity of urea from their dam's milk, but it was not reflected in their plasma concentration, which suggests that the protein metabolism could be different between groups. The plasma creatinine concentration in suckling lambs was similar between treatments, indicating a similar metabolism of muscle mass (Hegarty et al., 2006), so that the PAC of dams' diet had no effect on the use of amino acids of their lambs by reducing the ruminal degradation of dietary protein (Piluzza et al., 2014).

The antioxidant effect of PAC is well known (Soobrattee et al., 2005; López-Andrés et al., 2013), and some studies even report the transfer of dietary phenolic compounds from the milk to the meat of the suckling lamb, where they act as antioxidants (Vieira et al., 2022). However, in this study it was not observed in dams' milk, which was reflected in the similar plasma antioxidant activity of lambs from both treatments. The metabolism of PAC along the digestive tract is complex (Quijada et al., 2018), and the fact that the milk ingested by the lambs has already been processed in the mammary gland complicates the mechanism even further. In addition, (Leparmarai et al., 2019) suggested that most of the phenolic compounds ingested by sheep were catabolised before reaching the milk or blood, so that the actual amount received by the lambs through the milk could be very low.

The carcass characteristics, dressing percentage and fat cover was similar in the suckling lambs of both experimental groups. Differences observed in carcass weight, fatness score and perirenal fat weight are usually related to the quality of feeding sources (Karnezos and Matches, 1993; Bonanno et al., 2011), dry matter intake (Girard et al., 2016; Copani et al., 2016; Rivaroli et al., 2019), and/or age and weight at slaughter (Bonanno et al., 2011), all of which were similar in the present study. On average, the dressing percentage obtained in the suckling lambs was greater than the observed in fattened lambs, due to their lower development of the digestive tract of the former (Bonanno et al., 2011; Girard et al., 2016).

Unexpectedly, the reticulum–rumen and forestomach contents and the empty digestive compartments were heavier in lambs of the Sainfoin group. The higher weight of the digestive tract is usually associated with a worst dressing percentage (Dıaz et al., 2002). In this case, the greater weight of the digestive organs of the Sainfoin group lambs produced a numerically lower dressing percentage, although the differences were not statistically significant. On the other hand, the digestive content is closely related with the intake. Since the milk intake of the lambs of both treatments was similar, the cause of this result remains unclear.

Yellowness and SUM (an estimator of carotenoids) in animal fat and meat are mainly influenced by the carotenoid pigments coming from feedstuffs (Prache et al., 1990; Girard et al., 2016). The lack of differences observed in these parameters between treatments is ascribed to the similar sainfoin intake of their dams, and the consequent similar intake of secondary compounds. The lower L* value of caudal fat in Sainfoin lambs disagrees with the results observed by Rivaroli et al. (Rivaroli et al., 2019), who concluded that the mechanisms of PAC modifying the L* of caudal fat deposits are unclear. Brainard (2003) developed a method to estimate if instrumental colour differences (expressed as $\Delta E^*_{ab}$) are perceptible by human vision. In relation to this, Carrasco et al. (2009) reported that differences of colour ($\Delta E^*_{ab}$) of caudal fat lower than 5.2 between carcasses were imperceptible. In the present study, the difference between both treatments was 2.6; therefore, the practical implications were minimal. Fat L* values in suckling lambs of Churra Tensina, a similar local breed, ranged between 69 and 72 when their dams received a diet based on fresh forage or hay (Joy et al., 2012). On

the other hand, the colour parameters were similar to those obtained by Lobón et al. (2019) in fattened lambs previously raised by dams grazing sainfoin, despite their post–weaning finishing period and, consequently, heavier slaughter weight (23.4 kg BW).

Regarding the meat quality, the pH value of meat was within the normal range, close to 5.50 (Fusaro et al., 2020). The presence of PAC in the dams' diet did not have any effect on the chemical composition of the meat of their suckling lambs, as Gómez-Cortés et al. (2018) reported when grape pomace was included as a source of PAC in the diet of lactating ewes. The content of fat and protein in the meat were similar to those obtained in suckling lambs of dams fed with pasture (Fusaro et al., 2020) or supplemented with polyphenols (Giller et al., 2021).

In this study, the content of polyphenol in the meat of suckling lambs reflects the concentration in the milk of their dams. Proanthocyanidins and polyphenols are characterised by providing a great antioxidant power (Soobrattee et al., 2005) and studies show an improvement of antioxidant status which increases the shelf life of lamb meat (Vasta and Luciano, 2011; Valenti et al., 2019). However, this result was not reflected on the antioxidant capacity, probably because it was only measured on the first day post slaughter, when the oxidation process had hardly started.

In relation to haem pigments of *LTL*, Vieira et al. (2022) observed a reduction of MMb in the meat of suckling lambs whose dams were fed grape pomace (containing PAC). However, this reduction was observed from day 10 of meat storage, so the time of the present study could have been insufficient to show effects. Contrary to the present results of colour, Vasta et al. (2008) in a review pointed out that the meat of lambs fed with PAC was lighter than that of their counterparts fed with PEG, concluding that the mechanisms of action of PAC on meat colour are still unclear. Besides in the abovementioned review most of studies involved weaned lambs, whereas here we studied suckling lambs fed exclusively with maternal milk.

**Conclusions**

The inclusion of PAC from sainfoin in the dams' diet had no significant effect on the ADG, plasmatic antioxidant activity, and carcass and meat quality of their suckling lambs.

Therefore, fresh sainfoin can be fed to ewes during lactation to produce suckling lambs, achieving good performances and meat quality.

# Manuscript IV

## Sainfoin can be included up to 40% in the finishing concentrate of lambs without affecting their performance, ruminal fermentation, and carcass quality

C. Baila, S. Lobón, M. Blanco, I. Casasús, G. Ripoll, and M. Joy

**Abstract**

Sainfoin (*Onobrychis viciifolia*) is considered an excellent forage legume to be included in sheep diets as fresh forage, but its inclusion in concentrates fed to lambs reared under intensive systems has been scarcely studied. This study evaluated the effects of including different levels of dehydrated sainfoin in the concentrates fed to light lambs during the finishing period on animal performance, carcass traits, and ruminal fermentation. Twenty–six weaned male Rasa Aragonesa lambs (14.0 ± 0.49 kg body weight) were randomly grouped and individually fed *ad libitum* with isoproteic and isoenergetic pelleted concentrates containing 0% sainfoin (0SF; *n*=9), 20% sainfoin (20SF; *n*=9) or 40% sainfoin (40SF; *n*=8) for 40 days, from weaning to slaughter. The 40SF lambs had a higher dry matter intake ($P < 0.01$) and tended to show an improvement of average daily gain ($P < 0.10$). The diet did not affect most ruminal fermentation parameters ($P > 0.05$), except for pH, which was greater in 40SF lambs than in 20SF lambs ($P < 0.05$), and the proportion of acetic acid and the acetic/propionic ratio, both of which were higher in 40SF and 20SF lambs than in 0SF lambs ($P < 0.01$). The diet did not affect carcass weight, dressing percentage or the colour of *rectus abdominis* muscle and subcutaneous caudal fat ($P > 0.05$). The lack of detrimental effects on lamb performance and carcass traits suggests that the inclusion of up to 40% sainfoin in the concentrate of light lambs reared indoors would be advisable to promote the use of local forage resources.

**Keywords**

*Onobrychis viciifolia*, sheep, rumen, growth, metabolites, volatile fatty acids.

**Introduction**

Consumers from some Mediterranean regions, mainly Southern Europe, demand lamb meat with pale colour and white fat. To offer this type of meat, farmers must produce lambs under intensive systems, where light lambs (22–28 kg BW) are reared indoors, without grazing, and fed *ad libitum* on cereal–based concentrate plus cereal straw. However, this intensive system is facing some socio–economic challenges which are pushing towards some changes in production models. Global economic instability is forcing farmers to advocate for a system of local sourcing that provides greater self–

sufficiency and less environmental impact, making it a good alternative to the growing concern about the contribution of livestock farming to climate change (IPCC, 2022). Besides, there is also an increasing demand for healthier products by consumers, which is one of the most important current goals in animal production.

The inclusion of locally–produced forages has been widely studied as one of the strategies to simultaneously achieve greater sustainability and self–sufficiency and provide added value and higher quality to edible ruminant products (Buccioni et al., 2015; Huyen et al., 2020; Moorby and Fraser, 2021; Santos-Silva et al., 2023). Local forage legumes have shown beneficial results for ruminant production systems in terms of animal performance, product quality, health, and environmental sustainability (Waghorn et al., 1998; Rochon et al., 2004; Waghorn and Clark, 2006; Aufrère et al., 2008; Bonanno et al., 2011; Ripoll et al., 2012; Lobón et al., 2016; Johansen et al., 2018). In this sense, sainfoin (*Onobrichis viciifolia*) is a rustic forage legume, well adapted to cold and water scarcity, with high yields and quality in the first spring cut. All these characteristics along with the need to preserve the excess of production made this crop an attractive ingredient to be introduced in the concentrate of lambs reared under intensive systems.

It is known that the inclusion of forages in lamb diets can reduce the carcass fatness, which could be detrimental to meat quality (Priolo et al., 2002a). In addition, meat and carcass colour can also be affected at different extent depending on the tissue and the type of forage (Ponnampalam et al., 2017), which could lead to rejection by consumers. However, when the forage is preserved, the content of those secondary compounds can be reduced (Rufino-Moya et al., 2022), decreasing their potential effect on carcass colour. In view of the above, we hypothesise that the dehydrated sainfoin could be a good alternative for intensive light lamb production. Therefore, the objective of the present study was to evaluate the effects of increasing the level of dehydrated sainfoin inclusion in the pelleted concentrate (0%, 20%, and 40% of sainfoin) of light lambs during the finishing period on performance, metabolic and antioxidant blood status, ruminal fermentation, and carcass traits of finishing lambs.

## Material and methods

All the experimental procedures were accomplished according to the international guidelines of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimental purposes and were supervised and approved by the Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón (CEEA, 2017–07).

### *Animal management and experimental design*

The study was carried out at the facilities of the CITA in Zaragoza (Spain, 41°3' N, 0°47' W; 216 m above sea level). Twenty–six male lambs were selected from the experimental flock of the Rasa Aragonesa breed, reared with their dams, and managed identically until weaning. After, the lambs were randomly distributed into three groups balanced for age (30.0 ± 1.99 d) and body weight (14.0 ± 0.49 kg BW). The lambs were individually housed indoors with free access to concentrate, water, and mineral blocks. Each group received a concentrate with different inclusion of sainfoin for 40 days: a commercial cereal–based concentrate without sainfoin (0SF), concentrate with 20% sainfoin (20SF), and with 40% sainfoin (40SF). The chemical composition of sainfoin dehydrated pellets included in the concentrate can be found in Table 15.

**Table 15.** Chemical composition, proanthocyanidins (PAC) content and their fractions of the sainfoin pellets.

| | mean ± standard deviation |
|---|---|
| Chemical composition | |
| Crude protein, g/kg DM | 174 ± 4.3 |
| Ash, g/kg DM | 75.2 ± 2.51 |
| Neutral detergent fibre, g/kg DM | 263 ± 20.9 |
| Acid detergent fibre, g/kg DM | 129 ± 9.1 |
| Lignin, g/kg DM | 17.0 ± 3.25 |
| Net energy, MJ/kg DM | 18.1 ± 1.37 |
| Proanthocyanidins (PAC) [2] | |
| Total PAC | 1.32 ± 0.527 |
| Extractable PAC | 0.41 ± 0.152 |
| Protein–bound PAC | 0.77 ± 0.529 |
| Fibre–bound PAC | 0.15 ± 0.115 |

[1] g eq. sainfoin PAC/kg DM

**Table 16.** Ingredients, chemical composition, total carotenoids, polyphenols, proanthocyanidins (PAC) and their fractions of the diets [1]

| | 0SF | 20SF | 40SF |
|---|---|---|---|
| Dry matter (DM), g/kg | 905 | 904 | 903 |
| Ingredients, g/kg DM | | | |
| Barley | 310 | 252 | 50 |
| Corn | 250 | 189 | 250 |
| Wheat | 50 | 50 | 102 |
| Gluten feed | 60 | 60 | 130 |
| Soybean meal 47% | 173 | 138 | 159 |
| Bran | 25 | 81 | 0 |
| Palm oil | 10 | 10 | 15 |
| Calcium carbonate | 15 | 13 | 4 |
| Sodium chloride | 5 | 5 | 5 |
| Premix vitamin 0.2% | 2 | 2 | 2 |
| Sainfoin pellet | 0 | 200 | 400 |
| Straw | 100 | 0 | 0 |
| Chemical composition [2] | | | |
| Crude protein, g/kg DM | 174 ± 4.3 | 175 ± 6.5 | 173 ± 5.2 |
| Ether extract, g/kg DM | 32.6 ± 3.25 | 35.7 ± 3.60 | 38.0 ± 3.44 |
| Ash, g/kg DM | 75.2 ± 2.51 | 70.5 ± 2.06 | 78.5 ± 5.48 |
| Starch, g/kg DM | 426 ± 6.9 | 360 ± 13.8 | 296 ± 9.6 |
| Neutral detergent fibre, g/kg DM | 263 ± 20.9 | 292 ± 12.1 | 355 ± 16.4 |
| Acid detergent fibre, g/kg DM | 129 ± 9.1 | 168 ± 6.5 | 249 ± 10.4 |
| Lignin, g/kg DM | 17.0 ± 3.25 | 34.2 ± 3.17 | 59.6 ± 4.37 |
| Acid insoluble ashes, g/kg DM | 0.064 ± 0.2861 | 0.040 ± 0.1536 | 0.063 ± 0.2067 |
| Gross energy, MJ/kg DM | 18.1 ± 1.37 | 18.4 ± 1.25 | 18.4 ± 0.94 |
| Carotenoids, mg/kg DM | 7.72 ± 1.044 | 17.3 ± 1.355 | 29.9 ± 3.355 |
| Polyphenols [3] | 7.85 ± 0.710 | 12.07 ± 0.586 | 16.83 ± 0.960 |
| Proanthocyanidins (PAC) [4] | | | |
| Total PAC | 1.32 ± 0.527 | 3.04 ± 0.448 | 5.23 ± 0.550 |
| Extractable PAC | 0.41 ± 0.152 | 0.50 ± 0.169 | 0.75 ± 0.132 |
| Protein–bound PAC | 0.77 ± 0.529 | 2.07 ± 0.373 | 3.67 ± 0.508 |
| Fibre–bound PAC | 0.15 ± 0.115 | 0.47 ± 0.138 | 0.80 ± 0.118 |

[1] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the fattening concentrate.

[2] mean ± standard deviation

[3] g eq. tannic acid/kg DM

[4] g eq. sainfoin PAC/kg DM

The sainfoin used was cut at flowering stage in the spring, hot air dried, and pelleted. All the ingredients were mixed with the ground sainfoin pellets and pelleted (3.5–mm diameter) to avoid selection and were formulated to be isoenergetic and isoproteic. The ingredients and the chemical composition of the concentrates are presented in Table 16.

The inclusion of sainfoin in the concentrate reduced the cereal and cereal by–product contents from 795 g/kg DM in 0SF concentrate to 632 g and 532 g/kg DM, in 20SF and 40SF concentrates, respectively. The increased level of sainfoin inclusion was counterbalanced by decreases in barley contents and increases in wheat and gluten feed to meet the condition of isoproteic and isoenergetic among the three concentrates. Consequently, the increase of sainfoin inclusion in the concentrate led to a decrease in starch content and an increase in fibre and PAC fractions, although the content of the latter was low.

Concentrates were offered at +15% of the previous day's refusal to allow *ad libitum* feeding. The concentrate offered and refused was recorded daily per lamb to calculate the individual DMI. Samples were taken daily from each concentrate to obtain a weekly composite sample for chemical composition. Lambs were weighed once a week at 8:00 am using an electronic scale (0.1 kg precision) and the average daily gain (ADG) was calculated. Blood samples from jugular vein were obtained fortnightly (weeks 0, 2, 4, and 6) into heparin tubes (Vaccuette, Madrid, Spain), immediately centrifuged (3000 *g* for 15 min at 4 °C) and stored at −20 °C until metabolite analysis. Faecal samples (approximately 50 g) were collected by rectal stimulation at 09:00 h on the last 3 days of weeks 2, 4, and 6 to obtain one composite sample per week of collection. Samples were freeze–dried and stored at −20 °C until digestibility was determined by acid–insoluble ash (AIA) analysis.

***Slaughter procedures***

Lambs were slaughtered without prior fasting in the experimental abattoir of the CITA Research Centre, in accordance with Council Regulation (EC) Nº 1099/2009. The ruminal contents were extracted and filtered through a double cheesecloth before being stored

in sterile jars. Immediately, the pH of the ruminal liquid was measured using a micropH 2002 pH meter (Crison Instruments S.A., Barcelona, Spain). Samples were collected to determine ruminal ammonia ($NH_3$-N) and volatile fatty acids (VFA) contents. For $NH_3$-N analysis, 2.5 mL of rumen liquid was mixed with 0.1 N HCl in a 1/1 ratio (v/v) and stored at −20 °C, while for VFA analysis, 0.5 mL of liquid was mixed with 0.5 mL of deproteinising solution and 1 mL of distilled water and stored at −20 °C for subsequent determination. The rumens were then thoroughly cleaned and the colour was measured on the inner side (in contact with the ruminal papillae) of the ventral sac using a Minolta CM−2006d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan).

Carcasses were weighed without head and offal to obtain the hot carcass weight (HCW) and, after chilling at 4 °C for 24h, the cold carcass weight (CCW) was recorded. These data were used to calculate the dressing percentage (HCW × 100/slaughter weight) and the carcass shrinkage [(HCW-CCW) × 100/HCW]. The fatness degree of the carcasses was then scored following the Community Scale for the Classification of Carcasses of Ovine Animals (EC, 1249/2008): from 1 (low) to 5 (very high). Carcass colour was measured at the subcutaneous caudal fat at the tail root and at the *rectus abdominis* muscle using a Minolta CM−2006d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan). The absolute value of the sum of the translated spectrum (SUM), used as an estimator of carotenoid content, was calculated following an equation based on reflectance values, as explained in Prache and Theriez (1999). Finally, the perirenal fat deposits were extracted and weighed with an electronic scale (0.1 g precision).

***Chemical analyses***

All analyses of the chemical composition of the concentrates were performed in triplicate. Dry matter (DM) and ash contents were analysed in oven–dried samples, and crude protein content was determined by the Dumas Procedure using a nitrogen analyser (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain) according to the AOAC methods (AOAC, 2000). The total starch of the concentrates was measured using the commercial kit K–TSTA–100A (Neogen Corporation, Lansing, MI, USA) following the amyloglucosidase/α–amylase method (AOAC, 2000). Neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin contents of concentrates were

analysed following the sequential procedure of Van Soest et al. (1991) using the Ankom 200/220 fibre analyser (Ankom Technology Corporation, Fairport, NY, USA). The NDF was assayed with a heat stable amylase. Lignin was analysed in ADF residues by solubilisation of cellulose with sulfuric acid. All values were corrected for ash–free content. Ether extract was determined using an Ankom XT10 extractor (Ankom Technology Corporation, Fairport, NY, USA) following the Ankom procedure (AOCS, 2005. The net energy of the diets was calculated using the values of each ingredient set by FEDNA (2019) and an estimation for sainfoin pellets based on the calculation of Mertens (1983). The total carotenoid content of the concentrates was analysed as described in Blanco et al. (2019). The detailed procedure for the analysis of total polyphenols and extractable, protein–bound, and fibre–bound PAC of the concentrates can be found in Baila et al. (2022a).

The estimated organic matter digestibility (OMD) was calculated by the AIA method based on Shrivastava and Talapatra (1962). Briefly, to determine the AIA content, 2 g and 8 g of ground and freeze–dried samples of faeces and concentrates, respectively, were boiled with a mixture of 75 mL of 3N HCl and 25 mL of distilled water. After cooling, they were filtered and incinerated at 550 °C, and the AIA content of faeces and concentrates was determined by weighing. The estimated OM digestibility was calculated using the nutrient to marker ratio in the diet and faeces, as follows:

$$\text{Estimated digestibility coefficient (\%)} = 100 - [100 \times (AIA_{diet}/AIA_{faeces}) \times (OM_{faeces}/OM_{diet})]$$

where $OM_{faeces}$ and $OM_{diet}$ are the OM (%) in faeces and diet, respectively, and $AIA_{faeces}$ and $AIA_{diet}$ are the concentrations (%) of AIA in faeces and diet, respectively.

Plasma concentrations of glucose and urea were analysed by a kinetic method using an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India), whereas non–esterified fatty acids (NEFA) concentrations were determined by an enzymatic method using a commercial kit (Randox Laboratories Ltd., Crumlin Co., Antrim, UK). The concentration of polyphenols was obtained using a 1:25 (plasma: milli–Q water) dilution and following the method of Leal et al. (2019). Plasma 2,2–azinobis–(3–

ethylbensothiazoline)–6–sulfonic acid (ABTS) was studied as indicator of antioxidant activity, while lipid oxidation was determined through the determination of malondialdehyde (MDA). The method followed to analyse ABTS was based on Jiménez-Escrig et al. (2003) and the total MDA was determined as described in Bertolín et al. (2019).

The ruminal content of $NH_3$–N was analysed using the Berthelot reaction following the technique of Chaney and Marbach (1962) and ammonia was determined by the colorimetric method using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA), with the measurement set at 625 nm. The concentrations of VFA were determined using a Bruker Scion 460 gas chromatograph (Bruker, Billerica, MA, USA) equipped with a CP–8400 autosampler, flame ionisation detection, and a BR–SWax capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness, Bruker, Billerica, MA, USA). The technical specifications of the method are described in Rufino-Moya et al. (2019c).

### *Statistical analyses*

Data were analysed using SAS statistical software (v.9.3, SAS Inst. Inc., Cary, NC, USA). The lamb was considered as the experimental unit. The DMI, BW, ADG, total estimated OMD, carcass traits, and rumen fermentation parameters were analysed by a general linear model (GLM procedure) variance analysis with the diet (0SF, 20SF, and 40SF) as fixed effect. Plasma parameters were analysed with mixed models (MIXED procedure) for repeated measures with the diet, week (0, 2, 4, and 6), and their interaction as fixed effects and the lamb as the random effect. Degrees of freedom were adjusted using the Kenward–Rodger correction. Data were reported as least squares means and their associated standard errors of the mean (SEM). Tukey's correction was used for pairwise comparisons. Effects were considered significant at $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$.

## Results

### *Lamb performance and plasma metabolites*

The effect of the diet on performance and estimated OMD is presented in Table 17. The DMI of the lambs was affected by the effect of the diet ($P < 0.001$) with higher intake in lambs fed 40SF than their counterparts. Estimated OMD decreased with increasing levels of sainfoin in the concentrate ($P < 0.001$). The diet did not affect ADG ($P > 0.05$), despite 40SF lambs had a numerically higher weight at slaughter ($P = 0.10$).

**Table 17.** Effect of the diet [1] on the performance and the estimated organic matter digestibility (OMD) of the finishing lambs.

| | 0SF | 20SF | 40SF | SEM[2] | *P*-value |
|---|---|---|---|---|---|
| Dry matter intake, g/d | 741[b] | 745[b] | 895[a] | 17.8 | <0.001 |
| Estimated OMD, % | 71.0[a] | 62.3[b] | 54.9[c] | 5.84 | <0.001 |
| Average daily gain, g/d | 281 | 281 | 333 | 11.3 | 0.09 |
| Slaughter age, d | 70.0 | 70.8 | 71.0 | 1.95 | 0.54 |
| Slaughter weight, kg | 24.9 | 23.9 | 26.2 | 0.71 | 0.10 |

[1] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the fattening concentrate.

[2] Standard error of the mean

[a,b,c] Means within a row with different superscripts differ significantly at $P < 0.05$.

Plasma concentrations of metabolites, polyphenols, antioxidant activity, and lipid oxidation are shown in Figure 19. There was an interaction between the diet and the week on glucose ($P < 0.001$), NEFA ($P < 0.001$), and urea ($P < 0.05$) concentrations. Plasma glucose concentrations kept steady until week 4, but from this moment to the slaughter (week 6) it decreased in both diets with sainfoin, with lower glucose in 20SF compared to values of 0SF lambs ($P < 0.05$). Plasma concentrations of NEFA at the beginning of the experiment were lower in 0SF lambs than their counterparts ($P < 0.05$) but were similar among diets thereafter. The NEFA concentrations remained constant in 0SF lambs during the period studied ($P > 0.05$), whereas in 20SF and 40SF decreased from week 0 to 2 ($P < 0.05$), remaining steady the rest of the period. Regarding plasma urea concentration, all diets showed a decrease from week 0 to week 2 ($P > 0.01$), and thereafter 20SF and 40SF lambs remained steady, while 0SF lambs increased until the end of the study. Despite these differences, no significant effect was observed due to the diet within each week ($P > 0.05$).

Regarding polyphenols content, antioxidant capacity (ABTS), and lipid oxidation, any interaction was observed between diet and week ($P > 0.05$). The plasma polyphenols

content and the antioxidant activity were affected only by the week ($P < 0.001$), increasing as the period studied advanced. Lipid oxidation was affected by the diet ($P < 0.05$) and the week ($P < 0.001$). Although no effect was observed due to the diet in the different weeks studied, the average lipid oxidation was greater in 0SF than in 40SF lambs ($P < 0.05$) while 20SF lambs presented intermedium values (5.86 ± 0.096, 5.54 ± 0.102, and 5.46 ± 0.096, for 0SF, 20SF, and 40SF, respectively). Plasma lipid oxidation increased over time ($P < 0.001$).

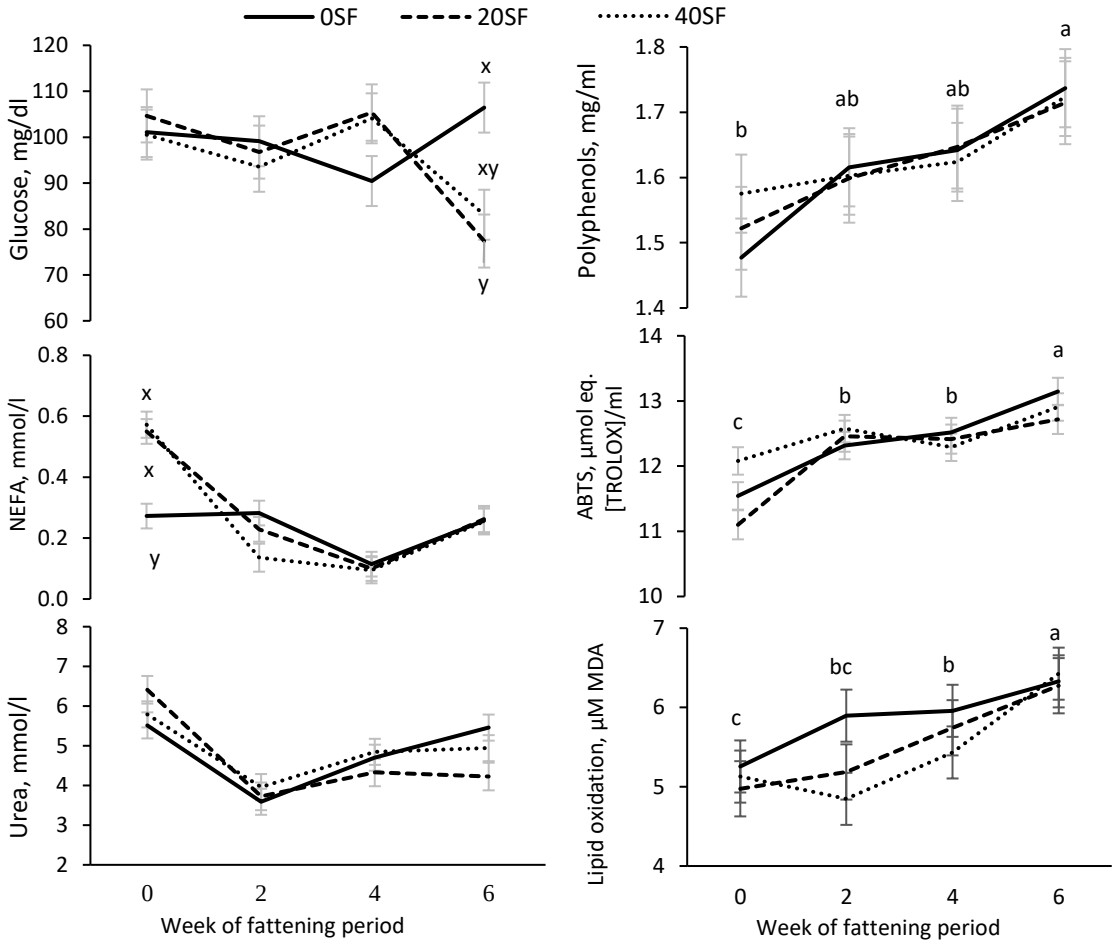


**Figure 19.** Effect of the diet [1] and the week of fattening on the concentrations of glucose, urea, and non–esterified fatty acid (NEFA), polyphenols, antioxidant activity [ABST: 2,2–azinobis–(3–ethylbensothiazoline)–6–sulfonic acid], and lipid oxidation, measured as malondialdehyde (MDA) in the plasma of the lambs.

[1] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the fattening concentrate.

[a,b,c] Values with different superscripts differ significantly among weeks at $P < 0.05$.

[x,y] Values with different superscripts differ significantly among treatments at $P < 0.05$.

***Rumen and fermentation parameters***

The colour of the rumen and the fermentation parameters at slaughter are shown in Table 18. The diet only affected the rumen redness ($P < 0.01$), ruminal pH ($P < 0.05$), proportion of acetic acid ($P < 0.01$), and acetic/propionic ratio ($P < 0.01$). The 40SF lambs presented higher redness of ruminal epithelium than their counterparts ($P < 0.05$), and greater pH values than 20SF lambs ($P < 0.05$), with intermediate values in 0SF lambs ($P > 0.05$). The proportion of acetic acid and acetic/propionic ratio were lower in 0SF than in both diets with sainfoin ($P < 0.05$), regardless of inclusion level. No effect of the diet was observed on $NH_3$–N, total VFA content, or individual proportions of VFA ($P > 0.05$).

**Table 18.** Effect of the diet [1] on the colour of the ruminal epithelium, pH, ammonia ($NH_3$–N), and volatile fatty acids (VFA) of the rumen of the finishing lambs.

| | 0SF | 20SF | 40SF | SEM[2] | *P*-value |
|---|---|---|---|---|---|
| Ruminal epithelium colour | | | | | |
| Lightness | 48.3 | 45.5 | 45.8 | 1.72 | 0.44 |
| Redness | 2.50$^{b}$ | 2.62$^{b}$ | 3.38$^{a}$ | 0.186 | 0.005 |
| Yellowness | 6.95 | 6.69 | 7.53 | 0.724 | 0.71 |
| Hue angle | 68.8 | 67.3 | 64.9 | 2.42 | 0.52 |
| Chroma | 7.44 | 7.22 | 8.30 | 0.689 | 0.51 |
| Ruminal fermentation parameters | | | | | |
| pH | 5.87$^{ab}$ | 5.68$^{b}$ | 6.34$^{a}$ | 0.164 | 0.03 |
| $NH_3$–N, mg/l | 45.5 | 69.1 | 66.3 | 16.08 | 0.53 |
| Total VFA, mM | 95.1 | 96.4 | 96.1 | 13.28 | 0.99 |
| Individual VFA, % total VFA | | | | | |
| Acetic acid | 49.2$^{b}$ | 60.6$^{a}$ | 58.5$^{a}$ | 2.24 | 0.003 |
| Propionic acid | 30.5 | 25.0 | 26.6 | 1.78 | 0.23 |
| Butyric acid | 15.5 | 10.2 | 10.9 | 2.12 | 0.49 |
| Valeric acid | 0.76 | 2.70 | 2.73 | 0.438 | 0.06 |
| Iso–butyric acid | 0.48 | 0.61 | 0.62 | 0.093 | 0.47 |
| Iso–valeric acid | 0.60 | 0.88 | 0.69 | 0.131 | 0.35 |
| Acetic/propionic ratio | 1.66$^{b}$ | 2.59$^{a}$ | 2.23$^{a}$ | 0.183 | 0.002 |

[1] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the fattening concentrate.

[2] Standard error of the mean

[a,b] Means within a row with different superscripts differ significantly among diets at $P < 0.05$.

***Carcass traits***

The diet did not affect any carcass traits and carcass colour ($P > 0.05$; Table 19), except for a trend towards a greater deposition of perirenal fat in 40SF than in 0SF lambs ($P < 0.10$).

**Table 19.** Effect of the diet [1] on the carcass traits and colour of the finishing lambs.

| | 0SF | 20SF | 40SF | SEM[2] | *P*-value |
|---|---|---|---|---|---|
| Carcass traits | | | | | |
| HCW [3], kg | 12.3 | 12.0 | 13.3 | 0.46 | 0.16 |
| CCW [4], kg | 12.0 | 11.6 | 12.9 | 0.44 | 0.13 |
| Dressing percentage [5], % | 49.4 | 50.1 | 50.5 | 0.75 | 0.61 |
| Carcass shrinkage [6], % | 2.64 | 3.19 | 2.60 | 0.257 | 0.23 |
| Fatness score [7] | 2.11 | 2.29 | 2.22 | 0.092 | 0.39 |
| Perirenal fat weight, g | 91 | 115 | 139 | 15.0 | 0.09 |
| *Rectus abdominis* muscle | | | | | |
| Lightness | 50.4 | 50.4 | 50.0 | 0.68 | 0.87 |
| Redness | 9.81 | 9.54 | 9.85 | 0.478 | 0.88 |
| Yellowness | 11.3 | 11.7 | 11.2 | 0.40 | 0.62 |
| Hue angle | 49.0 | 50.8 | 48.6 | 1.84 | 0.69 |
| Chroma | 15.0 | 15.2 | 15.1 | 0.40 | 0.91 |
| Metmyoglobin (MMb) | 15.4 | 17.1 | 16.3 | 0.62 | 0.19 |
| Oxymyoglobin (OMb) | 12.4 | 11.8 | 9.6 | 3.02 | 0.79 |
| Deoxymyoglobin (DMb) | 72.2 | 71.1 | 75.1 | 2.91 | 0.60 |
| Subcutaneous caudal fat | | | | | |
| Lightness | 69.1 | 69.4 | 68.1 | 0.77 | 0.47 |
| Redness | 3.15 | 3.27 | 3.15 | 0.285 | 0.95 |
| Yellowness | 10.4 | 10.6 | 11.6 | 0.54 | 0.24 |
| Hue angle | 73.1 | 75.0 | 74.8 | 1.42 | 0.56 |
| Chroma | 10.9 | 11.1 | 12.1 | 0.57 | 0.29 |
| SUM [8] | 81.9 | 97.4 | 109.7 | 9.32 | 0.12 |

[1] 0SF- 0% sainfoin; 20SF- 20% sainfoin; 40SF- 40% sainfoin in the fattening concentrate.

[2] Standard error of the mean

[3] HCW: Hot carcass weight

[4] CCW: Cold carcass weight

[5] HCW × 100/Slaughter weight

[6] (HCW – CCW) ×100/HCW

[7] Scale 1 (low) to 5 (high)

[8] Estimator of carotenoids as calculated in Prache and Theriez (1999).

## Discussion

### *Lamb performance and plasma metabolites*

The ADG of lambs was greater than 280 g in all diets, which is in concordance with the expected growth for this breed. Therefore, the lambs performed satisfactorily regardless of the diet. The greater DMI recorded in the 40SF lambs was not reflected in a statistically significant greater ADG, which can be related to the concomitant decrease of the OMD as inclusion of sainfoin increased. Increasing the proportion of forage in the diet caused a linear decrease in OMD due to the higher fibre content (Fimbres et al., 2002). Thus, the effect of the incorporation of forage in the concentrate produces similar effects on OMD. Besides, the presence of PAC in the diet has been linked to a decrease of diet digestibility (Priolo et al., 2002b; Waghorn, 2008). In the present study, PAC content were low in all diets, despite differences among them, compared with the content of PAC in fresh sainfoin (Baila et al. 2022a; Rufino-Moya et al., 2022). However, it must be highlighted that the minimum threshold of PAC concentration necessary to cause changes in the organism is not well defined and must be deeply studied. The 40SF lambs presented greater DMI and higher PAC content in the diet but lower OMD, together contributing to the differences in ADG being only a statistical trend. Related to this, Dey et al. (2008) and Bonanno et al. (2011) recorded higher intake and an improvement in the lambs' growth when lambs were fed diets including moderate to low concentrations of PAC (15 g/kg DM and 20 g/kg DM, respectively). However, the intake of PAC by 20SF lambs does not appear to have been sufficient to influence DMI or ADG.

Differences among treatments were found in plasma NEFA concentrations at the beginning of the study. NEFA act as an alternative pathway to glucose to provide energy when blood glucose decreases (Leroy et al., 2008), but also can be raised under adrenaline releasing in response to stress (Stewart et al., 2007). Lambs belonging to 0SF diet were the first bled and the elapsed time may be too short to cause an increase of NEFA as an answer to stress. In contrast 20SF and 40SF lambs were bled later, spending

longer time under stress condition, which could cause the differences in NEFA concentration between 0SF and the rest of diets.

The diet did not have effect on glucose concentration during the study except at slaughter moment. At this sampling there were differences between the 0SF and 20SF diets, with intermedium values 40SF. According to Ramos et al. (1994), both diets with sainfoin showed lower values outside the range considered normal for Rasa Aragonesa lambs (87-122 mg/dl). The effect found between 0SF and 20SF lambs could be partially related to the content of starch of diet and to the stress suffered at slaughter time. Glucose is also an indicator of the liver's response to adrenaline during stress (Martin et al., 2011). The 0SF diet was a concentrate richer in starch whereas 20SF and 40SF had greater NDF content. The starch is efficiently and rapidly transformed into glucose, but fibre is associated with great glycemic control and even a decrease in blood glucose (Farrer et al. 1995), since the main end products of cellulose fermentation are short-chain fatty acids instead of glucose (Kaneko et al., 2008). The diet did not affect plasma urea concentrations, which is related to compliance with the condition of isoproteic diets. A reduction in plasma urea has been reported in fresh sainfoin-fed ewes compared to those receiving sainfoin + PEG (a blocker of PAC), suggesting a reduced protein degradation due to the effect of PAC (Baila et al., 2022a). The lack of effect in the current study, indicates that the PAC intake from sainfoin included in the concentrate was not enough to produce an effect on the protein metabolism.

In the same line, plasma polyphenol concentration and antioxidant activity parameters were similar among diets, contrary to that expected as sainfoin is known for its content of antioxidant compounds, including polyphenols, which should improve the antioxidant activity in lambs (Leal et al., 2019). This find could be explained by the low content of antioxidant compounds in the pelleted sainfoin in this study, caused by its deterioration at high temperatures (Maillard and Berset, 1995) during the dehydration and pelleting processes.

***Rumen and fermentation parameters***

Previous research with Rasa Aragonesa lambs indicated that those grazing alfalfa had light brown rumen epithelium while concentrate–fed lambs had dark and grey

epithelium (Álvarez-Rodríguez et al., 2012). Although the decrease in pH has been associated with darker rumen colour (Álvarez-Rodríguez et al., 2012; Blanco et al., 2015), in the present study only a higher redness value was observed in 40SF compared to 20SF and 0SF, suggesting that differences in the proportion of fibre or secondary compounds among concentrates may have been sufficient to cause this effect, but not the rest of colour parameters. In that sense, Blanco et al. (2015) observed higher redness value in rumen epithelium of lambs fed alfalfa hay, compared to lambs concentrate–fed with barley straw up to 25%. This suggests that the effect on rumen redness may be due to the deposition of some forage compounds (such as carotenoids) in the rumen wall, which were more abundant in the 40SF concentrate in the current experiment.

The values observed in ruminal pH agree with those recorded by Álvarez-Rodríguez et al. (2010), ranging from 5.5 in lambs fed concentrate (close to those obtained in 0SF and 20SF lambs) to 6.5 in lambs grazing alfalfa plus concentrate (similar to the pH of 40SF lambs). Higher values of pH improve the growth conditions for cellulolytic bacteria that need a ruminal pH range of 6.2–7.2 (Van Soest, 1994). The increase in ruminal pH, in turn, is related to the fibre content which is in line with the pH value of 40SF lambs, however, this result was not reflected in 20SF lambs.

In ruminants, VFA are the main source of energy, and ammonia reflects protein intake (Hatfield et al., 1998). In the present study no effect was observed in the total VFA and $NH_3$–N contents, reflecting similar energy and protein utilization, which is consistent with the results observed for plasma metabolites. In turn, the diet affected the proportion of acetic acid, which was increased when sainfoin was included in the concentrate, consequently increasing the acetic/propionic ratio. This can be explained by the greater NDF and lower starch content in the 40SF and 20SF diets due to sainfoin inclusion, which favour the development of cellulolytic bacteria, responsible for the acetic acid production. The lack of effect of the diet on the ruminal $NH_3$–N content reflected the similar CP content of all diets, rather to the presence of sainfoin. Moreover, although the presence of PAC can reduce ruminal ammonia concentrations by decreasing protein degradability (Frutos et al., 2004), the content of PAC in sainfoin concentrates was not sufficient to modify ammonia concentrations, as confirmed by the similar plasma urea concentrations observed among diets. A reduction in ruminal

ammonia was observed in animals fed diets containing 20% pelleted sainfoin with 223 g of PAC/kg DM (Grosse Brinkhaus et al., 2016), which implies a dietary PAC concentration almost 10 and 7 times higher than those of the 20SF and 40SF diets, respectively.

***Slaughter and carcass traits***

Forage diets are related to greater digestive development than high–concentrate diets (Borton et al., 2005; Joy et al., 2008), therefore a decrease in dressing percentage would have been expected due to the sainfoin inclusion in the diet, however the size of the fibre was too small to be considered "physically effective" (Banakar et al., 2018) and no effect was observed.

Carcass characteristics were similar among the diets, with no differences in carcass fatness degree, which is one of the major concerns of consumers (Bernués et al., 2012). Carcass colour, measured in the rectus abdominis muscle and in the subcutaneous caudal fat, either was not affected by the diet. Grazing systems leads to increase redness and chroma in rectus abdominis colour (Carrasco et al., 2009; Ripoll et al., 2012) and yellowness values in subcutaneous caudal fat (Joy et al., 2008; Ripoll et al., 2008) compared to lambs concentrate–fed. These colour changes are related to the deposition of carotenoids present in fresh forages but, when forages are preserved, the carotenoid content decreases (Rufino-Moya et al., 2022). In the present study, sainfoin was dehydrated and, despite the differences in carotenoid concentration among the diets, their presence was insufficient to induce significant changes on fat colour. Besides, the colour can also be affected by the presence of PAC in the diet, liked to a delay in metmyoglobin formation leading to a lighter meat with an increase in colour stability (Priolo et al., 2005; Luciano et al., 2011; Lobón et al., 2017b). Nevertheless, herein, no effect on haem pigments formation in *rectus abdominis* was observed among diets. It is important to highlight that meat and fat colour are one of the major characteristics that determine the purchase and, so, the lack of differences in these parameters in the present study confirms that the effect of the inclusion of sainfoin in the concentrate made it possible to produce homogeneous carcasses, as demanded by consumers.

## Conclusions

Performance of finishing lambs fed 20% or 40% sainfoin included in pelleted concentrates was comparable to that of commercial concentrates without affecting carcass characteristics. Therefore, the inclusion of up to 40% sainfoin in the concentrate of light lambs can be used without affecting their performance, ruminal fermentation, and carcass characteristics. However, to better understand the implications of the present study, it would be advisable to carry out a test under commercial conditions and to evaluate the effects of the diets on meat quality.

# Manuscript V

## Inclusion of sainfoin in the concentrate of finishing lambs: Fatty acid profile of rumen, plasma, and muscle

C. Baila, M. Joy, J.R. Bertolín, S.P. Alves, R.J.B. Bessa, M. Blanco, and S.Lobón

**Abstract**

The effects of the level of inclusion of sainfoin (*Onobrychis viciifolia*) in the finishing concentrate for light lambs on the fatty acid (FA) composition of the ruminal digesta, plasma, and meat were evaluated. Twenty-six weaned male lambs were divided into three groups and fed individually *ad libitum* during 40 days with one of three concentrates differing in the level of sainfoin inclusion: 0% (0SF), 20% (20SF), and 40% (40SF). The rumen digesta showed an increase in C18:3 n-3 concentration and a decrease in C18:1 t10 concentration when sainfoin was included in the concentrate regardless the level of inclusion. However, the highest C18:1 t11 and the lowest C18:2 n-6 proportions were only obtained in the 40SF rumen, showing a stronger t11 biohydrogenation pathway. In plasma, most effects were associated with changes in levels of polyunsaturated FA (PUFA) n-3. The meat FA profile of 40SF lambs presented higher percentages of PUFA n-3 and CLA c9,t11 and a lower PUFA n-6/PUFA n-3 ratio compared with that in meat from 0SF or 20SF diets because of the potentiation of the ruminal t11 pathway. Inclusions of 20% and 40% sainfoin both showed beneficial effects on meat quality compared with that of the commercial concentrate without sainfoin; furthermore, these effects were most marked in the 40% sainfoin diet.

**Keywords**

*Onobrychis viciifolia,* rumen biohydrogenation, lipid metabolism, meat quality, local forage legume

**Introduction**

Current guidelines advise monitoring the intake of ruminant meat because of the high concentrations of saturated fatty acids (SFA) (Willett et al., 2019) although this has a healthier n-6/n-3 FA ratio than that of pork or chicken (Woods and Fearon, 2009). The high concentration of SFA in ruminant edible products is related to the ruminal biohydrogenation (BH), a complex process comprising multiple pathways that sequentially isomerize and hydrogenate the dietary unsaturated FAs, producing a more SFA profile (Jenkins et al., 2008; Vasta and Bessa, 2012). Although this goes against the current consumer desire to have healthy animal products in their diets (Parodi, 2016),

this microbial process also produces beneficial bioactive FAs such as C18:1 t11 and C18:2 c9,t11 that are mostly related to ruminant products consumption (Vahmani et al., 2020; Álvarez-Rodríguez et al., 2022). Hence, a deep understanding of the ruminal BH is needed to know and anticipate how thus can affect the metabolic availability of FA and their deposition in tissues and milk (Shingfield et al., 2013).

Light lamb production systems in several Mediterranean countries, such as Spain and Portugal, are based on indoors concentrate-fed from weaning (12–14 kg BW) to slaughter (22–28 kg BW and 70–90 days old). The inclusion of locally produced forages has been commonly studied as a strategy to simultaneously achieve greater sustainability and self-sufficiency in intensive systems and improve animal welfare and the meat FA profile (Moorby and Fraser, 2021; Santos-Silva et al., 2023). Forages are rich in C18:3 n-3 and promote the BH pathway to produce C18:1 t11 instead of the formation of C18:1 t10 isomer, which is associated with concentrate-rich diets (Griinari et al., 1998), producing more beneficial meat for consumers.

Sainfoin (*Onobrychis viciifolia*) is a Mediterranean forage legume with a high crude protein concentration and with a medium content of proanthocyanidins (PAC) (Rufino-Moya et al., 2022), also known as condensed tannins. Sainfoin is commonly preserved because its production is mainly obtained in the first spring cut. Thus, in this type of production system, it would be interesting to preserve sainfoin as pellets for inclusion in concentrates. In addition to the changes in ruminal BH caused by the inclusion of a forage in the concentrate, the presence of PAC can have a modulating effect on the specific BH pathways or on the rumen microbial population. This can produce changes in the concentration of polyunsaturated FA (PUFA) and FA intermediates (C18:3, C18:2, and C18:1 isomers) in the rumen (Frutos et al., 2020), which are potentially deposited on to ruminant products, such as meat (Lobón et al., 2017a).

We hypothesised that including dehydrated sainfoin forage into finishing diets for light lambs would promote the rumen BH pathways producing C18:1 t11 and C18:2 c9,t11 and thereby improve the FA profile of lamb meat. The study sought to evaluate the effects of the inclusion of sainfoin in two different rates in the finishing pelleted concentrate for lambs on the ruminal BH and on blood and meat FA profiles. Results

from the present experiment will provide a better understanding of the changes occurring in lipid metabolism from the diet to the meat FA profile.

## Materials and methods

All the experimental procedures performed in this trial were approved by the Animal Ethics Committee of the CITA Research Centre (CEEA, 2017-07), 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.

### *Animal management and experimental design*

The study was conducted during autumn 2020 at the CITA facilities (41°3’ N, 0°47’ W and 216 m above sea level) in Zaragoza, Spain. After weaning, 26 male Rasa Aragonesa lambs were randomly separated into three homogeneous groups considering their weaning age (30 ± 2.0 d) and weight (14.0 ± 0.49 kg). Lambs were individually penned indoors for 40 days until slaughter. Each group received a pelleted concentrate with a different level of sainfoin inclusion: a cereal-based concentrate without sainfoin (0SF), a concentrate with 20% of sainfoin (20SF), and a concentrate with 40% of sainfoin (40SF). Lambs had free access to the concentrates, water, and minerals. The sainfoin used in the 20SF and 40SF concentrates was cut at the flowering stage in spring 2019, dehydrated, and kept pelleted until early autumn, when the sainfoin pellets were ground and introduced into the concentrates (3.5–mm diameter pellets). The pelleted concentrates were formulated to be isoenergetic and isoproteic. The ingredients and chemical composition are detailed in Table 20.

**Table 20.** Ingredients and chemical and fatty acid (FA) composition (mean ± standard deviation) of the experimental diets.

| | Diets [1] | | |
|---|---|---|---|
| | 0SF | 20SF | 40SF |
| Ingredients, g/kg DM | | | |
| Barley | 310 | 252 | 50 |
| Corn | 250 | 189 | 250 |
| Wheat | 50 | 50 | 102 |
| Gluten feed | 60 | 60 | 130 |
| Soybean meal 47% | 173 | 138 | 159 |
| Bran | 25 | 81 | 0 |
| Palm oil | 10 | 10 | 15 |
| Calcium carbonate | 15 | 13 | 4 |
| Sodium chloride | 5 | 5 | 5 |
| Premix vitamin 0.2% | 2 | 2 | 2 |
| Sainfoin pellet | 0 | 200 | 400 |
| Straw | 100 | 0 | 0 |
| Chemical composition, g/kg DM | | | |
| Dry matter (DM), g/kg as fed | 905 ± 2.5 | 904 ± 4.5 | 903 ± 4.2 |
| Crude protein | 174 ± 4.3 | 175 ± 6.5 | 173 ± 5.2 |
| Ether extract | 32.6 ± 3.25 | 35.7 ± 3.60 | 38.0 ± 3.44 |
| Ash | 75.2 ± 2.51 | 70.5 ± 2.06 | 78.5 ± 5.48 |
| Starch | 426 ± 6.9 | 360 ± 13.8 | 296 ± 9.6 |
| NDF | 263 ± 20.9 | 292 ± 12.1 | 355 ± 16.4 |
| ADF | 129 ± 9.1 | 168 ± 6.5 | 249 ± 10.4 |
| ADL | 17.0 ± 3.25 | 34.2 ± 3.17 | 59.6 ± 4.37 |
| Gross energy, MJ/kg DM | 18.1 ± 1.37 | 18.4 ± 1.25 | 18.4 ± 0.94 |
| Proanthocyanidins (PAC) [2] | | | |
| Total | 1.32 ± 0.527 | 3.04 ± 0.448 | 5.23 ± 0.550 |
| Extractable | 0.41 ± 0.152 | 0.50 ± 0.169 | 0.75 ± 0.132 |
| Protein bound | 0.77 ± 0.529 | 2.07 ± 0.373 | 3.67 ± 0.508 |
| Fibre bound | 0.15 ± 0.115 | 0.47 ± 0.138 | 0.80 ± 0.118 |
| Delphinidin/cyanidin ratio | 51:49 ± 1.56 | 68:32 ± 0.65 | 71:29 ± 1.06 |
| Total FA, mg/g DM | 44.6 ± 1.13 | 45.9 ± 1.39 | 46.3 ± 1.97 |
| Individual FA, g /100 g total FA | | | |
| C12:0 | 0.08 ± 0.017 | 0.07 ± 0.011 | 0.11 ± 0.027 |
| C14:0 | 0.50 ± 0.022 | 0.51 ± 0.014 | 0.61 ± 0.022 |
| C15:0 | 0.06 ± 0.011 | 0.08 ± 0.009 | 0.07 ± 0.008 |
| C16:0 | 27.6 ± 0.23 | 28.0 ± 0.18 | 30.0 ± 0.30 |
| C16:1 c9 | 0.20 ± 0.069 | 0.22 ± 0.055 | 0.27 ± 0.061 |
| C18:0 | 7.34 ± 0.286 | 6.98 ± 0.270 | 7.42 ± 0.339 |
| C18:1 c9 | 24.6 ± 0.27 | 24.1 ± 0.32 | 26.8 ± 0.89 |
| C18:1 c11 | 0.28 ± 0.205 | 0.26 ± 0.176 | 0.28 ± 0.141 |
| C18:2 n-6 | 36.8 ± 0.25 | 35.0 ± 0.32 | 27.8 ± 0.58 |
| C18:3 n-3 | 2.49 ± 0.137 | 4.77 ± 0.193 | 6.71 ± 0.444 |
| SFA [3] | 35.6 ± 0.41 | 35.6 ± 0.39 | 38.2 ± 0.53 |
| MUFA [4] | 25.1 ± 0.18 | 24.6 ± 0.24 | 27.3 ± 0.82 |
| PUFA [5] | 39.3 ± 0.35 | 39.8 ± 0.29 | 34.5 ± 0.57 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate.

[2] g eq. of sainfoin PAC/kg DM. [3] Saturated FA. [4] Monounsaturated FA. [5] Polyunsaturated FA

***Sampling procedures and slaughter***

Composite samples of the concentrates were obtained weekly per animal to determine the chemical and FA composition. At day 40, without prior fasting, the lambs were slaughtered in the CITA experimental slaughterhouse adjacent to the lamb housing facilities. Lambs were stunned by a captive bolt pistol and exsanguinated, using standard commercial procedures and according to Council Regulation (EC) Nº 1099/2009. Blood samples were collected from the jugular vein of lambs into tubes containing heparin (Vaccuette, Spain), immediately centrifuged (3000 *g* for 15 min at 4 °C) and stored at −20 °C. Ruminal digesta was extracted (nonfiltered), kept in flasks, freeze–dried, and preserved at −20 °C. The *longissimus thoracis et lumborum* muscles corresponding to the right side of the carcass, after 24h *post-mortem* at 4 °C, were excised and sliced between the $4^{th}$ and $6^{th}$ lumbar vertebrae to study the intramuscular fat (IMF), cholesterol concentrations, and the FA profile of meat. All samples were freeze–dried (Lyobeta 25, Azbil Telstar, Japan) and kept at −20 °C.

***Chemical analyses***

All analyses of the chemical composition of the concentrates were run in duplicate. The techniques used for the chemical analyses of the concentrates are detailed in Baila et al. (2022a). The total starch of the concentrates was measured with the commercial kit K-TSTA-100A (Neogen Corporation, Lansing, MI, USA) following the amyloglucosidase/α-amylase method.

The dry matter (DM) and IMF of meat was measured by using NIRs (FoodScanTM2, Foss Analytics, Hilleroed, Denmark), and the amount of cholesterol in the meat was determined following the method of Bertolín et al. (2019) using an Acquity UPLC H-Class liquid chromatograph (Waters, Mildford, KA, USA) with a silica-based bonded phase column (Acquity UPLC HSS T3, 150 mm × 2.1 mm × 1.8 µm, Waters, Mildford, KA, USA), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ Detector, Waters), and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters, Mildford, KA, USA) and controlled with a Empower 3 software (Waters, Mildford, KA, USA). Evaluation of the FA profile of concentrates, plasma, and meat samples was performed at CITA (Spain). The FA profile of the concentrates was analysed using 500 mg of lyophilised

samples following the method described by Rufino-Moya et al. (2022). For plasma and meat FA profiles, 2 ml and 500 mg of lyophilised samples, respectively, were extracted according to Lee et al. (2012). Afterwards, all the samples were methylated as FA methyl esters using 4 ml of 0.5 M CH3ONa/CH3OH solution followed by 4 ml of acetyl chloride/CH3OH (1/10, v/v) and extracted in 3 ml of heptane. After, the FA concentration was determined in a Bruker Scion 436-GC gas chromatograph (Bruker, Billerica, MA, USA) gas chromatograph with a flame ionisation detector equipped with a CP-8400 autosampler and a SP-2560 capillary column (100 m × 0.25 mm ID × 0.20 µm for concentrate samples and 200 m × 0.25 mm ID × 0.20 µm for plasma and meat samples; Sigma Aldrich, Saint Louis, MO, USA). The technical conditions of the chromatographic conditions followed for the FA analyses of the concentrates can be found in detail in Rufino-Moya et al. (2022). For plasma and meat, the oven temperature was 70 °C for 1 min followed by 5 °C/min for 2 min to 225 °C maintained for 17 min with a total time of 80 min. The injector and detector temperatures were maintained at 260 °C and 250 °C, respectively. Identification of the FAs of concentrates, plasma, and meat were 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 (Kramer et al., 1997; Alves and Bessa, 2009; Bravo-Lamas et al., 2016). 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 concentration was 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 for concentrates and plasma and C23:0 (methyl tricosanoate N-23-M from Nu-Chek Prep, Inc., Elysian, MN, USA) for meat samples.

The analyses involving the FA determination of ruminal digesta were performed at the Laboratory of Animal Production and Nutrition of the Faculty of Veterinary Medicine, University of Lisbon (Portugal) as described in Alves et al. (2017). Briefly, freeze-dried rumen concentrations (250 mg) were directly transesterified according to the method described in Alves et al. (2013). The methyl nonadecanoate (C19:0) (internal standard) was used for quantification by gas chromatography with flame ionisation detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m × 0.25 mm × 0.20 µm film thickness, Supelco, Bellefonte, PA, USA)

capillary column with the chromatographic conditions described in Alves et al. (2018). The FA determinations were performed by comparison with ruminal chromatograms of Alves et al. (2013) and Alves and Bessa (2014). Calculations estimating the biohydrogenation extent of C18 dietary FAs in rumen provide an estimation of the degree of ruminal BH to which the main C18 dietary FAs have been subjected, whereas the completeness (%) in the rumen reflects an estimation of the BH extent that has occurred, considering the maximum BH that could be achieved if the entire dietary FAs were completely biohydrogenated (Alves et al., 2017). The sums, ratios, and enzymes activity relatives to the FA profiles are detailed in Baila et al. (2023).

### *Statistical analyses*

Data were analysed with SAS statistical software (v.9.3; SAS Inst. Inc., Cary, NC, USA), considering the animal as the experimental unit. The FA profiles of ruminal digesta, plasma, and meat were analysed using analysis of variance with a mixed model (MIXED procedure) and the diet (0SF, 20SF, and 40SF) as a fixed effect. When significant, the group statement was included in the model to adjust the variance heterogeneity. The degrees of freedom were adjusted with the Kenward–Roger correction. The least square means and their associated standard errors were obtained, and Tukey's correction was used for pairwise comparisons. The effects were considered significant at $P < 0.05$.

## Results

### *FA composition of ruminal digesta*

The main differences in the FA profile and the BH extent and completeness of the ruminal concentration due to the diets are shown in Table 21. The diet did not affect the total FA content of ruminal digesta ($P > 0.05$). The total SFA percentage was similar among diets ($P > 0.05$) but diet had effect on C16:0, C20:0, C22:0, C24:0, and C28:0 ($P < 0.001$), with higher percentages in 40SF lambs than that in their counterparts, except for C20:0, which increased with the level of sainfoin inclusion. The percentage of total branched-chain FA (BCFA) in rumen was affected by the diet ($P < 0.01$), being greater in 0SF than that in 20SF lambs. The total percentage of iso-BCFA did not differ among the

diets ($P > 0.05$) whereas the percentage of total *anteiso*-BCFA was higher in 0SF than that in 20SF and 40SF ($P < 0.05$).

Although the total ruminal percentage of monounsaturated FA (MUFA) was similar among diets ($P > 0.05$), 0SF lambs had lower percentages of total *cis*-MUFA, C18:1 c9, C18:1 c16, and C18:1 t11 than those in 40SF lambs and greater percentages of C18:1 t10 and C18:1 t12 than those in either 20SF or 40SF lambs ($P < 0.05$). Concerning the percentage of total *trans*-MUFA in the rumen, the 20SF diet reduced their values when compared with that obtained with the 0SF diet ($P < 0.05$).

The diet changed the percentages of total PUFA, C18:2 n-6, and CLA t10,c12 ($P < 0.01$), showing the rumen of 40SF lambs lower values than those of their counterparts. The percentages of C18:3 n-3 and C18:3 c9,t11,c15 were lower in the rumen of 0SF lambs than those of their counterparts ($P < 0.05$), which presented similar percentages ($P > 0.05$). The ruminal C18:2 t11,c15/t10,c15 percentage was lower in 0SF compared with those in 40SF ($P < 0.05$), whereas 20SF lambs presented intermediate values ($P > 0.05$). The effect of the diet was also significant for the percentage of C18:0 oxo-12/13 ($P < 0.01$), with lower values detected in 20SF lambs than that in their counterparts.

The C18:1 t10/C18:1 t11 ratio was affected by the diet ($P < 0.001$), decreasing as the level of sainfoin increased in the diet but the biohydrogenation intermediates (BI) remained unaffected ($P > 0.05$). Consequently, the proportion of BI caused by the presence of C18:1 t10 also decreased with the presence of sainfoin ($P < 0.001$). The diet did not affect the extent of BH (completeness) ($P > 0.05$) but did affect the BH percentage of C18:3 n-3, which increased with the level of inclusion of sainfoin ($P < 0.001$).

**Table 21.** Effect of the diet on fatty acid (FA) profile (% of total FA identified) and C18 rumen biohydrogenation extent and completeness (%) of the ruminal digesta of lambs.

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| Total FA, mg FA/g DM | 43.5 ± 1.24 | 47.3 ± 1.31 | 43.6 ± 1.24 | 0.08 |
| Individual FA | | | | |
| SFA [2] | 40.7 ± 2.97 | 48.1 ± 3.15 | 45.9 ± 2.97 | 0.23 |
| C12:0 | 0.12 ± 0.016 | 0.12 ± 0.016 | 0.15 ± 0.016 | 0.38 |
| C13:0 | 0.05 ± 0.011 | 0.06 ± 0.012 | 0.04 ± 0.011 | 0.55 |
| C14:0 | 0.68 ± 0.055 | 0.62 ± 0.058 | 0.69 ± 0.055 | 0.62 |
| C15:0 | 0.36 ± 0.034 | 0.29 ± 0.037 | 0.37 ± 0.034 | 0.25 |
| C16:0 | $24.9^{b}$ ± 0.34 | $24.5^{b}$ ± 0.36 | $29.3^{a}$ ± 0.34 | <0.001 |
| C17:0 | 0.27 ± 0.017 | 0.27 ± 0.019 | 0.27 ± 0.017 | 0.99 |
| C18:0 | 13.3 ± 3.06 | 21.1 ± 3.24 | 13.7 ± 3.06 | 0.17 |
| C20:0 | $0.35^{c}$ ± 0.013 | $0.41^{b}$ ± 0.014 | $0.46^{a}$ ± 0.013 | <0.001 |
| C21:0 | 0.02 ± 0.008 | 0.02 ± 0.008 | 0.01 ± 0.008 | 0.67 |
| C22:0 | $0.24^{b}$ ± 0.007 | $0.26^{b}$ ± 0.007 | $0.30^{a}$ ± 0.007 | <0.001 |
| C23:0 | 0.14 ± 0.030 | 0.10 ± 0.009 | 0.08 ± 0.019 | 0.26 |
| C24:0 | $0.25^{b}$ ± 0.022 | $0.29^{b}$ ± 0.017 | $0.36^{a}$ ± 0.008 | <0.001 |
| C26:0 | 0.09 ± 0.00 5 | 0.11 ± 0.009 | 0.13 ± 0.016 | 0.14 |
| C28:0 | $0.01^{b}$ ± 0.010 | $0.04^{b}$ ± 0.010 | $0.12^{a}$ ± 0.010 | <0.001 |
| BCFA [3] | $1.84^{a}$ ± 0.137 | $1.22^{b}$ ± 0.145 | $1.36^{ab}$ ± 0.137 | 0.01 |
| *iso*-BCFA | 0.57 ± 0.080 | 0.44 ± 0.085 | 0.54 ± 0.080 | 0.52 |
| *iso*-C13:0 | $0.007^{b}$ ± 0.0062 | $0.004^{b}$ ± 0.0065 | $0.031^{a}$ ± 0.0062 | 0.009 |
| *iso*-C14:0 | 0.13 ± 0.014 | 0.09 ± 0.015 | 0.10 ± 0.014 | 0.21 |
| *iso*-C15:0 | 0.20 ± 0.046 | 0.13 ± 0.017 | 0.20 ± 0.034 | 0.12 |
| *iso*-C16:0 | 0.19 ± 0.047 | 0.20 ± 0.050 | 0.15 ± 0.047 | 0.77 |
| *iso*-C17:0 | 0.04 ± 0.011 | 0.03 ± 0.011 | 0.05 ± 0.011 | 0.39 |
| *anteiso*-BCFA | $1.27^{a}$ ± 0.073 | $0.78^{b}$ ± 0.077 | $0.82^{b}$ ± 0.073 | <0.001 |
| *anteiso*-C15:0 | $1.02^{a}$ ± 0.065 | $0.64^{b}$ ± 0.068 | $0.73^{b}$ ± 0.065 | <0.01 |
| *anteiso*-C17:0 | $0.24^{a}$ ± 0.034 | $0.15^{ab}$ ± 0.036 | $0.09^{b}$ ± 0.034 | 0.02 |
| MUFA [4] | 35.7 ± 2.19 | 29.1 ± 2.33 | 33.9 ± 2.19 | 0.13 |
| *cis*-MUFA | $13.9^{b}$ ± 0.82 | $14.8^{ab}$ ± 0.87 | $17.4^{a}$ ± 0.82 | 0.02 |
| C16:1 c7/t3 [5] | $0.11^{a}$ ± 0.026 | $0.07^{ab}$ ± 0.023 | $0.04^{b}$ ± 0.010 | 0.02 |
| C16:1 c9 | 0.12 ± 0.013 | 0.11 ± 0.014 | 0.10 ± 0.013 | 0.45 |
| C18:1 c9 [6] | $12.6^{b}$ ± 0.83 | $13.2^{ab}$ ± 0.88 | $15.8^{a}$ ± 0.83 | 0.03 |
| C18:1 c11 | 0.90 ± 0.062 | 0.83 ± 0.065 | 0.92 ± 0.062 | 0.54 |
| C18:1 c12 | 0.02 ± 0.014 | 0.00 ± 0.015 | 0.02 ± 0.014 | 0.57 |
| C18:1 t16/c14 [7] | $0.03^{b}$ ± 0.014 | $0.12^{a}$ ± 0.028 | $0.22^{a}$ ± 0.061 | 0.002 |
| C18:1 c15 | $0.13^{b}$ ± 0.054 | $0.45^{a}$ ± 0.117 | $0.24^{b}$ ± 0.032 | 0.047 |
| C18:1 c16 | $0.01^{b}$ ± 0.002 | $0.02^{ab}$ ± 0.009 | $0.04^{a}$ ± 0.009 | <0.001 |

**Table 21**. (Continued)

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| *trans*-MUFA | 21.9$^{a}$ ± 1.8 | 14.5$^{b}$ ± 1.91 | 16.7$^{ab}$ ± 1.8 | 0.03 |
| C18:1 t6/t7/t8 [8] | 0.80 ± 0.259 | 1.43 ± 0.124 | 1.20 ± 0.117 | 0.09 |
| C18:1 t9 | 0.41 ± 0.155 | 0.80 ± 0.081 | 0.62 ± 0.056 | 0.08 |
| C18:1 t10 | 19.5$^{a}$ ± 1.88 | 10.3$^{b}$ ± 2.00 | 9.24$^{b}$ ± 1.883 | 0.01 |
| C18:1 t11 | 0.47$^{b}$ ± 0.108 | 1.14$^{b}$ ± 0.345 | 4.70$^{a}$ ± 0.6080 | <0.001 |
| C18:1 t12 | 0.68$^{a}$ ± 0.066 | 0.39$^{b}$ ± 0.070 | 0.41$^{b}$ ± 0.066 | 0.008 |
| C18:1 t15 | 0.09$^{b}$ ± 0.051 | 0.30$^{a}$ ± 0.054 | 0.32$^{a}$ ± 0.051 | 0.008 |
| PUFA [9] | 17.3$^{a}$ ± 1.03 | 16.9$^{a}$ ± 1.09 | 12.8$^{b}$ ± 1.03 | 0.009 |
| C18:2 n-6 | 15.6$^{a}$ ± 0.87 | 14.3$^{a}$ ± 0.93 | 9.97$^{b}$ ± 0.874 | <0.001 |
| C18:2 t9,c12 | 0.02 ± 0.010 | 0.06 ± 0.022 | 0.11 ± 0.037 | 0.05 |
| C18:2 t11,c15/t10,c15 [10] | 0.35$^{b}$ ± 0.035 | 0.59$^{ab}$ ± 0.146 | 0.69$^{a}$ ± 0.129 | 0.03 |
| C18:3 n-3 | 1.25$^{b}$ ± 0.105 | 1.76$^{a}$ ± 0.112 | 1.78$^{a}$ ± 0.105 | 0.002 |
| C18:3 c9,t11,c15 | 0.01$^{b}$ ± 0.005 | 0.11$^{a}$ ± 0.005 | 0.16$^{a}$ ± 0.020 | <0.001 |
| CLA [11] | 0.10 ± 0.017 | 0.12 ± 0.018 | 0.08 ± 0.017 | 0.30 |
| CLA c9,t11 | 0.07 ± 0.015 | 0.07 ± 0.016 | 0.07 ± 0.015 | 0.99 |
| CLA t10,c12 | 0.03$^{a}$ ± 0.007 | 0.04$^{a}$ ± 0.08 | 0.00$^{b}$ ± 0.007 | 0.002 |
| CLA t11,c13 | 0.00 ± 0.004 | 0.01 ± 0.005 | 0.01 ± 0.005 | 0.20 |
| oxo-FA | 2.41 ± 0.283 | 1.97 ± 0.196 | 3.61 ± 0.649 | 0.05 |
| C18:0 oxo-12/-13 [12] | 1.41$^{a}$ ± 0.142 | 0.94$^{b}$ ± 0.117 | 2.07$^{a}$ ± 0.416 | 0.009 |
| C18:0 oxo-10 | 1.00 ± 0.219 | 1.03 ± 0.232 | 1.54 ± 0.219 | 0.17 |
| C18:1 t10/C18:1 t11 | 54.5$^{a}$ ± 18.83 | 13.2$^{b}$ ± 2.80 | 1.98$^{c}$ ± 0.279 | <0.001 |
| BI [13] | 24.9 ± 2.10 | 17.7 ± 2.23 | 21.6 ± 2.10 | 0.089 |
| C18:1 t10 (% BI) [14] | 75.7$^{a}$ ± 4.15 | 55.8$^{b}$ ± 4.15 | 40.2$^{c}$ ± 4.15 | <0.001 |
| Biohydrogenation extent, % | | | | |
| C18:1 c9 | 46.5 ± 3.50 | 43.6 ± 3.71 | 36.0 ± 3.50 | 0.12 |
| C18:2 n-6 | 55.8 ± 2.64 | 57.9 ± 2.80 | 61.3 ± 2.64 | 0.35 |
| C18:3 n-3 | 47.6$^{c}$ ± 2.36 | 61.9$^{b}$ ± 2.50 | 71.4$^{a}$ ± 2.36 | <0.001 |
| Completeness, % | 34.2 ± 6.55 | 51.9 ± 6.95 | 37.2 ± 6.55 | 0.17 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate. [2] Saturated FA. [3] Branched-chain FA. [4] Monounsaturated FA. [5] C16:1 c7 and C16:1 t3 might coelute. [6] C18:1 c9 might coelute with the pair C18:1 t13 and t14. [7] C18:1 t16 coelutes with C18:1 c14 as minor isomer. [8] C18:1 t6, C18:1 t7, and C18:1 t8 might coelute. [9] Polyunsaturated FA. [10] C18:2 t11,c15 and C18:2 t10,c15 might coelute. [11] Conjugated linoleic acid. [12] C18:0 oxo-12 and C18:0 oxo-13 might coelute. [13] Biohydrogenation intermediates: all C18 FA except C18:0, C18:1 c9, C18:1 c11, C18:2 n-6, and C18:3 n-3. [14] Proportion of BI explained by C18:1 t10. The different lowercase letters indicate differences among groups at $P < 0.05$; standard error of means (±).

***FA composition of plasma***

The effect of diet on the FA profile of plasma is shown in Table 22. The diet had effect on the total FA content in plasma ($P < 0.01$), which was high in 0SF, intermediate in 20SF, and low in 40SF lambs ($P < 0.05$). The diet did not affect the total SFA percentage or that of individual SFA ($P > 0.05$), except for C15:0 ($P < 0.001$), which was greater in 40SF.

The diet did not change the total percentage of MUFA and *cis*- and *trans*-MUFA ($P > 0.05$) but did affect the percentage of four individual MUFA ($P < 0.05$). Thus, the plasma of 0SF lambs had greater percentages of C16:1 c9 and C24:1 c15 compared with those of 40SF lambs and a greater percentage of C18:1 t11 compared with that of 20SF while the C22:1 c13 percentage was greater in 40SF lambs than that in 20SF lambs ($P < 0.05$).

The diet did not show effect on the percentages of PUFA, PUFA n-6, or its major FA, C18:2 n-6 ($P > 0.05$) but did affect percentage of C18:3 n-6, C20:2 n-6, C20:3 n-6, and C20:4 n-6 ($P < 0.05$). The plasma of 0SF lambs had greater percentage of C18:3 n-6 and C20:4 n-6 than that of 40SF lambs and lower percentage of C20:3 n-6 than that of 20SF lambs while the percentage of C20:2 n-6 was greater in 20SF than that in 40SF lambs.

The diet affected the total percentage of PUFA n-3 ($P < 0.001$), C18:3 n-3 ($P < 0.001$), C22:5 n-3 ($P < 0.001$), and C22:6 n-3 ($P < 0.05$), with lower percentages in the plasma of 0SF lambs than those of their counterparts, which presented similar percentages between them ($P > 0.05$), except for C18:3 n-3, the percentage of which increased with the higher inclusion of sainfoin ($P < 0.001$).

**Table 22.** Effect of the diet on total FA content and FA profile (% of total FA identified) of plasma lambs.

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| Total FA, mg FA/ml plasma | 2.68$^{a}$ ± 0.102 | 2.67$^{ab}$ ± 0.108 | 2.31$^{b}$ ± 0.102 | 0.004 |
| Individual FA | | | | |
| SFA [2] | 78.9 ± 0.98 | 80.1 ± 1.03 | 80.0 ± 0.98 | 0.66 |
| C12:0 | 1.37 ± 0.086 | 1.46 ± 0.092 | 0.71 ± 0.034 | 0.05 |
| C14:0 | 0.71 ± 0.034 | 0.77 ± 0.036 | 0.71 ± 0.034 | 0.34 |
| C15:0 | 0.17$^{b}$ ± 0.017 | 0.14$^{b}$ ± 0.018 | 0.25$^{a}$ ± 0.017 | <0.001 |
| C16:0 | 41.6 ± 0.42 | 41.3 ± 0.44 | 41.1 ± 0.42 | 0.76 |
| C17:0 | 0.37 ± 0.034 | 0.40 ± 0.036 | 0.44 ± 0.034 | 0.31 |
| C18:0 | 34.6 ± 0.65 | 35.9 ± 0.69 | 35.6 ± 0.65 | 0.37 |
| C20:0 | 0.07 ± 0.016 | 0.06 ± 0.012 | 0.11 ± 0.031 | 0.32 |
| C22:0 | 0.04 ± 0.012 | 0.06 ± 0.012 | 0.03 ± 0.012 | 0.18 |
| C24:0 | 0.04 ± 0.007 | 0.04 ± 0.008 | 0.03 ± 0.007 | 0.34 |
| MUFA [3] | 9.30 ± 0.571 | 8.94 ± 0.605 | 8.83 ± 0.571 | 0.84 |
| *cis*-MUFA | 7.01 ± 0.402 | 7.70 ± 0.427 | 7.10 ± 0.402 | 0.45 |
| C16:1 c9 | 0.35$^{a}$ ± 0.033 | 0.26$^{ab}$ ± 0.034 | 0.15$^{b}$ ± 0.033 | <0.001 |
| C18:1 c9 | 5.96 ± 0.360 | 6.76 ± 0.382 | 6.36 ± 0.360 | 0.33 |
| C18:1 c11 | 0.57 ± 0.044 | 0.57 ± 0.047 | 0.50 ± 0.044 | 0.37 |
| C20:1 c11 | 0.03 ± 0.013 | 0.05 ± 0.014 | 0.03 ± 0.013 | 0.67 |
| C22:1 c13 | 0.04$^{ab}$ ± 0.014 | 0.02$^{b}$ ± 0.005 | 0.05$^{a}$ ± 0.007 | 0.01 |
| C24:1 c15 | 0.05$^{a}$ ± 0.006 | 0.04$^{ab}$ ± 0.015 | 0.02$^{b}$ ± 0.006 | 0.005 |
| *trans*-MUFA | 2.29 ± 0.308 | 1.24 ± 0.326 | 1.73 ± 0.308 | 0.08 |
| C18:1 t10 | 1.59 ± 0.212 | 0.96 ± 0.225 | 1.26 ± 0.212 | 0.15 |
| C18:1 t11 | 0.70$^{a}$ ± 0.100 | 0.28$^{b}$ ± 0.106 | 0.47$^{ab}$ ± 0.100 | 0.02 |
| PUFA [4] | 11.8 ± 0.57 | 11.0 ± 0.61 | 11.2 ± 0.57 | 0.60 |
| PUFA n-6 | 11.3 ± 0.56 | 10.2 ± 0.59 | 10.2 ± 0.56 | 0.28 |
| C18:2 n-6 | 10.1 ± 0.53 | 9.10 ± 0.567 | 9.43 ± 0.53 | 0.42 |
| C18:3 n-6 | 0.14$^{a}$ ± 0.021 | 0.08$^{ab}$ ± 0.022 | 0.07$^{b}$ ± 0.021 | 0.04 |
| C20:2 n-6 | 0.05$^{ab}$ ± 0.008 | 0.08$^{a}$ ± 0.023 | 0.02$^{b}$ ± 0.007 | 0.008 |
| C20:3 n-6 | 0.05$^{b}$ ± 0.012 | 0.09$^{a}$ ± 0.013 | 0.08$^{ab}$ ± 0.013 | 0.046 |
| C20:4 n-6 | 0.95$^{a}$ ± 0.053 | 0.79$^{ab}$ ± 0.056 | 0.72$^{b}$ ± 0.057 | 0.02 |
| PUFA n-3 | 0.43$^{b}$ ± 0.065 | 0.80$^{a}$ ± 0.069 | 0.93$^{a}$ ± 0.065 | <0.001 |
| C18:3 n-3 | 0.27$^{c}$ ± 0.036 | 0.41$^{b}$ ± 0.038 | 0.60$^{a}$ ± 0.036 | <0.001 |
| C20:5 n-3 | 0.09 ± 0.025 | 0.10 ± 0.027 | 0.07 ± 0.027 | 0.77 |
| C22:5 n-3 | 0.01$^{b}$ ± 0.020 | 0.16$^{a}$ ± 0.022 | 0.18$^{a}$ ± 0.022 | <0.001 |
| C22:6 n-3 | 0.05$^{b}$ ± 0.017 | 0.12$^{a}$ ± 0.018 | 0.10$^{ab}$ ± 0.017 | 0.02 |
| C18:1 t10/C18:1 t11 | 2.55 ± 0.313 | 5.53 ± 1.638 | 5.96 ± 3.321 | 0.15 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate. [2] Saturated FA. [3] Monounsaturated FA. [4] Polyunsaturated FA. The different lowercase letters indicate differences among groups at $P < 0.05$; standard error of means (±).

***Fatty acid composition of longissimus lumborum muscle***

The meat FA profile is shown in Table 23. The diet had no effect on DM, IMF, and cholesterol ($P > 0.05$) percentages but affected the percentage of total FA ($P < 0.05$), which was higher in 0SF than that 40SF, whereas 20SF presented intermediate values.

Total SFA percentage and most individual SFA percentages were unaffected by the diet ($P > 0.05$), except for those of C15:0 ($P < 0.001$) and C17:0 ($P < 0.05$), which were greater in 0SF than those in 40SF lambs, and of C11:0 ($P < 0.01$), with lower values in 20SF than that in their counterparts. The diet affected the total concentration of BCFA, *iso*-BCFA, and *anteiso*-BCFA ($P < 0.001$). The percentages of total-BCFA and *iso*-BCFA decreased as the level of sainfoin inclusion increased in the diet whereas the percentage of total *anteiso*-BCFA decreased due to the presence of sainfoin in the diet. Similarly, percentages of *iso*-C16:0, *iso*-C17:0, total *anteiso*-BCFA, and *anteiso*-C17:0 were greater in 0SF than those in their counterparts ($P < 0.05$) regardless of the level of sainfoin inclusion, whereas the percentage of *anteiso*-C13:0 was greater in the 0SF meat than that in the 40SF lambs ($P < 0.05$).

The diet did not change the total percentage of MUFA, *cis*-MUFA or C18:1 c9 in meat ($P > 0.05$) but did affect the percentage of four minor individual *cis*-MUFA. The meat of 0SF lambs had a higher C24:1 c14 percentage than that of their counterparts regardless of the proportion of sainfoin and higher C18:1 c11 percentage only in that of 40SF lambs ($P < 0.05$). The percentages of C18:1 c12 and C20:1 c11 were also affected by the diet ($P < 0.01$ and $P < 0.001$, respectively), with greatest percentages in 40SF and 20SF lambs, respectively. The diet affected the total percentage of *trans*-MUFA and 6 of the 9 individual FA detected ($P < 0.05$). The meat of 0SF lambs had greater percentages of total *trans*-MUFA, C17:1 t9, and C18:1 t10 than those in the 40SF meat but had the lowest percentages of C16:1 t9 and C18:1 t9 than that in the meat of their counterparts regardless of the level of inclusion of sainfoin and had a lower percentage of C18:1 t12 than that in 40SF meat ($P < 0.05$). The percentage of C16:1 t10 was lower in the 20SF lambs than that in their counterparts ($P < 0.05$).

**Table 23.** Effect of the diet on intramuscular fat (IMF), cholesterol, total fatty acid (FA) content (g/100 g of fresh muscle), and FA profile (% of total FA identified) of the *longissimus lumborum* muscle of lambs.

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| Dry matter | 22.1 ± 0.05 | 22.0 ± 0.05 | 22.3 ± 0.05 | 0.66 |
| IMF | 3.09 ± 0.151 | 2.99 ± 0.160 | 2.98 ± 0.151 | 0.87 |
| Cholesterol | 0.136 ± 0.0038 | 0.136 ± 0.0040 | 0.140 ± 0.0038 | 0.74 |
| Total FA | $0.93^{a}$ ± 0.041 | $0.84^{ab}$ ± 0.045 | $0.79^{b}$ ± 0.041 | 0.046 |
| Individual FA | | | | |
| SFA [2] | 44.7 ± 0.47 | 44.6 ± 0.46 | 45.6 ± 0.46 | 0.28 |
| C10:0 | 0.49 ± 0.050 | 0.52 ± 0.053 | 0.46 ± 0.053 | 0.72 |
| C11:0 | $0.02^{a}$ ± 0.001 | $0.01^{b}$ ± 0.002 | $0.03^{a}$ ± 0.003 | 0.005 |
| C12:0 | 0.34 ± 0.029 | 0.36 ± 0.029 | 0.40 ± 0.029 | 0.34 |
| C13:0 | 0.08 ± 0.011 | 0.06 ± 0.012 | 0.06 ± 0.011 | 0.25 |
| C14:0 | 2.51 ± 0.142 | 2.31 ± 0.142 | 2.37 ± 0.141 | 0.59 |
| C15:0 | $0.72^{a}$ ± 0.030 | $0.57^{b}$ ± 0.032 | $0.49^{b}$ ± 0.030 | <0.001 |
| C16:0 | 21.1 ± 0.35 | 20.4 ± 0.35 | 21.3 ± 0.35 | 0.15 |
| C17:0 | $0.91^{a}$ ± 0.037 | $0.87^{ab}$ ± 0.039 | $0.77^{b}$ ± 0.037 | 0.04 |
| C18:0 | 10.9 ± 0.22 | 11.3 ± 0.23 | 11.4 ± 0.22 | 0.24 |
| C20:0 | 0.09 ± 0.007 | 0.07 ± 0.006 | 0.11 ± 0.028 | 0.06 |
| C21:0 | 0.02 ± 0.011 | 0.03 ± 0.012 | 0.04 ± 0.011 | 0.39 |
| C22:0 | 0.01 ± 0.001 | 0.02 ± 0.004 | 0.03 ± 0.016 | 0.06 |
| C24:0 | 0.02 ± 0.006 | 0.03 ± 0.006 | 0.04 ± 0.006 | 0.10 |
| BCFA [3] | $2.11^{a}$ ± 0.071 | $1.80^{b}$ ± 0.070 | $1.47^{c}$ ± 0.069 | <0.001 |
| *iso*-BCFA | $1.52^{a}$ ± 0.046 | $1.35^{b}$ ± 0.045 | $1.12^{c}$ ± 0.044 | <0.001 |
| *iso*-C15:0 | 0.77 ± 0.037 | 0.75 ± 0.037 | 0.66 ± 0.037 | 0.14 |
| *iso*-C16:0 | $0.09^{a}$ ± 0.005 | $0.05^{b}$ ± 0.005 | $0.05^{b}$ ± 0.005 | <0.001 |
| *iso*-C17:0 | $0.34^{a}$ ± 0.017 | $0.26^{b}$ ± 0.018 | $0.24^{b}$ ± 0.017 | <0.001 |
| *iso*-C18:0 | 0.28 ± 0.034 | 0.30 ± 0.036 | 0.20 ± 0.034 | 0.12 |
| *anteiso*-BCFA | $0.60^{a}$ ± 0.037 | $0.46^{b}$ ± 0.040 | $0.35^{b}$ ± 0.037 | <0.001 |
| *anteiso*-C13:0 | $0.20^{a}$ ± 0.024 | $0.17^{ab}$ ± 0.024 | $0.11^{b}$ ± 0.023 | 0.04 |
| *anteiso*-C15:0 | 0.09 ± 0.007 | 0.07 ± 0.021 | 0.07 ± 0.009 | 0.19 |
| *anteiso*-C17:0 | $0.32^{a}$ ± 0.014 | $0.22^{b}$ ± 0.015 | $0.18^{b}$ ± 0.014 | <0.001 |
| MUFA [4] | 28.4 ± 0.65 | 28.6 ± 0.64 | 28.7 ± 0.63 | 0.96 |
| *cis*-MUFA | 24.3 ± 0.68 | 25.0 ± 0.67 | 25.4 ± 0.66 | 0.52 |
| C16:1 c9 | 1.29 ± 0.077 | 1.23 ± 0.077 | 1.20 ± 0.076 | 0.70 |
| C16:1 c11 | 0.01 ± 0.004 | 0.02 ± 0.003 | 0.03 ± 0.008 | 0.30 |
| C16:1 c12 | 0.01 ± 0.004 | 0.03 ± 0.004 | 0.02 ± 0.004 | 0.08 |
| C18:1 c6/c8 [5] | 0.15 ± 0.018 | 0.12 ± 0.019 | 0.15 ± 0.018 | 0.40 |
| C18:1 c9 | 20.8 ± 0.66 | 21.1 ± 0.70 | 21.4 ± 0.66 | 0.82 |
| C18:1 c11 | $1.73^{a}$ ± 0.050 | $1.61^{ab}$ ± 0.050 | $1.50^{b}$ ± 0.049 | 0.02 |
| C18:1 c12 | $0.05^{b}$ ± 0.011 | $0.05^{b}$ ± 0.015 | $0.15^{a}$ ± 0.026 | 0.007 |

**Table 23**. (Continued)

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| C18:1 c13 | 0.01 ± 0.007 | 0.04 ± 0.007 | 0.03 ± 0.007 | 0.07 |
| C18:1 c14 | $0.04^{a}$ ± 0.005 | $0.01^{b}$ ± 0.005 | $0.02^{b}$ ± 0.005 | 0.01 |
| C18:1 c15 | 0.02 ± 0.007 | 0.04 ± 0.007 | 0.03 ± 0.007 | 0.15 |
| C20:1 c11 | $0.11^{b}$ ± 0.007 | $0.15^{a}$ ± 0.014 | $0.06^{c}$ ± 0.011 | <0.001 |
| C22:1 c13 | 0.02 ± 0.004 | 0.02 ± 0.004 | 0.01 ± 0.004 | 0.16 |
| C24:1 c15 | $0.08^{a}$ ± 0.013 | $0.03^{b}$ ± 0.010 | $0.02^{b}$ ± 0.005 | <0.001 |
| *trans*-MUFA | $3.41^{a}$ ± 0.246 | $2.69^{ab}$ ± 0.261 | $2.50^{b}$ ± 0.246 | 0.04 |
| C16:1 t9 | $0.06^{c}$ ± 0.005 | $0.07^{b}$ ± 0.006 | $0.12^{a}$ ± 0.014 | 0.002 |
| C16:1 t10 | $0.02^{ab}$ ± 0.004 | $0.01^{b}$ ± 0.001 | $0.02^{a}$ ± 0.003 | 0.007 |
| C17:1 t9 | $0.11^{a}$ ± 0.011 | $0.03^{ab}$ ± 0.011 | $0.06^{b}$ ± 0.011 | 0.01 |
| C18:1 t5 | 0.01 ± 0.002 | 0.005 ± 0.0017 | 0.01 ± 0.002 | 0.15 |
| C18:1 t6/t8 [6] | 0.04 ± 0.010 | 0.06 ± 0.010 | 0.05 ± 0.010 | 0.32 |
| C18:1 t9 | $0.10^{b}$ ± 0.012 | $0.15^{a}$ ± 0.013 | $0.16^{a}$ ± 0.012 | 0.003 |
| C18:1 t10 | $2.27^{a}$ ± 0.205 | $1.60^{ab}$ ± 0.217 | $1.32^{b}$ ± 0.205 | 0.01 |
| C18:1 t11 | 0.73 ± 0.074 | 0.64 ± 0.078 | 0.66 ± 0.074 | 0.71 |
| C18:1 t12 | $0.07^{b}$ ± 0.008 | $0.07^{b}$ ± 0.008 | $0.10^{a}$ ± 0.008 | 0.001 |
| PUFA [7] | 23.2 ± 0.34 | 23.7 ± 0.97 | 23.0 ± 0.63 | 0.78 |
| CLA [8] | 0.33 ± 0.026 | 0.33 ± 0.028 | 0.36 ± 0.026 | 0.64 |
| CLA t7,c9 | 0.02 ± 0.010 | 0.01 ± 0.003 | 0.01 ± 0.002 | 0.41 |
| CLA c9,t11 | $0.16^{b}$ ± 0.014 | $0.14^{b}$ ± 0.015 | $0.22^{a}$ ± 0.014 | 0.003 |
| CLA t9,c11 | 0.10 ± 0.011 | 0.10 ± 0.011 | 0.06 ± 0.011 | 0.05 |
| CLA t10,c12 | 0.01 ± 0.002 | 0.01 ± 0.002 | 0.01 ± 0.002 | 0.97 |
| PUFA n-6 | 18.5 ± 0.70 | 18.1 ± 0.69 | 16.7 ± 0.68 | 0.19 |
| C18:2 n-6 | 12.8 ± 0.29 | 12.9 ± 0.9 | 11.9 ± 0.48 | 0.26 |
| C18:3 n-6 | $0.12^{a}$ ± 0.007 | $0.12^{a}$ ± 0.008 | $0.09^{b}$ ± 0.093 | 0.02 |
| C20:2 n-6 | 0.15 ± 0.005 | 0.15 ± 0.018 | 0.13 ± 0.012 | 0.26 |
| C20:3 n-6 | 0.44 ± 0.013 | 0.43 ± 0.013 | 0.42 ± 0.013 | 0.37 |
| C20:4 n-6 | 4.32 ± 0.131 | 4.20 ± 0.131 | 3.97 ± 0.129 | 0.19 |
| C22:4 n-6 | $0.39^{a}$ ± 0.017 | $0.35^{a}$ ± 0.017 | $0.28^{b}$ ± 0.016 | 0.002 |
| C22:5 n-6 | 0.08 ± 0.014 | 0.09 ± 0.005 | 0.07 ± 0.008 | 0.13 |
| PUFA n-3 | $3.30^{c}$ ± 0.105 | $4.00^{b}$ ± 0.112 | $4.69^{a}$ ± 0.105 | <0.001 |
| C18:3 n-3 | $0.82^{c}$ ± 0.036 | $1.17^{b}$ ± 0.038 | $1.64^{a}$ ± 0.036 | <0.001 |
| C20:3 n-3 | 0.01 ± 0.004 | 0.02 ± 0.004 | 0.02 ± 0.004 | 0.14 |
| C20:5 n-3 | $0.74^{b}$ ± 0.042 | $0.91^{a}$ ± 0.044 | $1.02^{a}$ ± 0.042 | <0.001 |
| C22:5 n-3 | 1.30 ± 0.047 | 1.38 ± 0.047 | 1.38 ± 0.046 | 0.37 |
| C22:6 n-3 | $0.47^{b}$ ± 0.020 | $0.52^{ab}$ ± 0.018 | $0.60^{a}$ ± 0.042 | 0.03 |
| C18:1 t10/C18:1 t11 | 3.30 ± 0.411 | 2.86 ± 0.436 | 1.98 ± 0.411 | 0.09 |

**Table 23**. (Continued)

| | Diets [1] | | | *P*-value |
|---|---|---|---|---|
| | 0SF | 20SF | 40SF | |
| PUFA/SFA | 0.52 ± 0.010 | 0.53 ± 0.025 | 0.50 ± 0.018 | 0.60 |
| PUFA n-6/PUFA n-3 | 5.44$^{a}$ ± 0.206 | 4.57$^{b}$ ± 0.218 | 3.70$^{c}$ ± 0.206 | <0.001 |
| AI index [9] | 0.62 ± 0.022 | 0.60 ± 0.022 | 0.63 ± 0.021 | 0.55 |
| TI index [10] | 1.02 ± 0.020 | 0.96 ± 0.020 | 0.95 ± 0.019 | 0.07 |
| H:h index [11] | 1.73 ± 0.055 | 1.81 ± 0.054 | 1.73 ± 0.053 | 0.44 |
| $\Delta^9$-desaturase C16 (%) | 5.72 ± 0.176 | 5.68 ± 0.437 | 5.25 ± 0.211 | 0.26 |
| $\Delta^9$-desaturase C18 (%) | 65.6 ± 0.83 | 65.0 ± 0.88 | 65.2 ± 0.83 | 0.87 |
| Elongase (%) | 57.9 ± 0.70 | 60.1 ± 0.74 | 59.9 ± 0.70 | 0.07 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate. [2] Saturated FA. [3] Branched-chain FA. [4] Monounsaturated FA. [5] C18:1 c6 and C18:1 c8 might coelute. [6] C18:1 t6 and C18:1 t8 might coelute. [7] Polyunsaturated FA. [8] Conjugated linoleic acid. [9] Atherogenicity index. [10] Thrombogenicity index. [11] Hyper-hypocholesterolemic index. The different lowercase letters indicate differences among groups at $P < 0.05$; standard error of means (±).

**Discussion**

First, it is important to highlight that, in addition to the effect produced by the inclusion of sainfoin in the concentrate, differences in the cereal content among the diets can play an important role in the changes in ruminal BH. To maintain the isoproteic and isoenergetic conditions of the feeds, the inclusion of 20% and 40% sainfoin caused a significant decrease in the cereal content. These differences were especially evident in barley, whose content shows minor variations between 0SF and 20SF (310 and 252 g/kg DM, respectively) but were much lower in 40SF (50 g/kg DM). Consequently, these differences would have contributed to the effects obtained in the 40SF rumen compared with those of the other diets.

The significant effects on the rumen FA profile showed that the different diets interfered with the ruminal BH and/or with the rumen microbiota. Higher percentages of *anteiso*-BCFA were observed in the rumen of 0SF lambs probably because of the higher concentration of starch in their diet. Fievez et al. (2012) demonstrated that increased productions of *anteiso*-BCFA were linked to increased amylolytic populations. Moreover, although PAC have inhibitory effects on microbial growth and on proteolysis, reducing ruminal concentrations of BCFA (Costa et al., 2017), we suggest that the low

PAC content in the sainfoin concentrates compared with that in fresh sainfoin (Baila et al., 2022a; Rufino-Moya et al., 2022) due to the pelleting process could not be enough to justify the changes in BCFA ruminal concentrations.

Regarding the MUFA percentages in the rumen, C18:1 c9 may come from diet or from the BH of C18:2 n-6 (Kishino et al., 2013). In this study, a greater C18:1 c9 percentage was observed in the 40SF rumen, which would be related to its greater content in the diet, given the statistically similar BH extent of C18:2 n-6 and C18:1 c9 among diets. Toral et al. (2016) and Huyen et al. (2020) studied sainfoin *in vitro* and in dairy cows fed sainfoin silage and observed a low BH extent and higher C18:1 c9 ruminal percentages coming from the diet. However, because the C18:1 c9 could not be chromatographically resolved from the C18:1 t13/t14 pair, the proportion of C18:1 c9 may also be related to the eventual presence of C18:1 t13 formed from the BH of C18:3 n-3 (Alves et al., 2021), which was higher in 40SF meat.

Several of the most important effects produced by the sainfoin inclusion in the diet were observed in the *trans*-MUFAs. The study of this group of FA is important because *trans*-MUFA in food industry has been phased out due to their potential negative effects on human health, so that ruminant-derived products have become one of the main sources of consumption of this type of fats (Alves et al., 2021). The *trans*-FA present in ruminant meat and milk are produced during ruminal BH, and C18:1 t10 and C18:1 t11 are the main FA formed during this process. High ruminal concentrations of C18:1 t11 have been associated with forage–rich diets, whereas the formation of the C18:1 t10 isomer is linked with concentrate-rich diets (Griinari et al., 1998). As the high concentration of C18:1 t10 in products is undesirable, the study of the modification of ruminal BH to enhance the production of C18:1 t11 is a main target in ruminant nutrition because this is converted to CLA c9,t11 in meat through activity of $\Delta^9$-desaturase, having potential beneficial implications for human health (Palmquist et al., 2004). The predominance of the t11 or the t10 BH pathway is studied by using the C18:1 t10/C18:1 t11 ratio. The inclusion of sainfoin in the diet decreased C18:1 t10 ruminal concentrations; however, the concentration of C18:1 t11 and consequently the C18:1 t10/C18:1 t11 ratio only increased in the 40SF group. The reduction in the rumen of approximately 50% of C18:1 t10 concentration when sainfoin was included in the

concentrate suggests a greater presence of the t11 BH pathway in both sainfoin concentrates than those in the 0SF meat, which was also supported by the decrease in CLA t10,c12 ruminal percentage when sainfoin was included at 40%. Indeed, this FA is directly derived from the *trans*-10 shifted BH pathway of C18:2 n-6 (Griinari and Bauman, 1999) and has been associated with negative effects on lipid metabolism, specifically with milk fat depression in dairy cows (Tricon et al., 2004). The predominance of the t11 BH pathway with the inclusion of 40% sainfoin was confirmed with the increase in C18:1 t11 concentration, which was almost 6-fold higher in 40SF meat than that in 0SF or 20SF meat, and is produced as a result of the t11 BH pathways of C18:3 n-3 and C18:2 n-6 after the hydrogenation of the C18:2 t11,c15 and CLA c9,t11 intermediates, respectively (Griinari et al., 1998; Alves et al., 2017). This result confirms the efficacy of increasing C18:1 t11 as the main isomer formed in the rumen of animals fed forage-rich diets (Alves et al., 2013).

As expected, the numerically higher values of BI were negatively related to the values of C18:0 and BH completeness indicating a more incomplete BH process. However, the differences observed in C18:1 t10 (%BI) showed that as the presence of sainfoin in the concentrate increased, lower proportions of the BI were represented by C18:1 t10, thus supporting the predominance of the t11 pathway over the t10 pathway as indicated by the differences in the C18:1 t10/C18:1 t11 ratio and the C18:1 t10 values in rumen.

The observed decrease of approximately 40% of C18:2 n-6 in the ruminal content of 40SF lambs compared with that in the rest of diets could be associated with the 25% lower percentage of this FA in the diet. As C18:2 n-6 is the main FA in the diets and is on average 7-fold more abundant than C18:3 n-3, the lower percentage of C18:2 n-6 in the 40SF diet was reflected on the lower ruminal concentration of total PUFA. The inclusion of sainfoin in the diet also increased the BH extent of C18:3 n-3, and this effect was greater when sainfoin was included at 40%. It is frequently reported that an increase in C18 PUFA BH occurs when the intake of PUFA is high (Jenkins, 1993), as a response of their greater availability and as a defence against the potential toxicity of dietary PUFA to ruminal bacteria (Maia et al., 2007). The higher fibre content of the 40SF concentrate when compared with the rest of the diets could have contributed to the increase in C18:3 n-3 BH extent, producing a longer retention time of the feed in the rumen and a

better environment for cellulolytic bacteria, which would cause a greater extension of the BH process (Sackmann et al., 2003). However, increasing the BH extent of dietary PUFAs is not always positive since the disappearance of PUFAs can reduce their content in the final product. Otherwise, it is important to highlight that, in this study, the concentration of C18:3 n-3 found in 40SF rumen was equal to that of 20SF rumen and higher than that in the 0SF group, despite the large BH extent. Increased concentrations of C18:3 n-3 in the ruminal content when using sainfoin have already been found in previous studies (Campidonico et al., 2016; Grosse Brinkhaus et al., 2016; Toral et al., 2016; Huyen et al., 2020). Nevertheless, this finding is usually associated with the presence of PAC in sainfoin, which can affect the BH of dietary PUFA (Frutos et al., 2020). Herein, the lack of C18:3 n-3 BH inhibition may be associated with the low PAC content in the sainfoin diets and their low activity. Hence, the higher ruminal C18:3 n-3 concentration in the 20SF and 40SF groups was simply related to a higher availability of this FA in the diet because of the inclusion of sainfoin. Furthermore, this effect was even higher in 40SF probably because of the higher C18:3 n-3 intake observed in this group.

Regarding the FA profile of plasma samples, the effect of sainfoin inclusion on the FA profile was more diluted. Although the changes found for C18:3 n-3 and PUFA n-3 were close to those obtained in the ruminal digesta, the concentrations of MUFA, C18:1 t11, and C18:1 t10 did not reflect the effect obtained in the rumen. Therefore, in this case, plasma samples were not useful to predict what happened during ruminal BH. The lack of a clear link between rumen and plasma FA profile has been previously observed (Bauchart, 1993; Vasta et al., 2009a) and may be because of the transfer of long-chain FAs to tissues, which depends on numerous factors such as lipid transport and metabolism (Christie, 1981).

The meat FA profile reflects the final effect of the ruminal BH produced by the diet. The amount of IMF was similar or even higher than that found in light lambs slaughtered at similar weight (Lobón et al., 2017a; Natalello et al., 2019; Blanco et al., 2021). However, the FA content of meat was found to be very low in all groups compared with 1.7% of total FA obtained on average in lambs of 28.2 kg BW (Vasta et al., 2009b) and up to 2.6% of FA in meat from heavier lambs (38 kg BW) (Jerónimo et al., 2010). This indicates that a low percentage of the IMF was related to FA, being the IMF composed

mostly of phospholipids. Moreover, the FA content was even lower in the meat of lambs fed 40% sainfoin in the concentrate. Despite the differences observed in *cis*-MUFA, C18:2 n-6, PUFA n-6, and total PUFA between groups in the ruminal FA profile, no changes were found in meat. By contrast, the changes in rumen percentages of BCFA with sainfoin inclusion were partially reflected in lower percentages of total BCFA and *anteiso*-BCFA in meat. In addition, despite no differences in *iso*-BCFA in rumen, this group of FA decreased in concentration in meat with the inclusion of sainfoin. These results might not be desirable as some of the positive effects related to human health are attributed to BCFA intake (Ran-Ressler et al., 2011).

The higher concentrations of C18:3 n-3 obtained in the rumen of 40SF lambs produced a higher transference of this FA to animal tissues as previously reviewed with high PUFA n-3 diets (Álvarez-Rodríguez et al., 2022). This is because higher concentrations of this FA would escape from the rumen, be absorbed in the small intestine, and be deposited in the muscle (Scollan et al., 2006). Meat CLA c9,t11 concentrations of 40SF lambs were almost 40% higher than that in the rest of the diets, and enhancement of this FA is one of the most desirable goals in ruminant products (Palmquist, 2006). The CLA c9,t11 present in meat is derived from the following: i) ruminal synthesis as a BH product of C18:2 n-6 and subsequent transport to meat or ii) endogenous synthesis in the tissues from rumen-derived C18:1 t11 via $\Delta^9$-desaturase activity (Bessa et al., 2015). As higher concentrations of C18:1 t11 were found in the 40SF rumen, it can be assumed that the increase in the percentage of CLA c9,t11 in the meat came predominantly from the higher ruminal concentration of C18:1 t11. In this case, the promotion of t11 BH pathways of both C18:3 n-3 and C18:2 n-6 and the greater BH extent of C18:3 n-3 allowed higher concentrations of C18:1 t11 in rumen, thus promoting its conversion to CLA c9,t11 in meat. This result agrees with previous studies stating that approximately 93% of C18:1 t11 is converted to CLA c9,t11 in lamb meat (Palmquist et al., 2004). A higher CLA c9,t11 concentration in lamb meat of forage–fed animals compared with that from a grain–based diet has been previously reported (Lobón et al., 2017a; Álvarez-Rodríguez et al., 2018) and showed an increase in CLA isomers formation due to a higher PUFA BH extent. However, despite the increase in CLA c9,t11 concentration with the presence of 40% sainfoin, the percentages of this FA

with respect to total FA were low relative to those reported in other studies conducted with light lambs (Natalello et al., 2019; Blanco et al., 2021). This is probably because *trans*-C18, including CLA isomers, are preferentially incorporated into neutral lipids (Jerónimo et al., 2011). Lean meat with a low FA content is mostly composed of membrane phospholipids (polar lipids); thus, both CLA c9,t11 and C18:1 t11 might not have been preferentially deposited in the IMF. Possibly for the same reason, no effect on C18:1 t11 was observed in the meat even though differences were observed in the rumen. Therefore, this study shows that CLA c9,t11 promotion in meat should not only be focused on a high substrate supply coming from ruminal BH but should also be accompanied by a high IMF, rich in total FA as stated in Bessa et al. (2015).

The high C20:5 n-3 and C22:6 n-3 concentrations found in 40SF muscle is an indication that, even with a higher BH of C18:3 n-3 occurring in the rumen of these animals, greater amounts of C18:3 n-3 were absorbed and made available for the synthesis of both C20:5 n-3 and C22:6 n-3 as C18:3 n-3 is the precursor for the synthesis of long–chain PUFA n-3 (Scollan et al., 2014). Previous studies described similar results in muscle from lambs fed sainfoin silage (Girard et al., 2016) and lambs grazed with forage legumes (Gruffat et al., 2020). Furthermore, the lowest PUFA n-6/PUFA n-3 values obtained in the 40SF group demonstrate that it is possible to decrease that value to within the recommended limits (Vahmani et al., 2015) by including sainfoin in a concentrate–based diet.

**Conclusions**

In conclusion, the inclusion of sainfoin in the diet supported an increase in ruminal PUFA n-3 percentages and a decrease in PUFA n-6/PUFA n-3 and C18:1 t10/C18:1 t11 ratios, being this effect most apparent in the 40SF diet. This diet also produced a major decrease in the percentage of PUFA n-6 and an increase in that of C18:1 t11 and in CLA c9,t11 in the rumen. These ruminal changes were reflected in greater percentages of PUFA n-3 and CLA c9,t11 in the meat as the level of sainfoin inclusion increased, which resulted in higher amounts of those FA in 40SF meat even with a lower amount of total FA. Therefore, meat from lambs fed with 20% or 40% sainfoin in the diet achieved a healthier FA profile for humans. The more desirable results obtained in the 40SF diet

could be related to a higher C18:3 n-3 intake due to the 40% inclusion of sainfoin in the diet along with a stronger t11 biohydrogenation pathway in the rumen of these lambs.

Therefore, the inclusion of sainfoin in lamb finishing concentrate improved meat quality, especially when sainfoin was included up to 40% as the effects were greater. Although the total amounts of beneficial FA achieved in meat from the inclusion of sainfoin in the concentrate were not as high as in several studies where lambs were fed fresh, silage, or hay forages, this study demonstrates that it is possible to improve the FA profile of meat without modifying the typical southern European lamb production system where lambs are concentrate–fattened indoors.

**Supplementary Table S1.** Effect of the diet on the intake of fatty acids (FA) of the concentrates.

| | Diets [1] | | |
|---|---|---|---|
| | 0SF | 20SF | 40SF |
| total FA intake, g/d | 33.0 | 34.2 | 41.4 |
| Intake individual FA, g/d | | | |
| C12:0 | 0.03 | 0.02 | 0.05 |
| C14:0 | 0.17 | 0.17 | 0.25 |
| C16:0 | 9.12 | 9.57 | 12.43 |
| C16:1 c9 | 0.07 | 0.08 | 0.11 |
| C18:0 | 2.43 | 2.39 | 3.07 |
| C18:1 c9 | 8.13 | 8.24 | 11.11 |
| C18:1 c11 | 0.09 | 0.09 | 0.12 |
| C18:2 n-6 | 12.16 | 11.97 | 11.52 |
| C18:3 n-3 | 0.82 | 1.63 | 2.78 |

[1] 0SF, 0% of sainfoin; 20SF, 20% of sainfoin; 40SF, 40% of sainfoin in the finishing concentrate.

# Final Considerations

In recent years, the inclusion of local resources in livestock systems has been promoted to increase sustainability and self–sufficiency of farms and decrease dependence on imports for livestock feed sources (Moorby and Fraser, 2021). On the other hand, the effect of PAC (condensed tannins), secondary compounds found naturally in some legumes, have attracted much interest for their potential beneficial effects on the performance of the animals consuming them. In this framework, the present thesis was carried out to study the use of sainfoin, a local forage with a medium content of PAC, for feeding autochthonous breed meat sheep in two different lamb production systems.

**Study 1** was focused on evaluating the effect of PAC present in fresh sainfoin fed to Rasa Aragonesa dams rearing one suckling lamb on animal performance and product quality. Although the effect of PAC in dams' diets has been extensively studied (Wang et al., 1996; Frutos et al., 2004; Pascual et al., 2019), little is known about the passage of these compounds through milk and how they may affect the performance and meat quality of suckling lamb, whose diet is based exclusively on the dams' milk. The effect of PAC on milk production varies among studies, although some literature recorded an increase in milk production of ewes fed PAC–containing diets (Aerts et al., 1999; Pascual et al., 2019). However, it must be taken into account that high PAC concentrations in the diet can reduce dry matter intake, as had been observed in ewes fed with 3 g of quebracho tannin/kg DM (Hervás et al., 2003), which could lead to a reduction in the milk production of ewes. In the present study the dry matter intake was similar between the two treatments studied (sainfoin *vs*. sainfoin plus PEG), which was reflected in a similar milk yield of ewes between groups and, therefore, in similar growth in their suckling lambs. Lamb growth during suckling was comparable to the growth recorded in this breed with dams grazing alfalfa or indoors concentrate–fed (Joy et al., 2012; Lobón et al., 2019a).

Although feed intake increased as lactation advanced in response to higher requirements for milk production and recovery of rumen size after uterine involution, during the week 3 of lactation ewes from both groups had a peak of blood non esterified fatty acids (NEFA). This metabolic response was related to the lower forage quality during

that week, which was manifested by decreased gross energy, crude protein, PAC, and C16:1 c9 and C18:3 n-3 fatty acids and increased fibre and C16:0 and C18:0 contents of fresh sainfoin. The increase in blood NEFA was effective, counterbalancing the lower nutritional intake of the forage and not affecting the milk production of the ewes, although it did result in a lower percentage of milk fat in the ewes of both groups during the third week of lactation.

An interesting finding is the reduction of urea concentration in both plasma and milk due to the presence of PAC in dams' diet, suggesting a lower ruminal degradation of protein, as seen in a previous study using sainfoin as main feed of lactating cows (Grosse Brinkhaus et al., 2016). This could imply a more efficient utilisation of dietary protein by ewes, while reducing its urinary contribution to soil contamination through ammonia emissions. However, no other associated effects were observed related to the better use of protein, as it could be an increase in milk protein, milk production, milk lactose or higher blood glucose levels (Frutos et al., 2004).

One of the most studied effects of sainfoin is its potential as a natural anti–parasitic in the digestive tract (Hoste et al., 2015; Saratsis et al., 2016), thus this aspect was included in the approach of the present study (Study 1). No differences were observed in the amount of *Strongyloides* eggs secreted by either ewes or lambs during the entire experimental period. However, since the animals were dewormed prior to the beginning of the experiment and the ewe–lamb pairs were not in contact with other pairs, this could have hindered the collective transmission and development of these parasites, concluding in an absence of differences between the groups studied.

Despite the few effects observed on the productive parameters of ewes and lambs, the presence of sainfoin PAC produced several changes in the fatty acid profile of milk. Milk from ewes with presence of sainfoin PAC in the diet showed higher concentrations of PUFA n-3 and n-6 throughout lactation, which was previously observed in lactating ewes fed sainfoin hay (Pascual et al., 2019). This result suggests an effect of PAC focused on decreasing ruminal biohydrogenation of dietary PUFA, according to a previous review (Frutos et al., 2020). The canonical analyses carried out to see the major effects of the presence of PAC in the diet on the milk FA profile support this theory, indicating that the FA capable of differentiating the milk of both groups were mostly PUFA. Besides of these

effects on milk FA profile which are clearly beneficial from a consumer health point of view, some negative effects were also observed as the decrease of some intermediate fatty acids that are considered desirable, such as C18:1 t11, which was lower in ewes with sainfoin PAC during the whole lactation period studied, or CLA c9,t11, which decreased in the presence of PAC in week 1 of lactation. However, it should be highlighted that no rumen samples from ewes were analysed in this study, so the changes in ruminal biohydrogenation processes produced by PAC in the current study are not specifically known.

The effect of the presence of PAC on dams' diet did not show differences in carcass traits and meat quality of suckling lambs, except for the decrease of polyphenol meat content. This result was unexpected, since PAC are considered phenolic compounds, and not desirable, as they are involved in increasing antioxidant activity of tissues (López-Andrés et al., 2014). However, this result was in line with the lower polyphenol content observed in milk. Nevertheless, the differences observed between milk and meat polyphenols contents could be due to the inaccuracy of the Folin–Ciocalteu technique. This technique is accurate for polyphenol quantification in forage (Rufino-Moya et al. 2022), but in meat and milk could not be recommended given the high complexity of macromolecules of both matrices and the extraordinarily large variety of phenolic compounds detected in sainfoin (Regos and Treutter, 2010) could have been metabolised and, therefore, not detected. Moreover, the absence of differences between groups in the metabolites indicating the antioxidant capacity in the plasma of sheep and lambs (SOD, malondialdehyde, and ABTS) and in the ABTS of meat and milk supports the idea that the determination of polyphenol content in meat and milk by the Folin–Ciocalteu technique may not be correct.

**Study 2** was planned with the objective of studying the effect of the level of sainfoin inclusion (0%, 20%, and 40%) in the concentrate of Rasa Aragonesa lambs during the fattening period. Most of the lambs of the Rasa Aragonesa breed are raised under the standards specified by the Protected Geographical Indication (PGI) "Ternasco de Aragón". Among other requirements, the legislation defining the criteria for rearing these lambs stipulates that they must be fattened indoors with concentrates. Then, to include forage in the diet in the present study, it must be dehydrated before being

included in the concentrate to adapt to the requirement of the production system of PGI. In addition, the necessary dehydration of sainfoin plus the pelleting process of concentrate caused an important reduction of some sainfoin compounds, such as carotenoids or PAC. Total PAC were quantified at 31.4 g sainfoin PAC/kg DM on week 4 on fresh sainfoin, while sainfoin pellets made from the same fresh sainfoin after drying reduced its total PAC content to 17.3 g sainfoin PAC/kg DM (almost 50% of losses). When those sainfoin pellets were introduced at 40% in the concentrate, the PAC content of the concentrate was only of 5.23 g sainfoin PAC/kg DM. Therefore, as PAC were almost disappeared in the concentrates, the differences among treatments were more due to the inclusion of forage than to the presence of PAC, making difficult to assess the effect of PAC in finishing lambs. However, although the reductions in PAC fractions are evident, the minimum threshold of PAC concentration necessary to cause changes in the organism is not well defined and must be deeply studied. The pelleting process not only can damage the total amount of PAC, but also the availability of PAC, *i.e.* the different fractions of PAC showing whether these compounds are free or bound to proteins or fibres, can be modified. Previous studies (Rufino-Moya et al., 2019a; Rufino-Moya et al., 2022) have already shown how can the different fractions be affected by the conservation status of forages, which can modify the potential effects that PAC can produce in the animals that consume them.

The inclusion of 40% sainfoin in the diet decreased the digestibility of this concentrate due to the higher proportion of fibre, which could impair growth of lambs of this treatment. However, the lambs counterbalanced this fact increasing their dry matter intake and thus achieved similar growths compared to the other groups. There were no differences among treatments concerning the final weight or in the carcass weight of lambs, although both values were numerically higher in the animals fed with the 40% sainfoin concentrate. The low number of animals in the present study does not allow to confirm a clear conclusion, and thus a trial under commercial circumstances would be necessary to discern this aspect. If this effect towards higher final weights is confirmed, lambs fed 40% sainfoin could be more profitable.

The lack of negative effects on animal performance of the inclusion of the greater proportion of sainfoin in the concentrate, together with the lack of changes in the carcass

and subcutaneous fat colour are considered positive findings for producers, as shows that the inclusion up to 40% sainfoin in the concentrate allowed the production of homogeneous carcasses with similar characteristics to those required by the PGI. In contrast to the present results, changes in colouration have been observed mainly in animals fed fresh forage (Carrasco et al., 2009; Ripoll et al., 2012; Lobón et al., 2017). The absence of differences between groups in the current study can be related to the pelleting process as the main compounds responsible of colour changes are mainly carotenoids, which had been reduced from fresh sainfoin to concentrate. Forage preservation as silage or hay have previously shown to decrease carotenoid concentrations in sainfoin (Rufino-Moya et al., 2022). Nevertheless, despite heat treatment, carotenoid content was three times higher in the concentrate with 40% sainfoin, so that it cannot be dismissed that differences could appear among groups with a longer experimental period, as in this study the animals reached the market slaughter weight at 40 days, assuming a short fattening period.

Regarding changes in ruminal fermentation parameters, the inclusion of sainfoin in the concentrate, regardless of the inclusion level, produced an increase in the percentage of acetic acid formation in the rumen and, consequently, a higher acetic/propionic ratio. This effect was expected since the formation of this volatile fatty acid is enhanced when the proportions of forage in the ration increase. On the other hand, another effect associated with the inclusion of forage is the increase in ruminal pH, although this was only greater when sainfoin was introduced at 40%.

Despite slight changes in ruminal fermentation, the inclusion of sainfoin in the concentrate showed many differences in ruminal biohydrogenation (BH). The inclusion of sainfoin in the diet, both at 20% and 40%, produced an increase in the percentage of ruminal C18:3 n-3, since this is the major fatty acid (FA) of forages. Sainfoin inclusion in silages mixtures also led to higher C18:3 n-3 ruminal accumulation in lambs (Campidonico et al., 2016). On the other hand, diets rich in concentrates enhance the t10 BH pathway (resulting in higher formations of the C18:1 t10 isomer), while the inclusion of forage in the diet is characterised by promoting the t11 BH pathway (leading to C18:1 t11 isomer formation) (Griinari et al., 1998). When sainfoin was included at 20% or 40% in the diet, a decrease in the C18:1 t10 isomer was observed, indicating a lower

existence of the t10 BH pathway due to the lower cereal percentages in the ration. However, increased C18:1 t11 isomer formation in the rumen of lambs was only observed when sainfoin was included at 40%, suggesting that a lower inclusion of sainfoin might not be sufficient to enhance the t11 BH pathway. This is important, as ruminal t11 BH pathway is what gives rise to FA that have been shown to be beneficial for human health, such as C18:1 t11 and CLA c9,t11. Thus, the combination of increased C18:3 n-3 intake related to sainfoin inclusion in the diet and the enhancement of the t11 BH pathway in the presence of 40% sainfoin resulted in higher concentrations of C18:3 n-3, total PUFA n-3, and CLA c9, t11 in meat compared to that of lambs in the other two groups. Greater meat concentration of PUFA was also observed in lambs fed sainfoin silage compared to lambs fed red clover or alfalfa (Girard et al., 2016) and sainfoin pellet supplementations showed to be effective in increasing C18:3 n-3 in lambs' meat (Gruffat et al., 2020). This improvement in meat FA profile, only achieved with 40% sainfoin inclusion, together with the numerically higher final BW of lambs recorded, lead to the conclusion that the inclusion of 40% sainfoin produced the best results, but when sainfoin was included at 20% the results were less clear. This finding could contribute to a better understanding of the BH processes associated with the inclusion of forage in lamb diets and could be very promising in the production of heavier lambs with fattier meats.

In this Study, the plasma FA profile was also analysed to see if it could reflect or approximate the rumen FA profile or predict that of the meat. The plasma FA profile showed a higher amount of FA in lambs without sainfoin in the concentrate compared to those receiving 40% sainfoin, which coincides with the results obtained in the meat. In addition, it also gave an approximate picture of what happened with total PUFA n-3 and C18:3 n-3 in both rumen and meat, which showed higher concentrations with the inclusion of sainfoin in the concentrate. For this reason, although plasma has been reported as a tool to predict the t10/t11 ratio in the rumen (Alves et al., 2017), in the present study, plasma concentrations of these isomers did not reflect the effects obtained in rumen. Nevertheless, it would be interesting to stablish correlations between the FA of the plasma and rumen through statistical analyses.

The general conclusion from both studies is complicated due to the different objectives of them. While Study 1 was focused on the effect of the inclusion of PAC in the diet, the objective of Study 2 was the study of the sainfoin inclusion. Therefore, the absence of a control group in Study 1 prevented the analysis of the effects of feeding fresh sainfoin to lactating ewes, while the lack of PEG to block the effect of PAC prevented its study in Study 2. Despite this, there are certain connections between the studies due to the similarity of the effects observed on the FA profile of milk in Study 1 and meat in Study 2. Both the presence of PAC in the dams' diet and the inclusion of 40% sainfoin in the lambs' fattening concentrate produced an increase in the concentrations of PUFA n-3 and C18:3 n-3, reduced the concentrations of BCFA and did not produce changes in the percentage of C18:1 c9 and total CLA in the milk of the ewes and in the meat of the fattening lambs. However, it should be highlighted that the results obtained on meat FA of lambs fed 40% sainfoin, which are related to the inclusion of higher amounts of PUFA and a promotion of t11 BH pathway, are surprising, as the percentage of total PUFA and C18:3 n-3 in this concentrate was considerably lower than the concentrations existing in the fresh sainfoin of Study 1 (47% *vs.* 6.71% in fresh sainfoin vs 40% sainfoin concentrate, respectively). This reduction occurs because unsaturated FA are highly susceptible to degradation by the heat treatments used in the pelleting process (approximately 20% reduction of C18:3 n-3 during the pelleting process). However, it should be considered that changes in ruminal BH of lambs fed 40% sainfoin, could also be affected by changes in the rumen population as shown by the differences obtained in BCFA, which are synthetised by ruminal bacteria (Fievez et al., 2012) or by an increase in the percentage of acetate, which is a precursor of fatty acid synthesis (Bauman et al., 1973; Smith and Crouse, 1984). Nevertheless, in order to know the exact similarities between the rumen BH processes of both studies, it would have been necessary to analyse and study samples of the rumen content of the ewes in Study 1, which was not taken into account in this thesis.

# Implications

The suckling lamb system studied showed that ewes fed fresh sainfoin during the rearing of suckling lamb allowed good animal performance in both ewes and suckling lambs, whether the sainfoin contained active proanthocyanidins or not. However, the expected positive effects on some parameters were not recorded, as the decrease of parasitic load or the improvement of antioxidant activity in dams or lambs. The presence of PAC may have promoted some beneficial changes that occur during the ruminal biohydrogenation process, resulting in the presence of more desirable milk fatty acids. However, the exact mechanisms by which the changes occurred have not been studied, so that the effect of PAC present in the dams' diet on the milk FA profile should be deeply studied.

The fattening lamb system studied proved that the inclusion of dehydrated sainfoin up to 40% in the concentrate of finishing lambs resulted in similar productive results to those observed in lambs fed with a cereal–based concentrate. Both sainfoin–containing concentrates (20% and 40%) showed an improvement of the meat FA profile through the potentiation of t11 biohydrogenation, the pathway related to higher proportion of forage in diets. Thus, this effect was greater when sainfoin was included at 40% in the diet. Therefore, in the present study, it can be stated that the inclusion of 40% sainfoin in the concentrate of finishing lambs obtained better overall results than those obtained by lambs fed with 20% sainfoin or with a commercial concentrate without sainfoin inclusion.

Moreover, in accordance with the present results and with the current promotion of the use of fodder legumes through incentives set in the European legislative framework of the Common Agricultural Policy (CAP), the inclusion of sainfoin in the concentrate during the finishing period of light lambs could be a promising option during the rearing of light lambs. However, the results of this PhD thesis must be confirmed in commercial farms in order to allow the introduction of forage in the diet of lambs included in the PGI category of "Ternasco de Aragón". Considering the results, the use of forage legumes could be allowed to feed during fattening and could be a suitable market option for lambs reared under sustainable conditions and similar conditions of PGI. This, in turn, would make it possible to achieve the social and environmental circumstances demanding greater sustainability of livestock systems.

# Conclusions

Under the conditions of the present studies, it can be concluded that:

1. The presence of sainfoin proanthocyanidins (PAC) in the diet of dams rearing a suckling lamb did not affect their dry matter intake, body weight, body condition score, parasitism, and milk yield. However, it reduced the urea contents in milk and plasma, and polyphenols in milk.

2. The week of lactation affected all parameters related to performance, milk yield, milk composition, and plasma metabolites. At the beginning of lactation, the intake, milk yield, and urea and polyphenols contents in milk were lower than those recorded at the end of study (week 4). In contrast, the protein content was greater at the beginning of lactation.

3. Milk fatty acid (FA) profile was more related to changes in ruminal metabolism than to evolution of sainfoin FA profile. The presence of sainfoin PAC in the diet of dams increased C18:0, C18:2 n-6, C18:3 n-3, and total polyunsaturated FA (PUFA) n-6 and n-3, and decreased C18:1 t11, branched- and odd-chain fatty acid contents in milk. These results suggest that the inhibition of rumen biohydrogenation by sainfoin PAC seemed to occur in the early stages of this process.

4. The presence of sainfoin PAC in dams' diet did not affect the growth, blood metabolites, carcass, or meat quality of their suckling lambs.

5. The inclusion of dehydrated sainfoin in diet of finishing lambs caused a decrease of the estimated digestibility of the concentrates. However, despite the lower digestibility, the 40% sainfoin inclusion increased the intake, although this finding was not reflected on statistical differences concerning slaughter and carcass weights.

6. Ruminal fermentation was slightly affected by sainfoin inclusion in the diet, although it increased the acetic percentage in the rumen.

7. The inclusion of sainfoin in the finishing concentrate strongly affected the ruminal biohydrogenation. The rumen digesta showed an increase of C18:3 n-3 and a decrease of C18:1 t10 when sainfoin was included, regardless the level of inclusion. However, the highest C18:1 t11 and the lowest C18:2 n-6 proportions were only obtained with 40% sainfoin inclusion, showing a stronger t11 biohydrogenation pathway.

8. The plasma FA profile was barely affected by sainfoin inclusion in the concentrate. Differences among groups concerning PUFA in plasma were close to those obtained in rumen and meat, while the plasma t10/t11 ratio did not reflect the different rumen patterns obtained among diets.

9. The meat FA profile of lambs fed with 40% sainfoin inclusion presented greater percentages of PUFA n-3 and CLA c9,t11 and lower PUFA n-6/n-3 ratio.

10. From the present thesis, we can conclude that sainfoin with moderate content of PAC can be used as the main ingredient for dams' diet during lactation to produce suckling lamb without deleterious effects on performances. The inclusion of dehydrated sainfoin in the concentrate fed to finishing lambs had not negative effects on the performance and improved the meat FA profile due to changes in ruminal BH, especially when sainfoin was included at 40%.

# Conclusiones

Bajo las condiciones de los presentes estudios, se puede concluir:

1. La presencia de proantocianidinas (PAC) de la esparceta en la dieta de ovejas durante la fase de cría de un cordero lechal no afectó a su ingestión de materia seca, peso, condición corporal, parasitación o rendimiento lechero. Sin embargo, redujo las concentraciones de urea en leche y plasma, así como el contenido en polifenoles de la leche.

2. La semana de lactación afectó a todos los parámetros relacionados con el estado corporal, rendimiento lechero, composición de la leche y metabolitos de las ovejas durante el periodo de lactación estudiado. Al inicio de la lactación, la ingestión, rendimiento lechero y los contenidos de urea y polifenoles de la leche fueron menores a los obtenidos al final del estudio (semana 4). En cambio, la concentración de proteína de la leche fue superior al inicio de la lactación.

3. El perfil de ácidos grasos (AG) de la leche estuvo más ligado a cambios en el metabolismo ruminal que a la evolución en el contenido de AG de la esparceta. La presencia de PAC de esparceta en la dieta de las ovejas incrementó las concentraciones de C18:0, C18:2 n-6, C18:3 n-3 y AG poliinsaturados (AGPI) n-6 y n-3, mientras que redujo los porcentajes de C18:1 t11 y AG ramificados e impares en la leche. Estos resultados sugieren que la inhibición de la biohidrogenación ruminal debido a la presencia de PAC en la dieta tuvo lugar en fases iniciales del proceso.

4. La presencia de PAC de esparceta en la dieta de ovejas no afectó al crecimiento, metabolitos sanguíneos y calidad de la canal y de la carne de sus corderos lechales.

5. La inclusión de esparceta deshidratada en la dieta de corderos de cebo produjo una reducción de la digestibilidad estimada de los piensos. Sin embargo, pese a la menor digestibilidad, la inclusión de un 40% de esparceta en el pienso aumentó la ingestión de materia seca, aunque dicho resultado no se reflejó en diferencias estadísticas de los pesos al sacrificio y de la canal.

6. La fermentación ruminal de los corderos se vio escasamente afectada por la inclusión de esparceta en el pienso, aunque incrementó el porcentaje de ácido acético en el rumen.

7. La inclusión de esparceta en el pienso de cebo de corderos afectó en gran medida a la biohidrogenación ruminal. El contenido ruminal mostró un incremento de C18:3 n-3 y una disminución de C18:1 t10 ante la presencia de esparceta, independientemente del nivel de inclusión. Sin embargo, los mayores porcentajes de C18:1 t11 y los menores porcentajes de C18:2 n-6 sólo se obtuvieron con la inclusión de un 40% de esparceta, mostrando un efecto más marcado de la ruta t11 de biohidrogenación ruminal.

8. El perfil de AG del plasma se vio poco afectado por la inclusión de esparceta en el pienso. Las diferencias en los contenidos de AGPI del plasma mostraron una dinámica similar a la observada en el rumen y la carne, mientras que la ratio t10/t11 del plasma no reflejó los diferentes patrones de biohidrogenación observados en los diferentes grupos.

9. El perfil de AG de la carne de los corderos alimentados con una inclusión del 40% de esparceta en el pienso presentó mayores porcentajes de AGPI n-3 y CLA c9,t11 y una menor ratio de AGPI n-6/AGPI n-3.

10. A partir de la presente tesis, podemos concluir que la esparceta con un contenido moderado de PAC puede utilizarse como ingrediente principal de la dieta de las ovejas durante la fase de cría de un cordero lechal sin producir efectos perjudiciales sobre el rendimiento de los animales. La inclusión de esparceta deshidratada en el concentrado de corderos de cebo no presentó efectos negativos sobre el rendimiento y mejoró el perfil de AG de la carne a causa de los cambios ocurridos en la BH ruminal, especialmente cuando la esparceta se incluyó al 40%.

# Appendix

**Publications to which the Thesis has given rise (in chronological order)**

**Publications included in the Journal Citation Reports (ISI)**

**Baila, C., Joy, M., Blanco, M., Casasús, I., Bertolín, J. R., and Lobón, S. 2022.** Effects of feeding sainfoin proanthocyanidins to lactating ewes on intake, milk production and plasma metabolites. Animal, 16(1), 100438. https://doi.org/10.1016/j.animal.2021.100438

**Baila, C., Lobón S., Blanco, M., Casasús, I., Ripoll, G., and Joy. M. 2022**. Sainfoin in the dams' diet as a source of proanthocyanidins: effect on the growth, carcass and meat quality of their suckling lambs. Animals 2022, 12(4), 408; https://doi.org/10.3390/ani12040408

**Pelegrin-Valls, J., Álvarez-Rodríguez, J., Martín-Alonso, M. J., Ramírez, G. A., Baila, C., Lobon, S., Joy, M., and Serrano-Pérez, B. 2022.** Effect of Maternal Dietary Condensed Tannins from Sainfoin (*Onobrychis viciifolia*) on Gut Health and Antioxidant-Immune Crosstalk in Suckling Lambs. Agriculture, 12, 1694.doi:10.3390/agriculture12101694.

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

**Publications not included in the Journal Citation Reports (ISI)**

**Baila C., Joy M., Blanco M., Casasús I., Bertolín, JR. y Lobón S. 2020.** Interés de los taninos condensados de la esparceta en la alimentación de ovino en lactación. Albéitar, 235: 30-33.

**Baila, C., Joy, M., Blanco, M., Casasús, I., Domínguez, A., Legua, M. A., Bertolín, J. R. y Lobón, S. 2022**. Inclusión de la esparceta en la alimentación del ovino en lactación y cebo. Tierras, 38: 60-63.

**Baila, C., Lobón S., Blanco, M., Casasús, I., Ripoll, G., Bertolín, J. R. y Joy. M. 2022.** Efecto de la inclusión de esparceta en el pienso de cebo de ternascos. Mundo Ganadero, 309: 38-41.

**Contributions to congress conferences**

**Blanco, M., Baila, C., Joy, M., Bertolín, J.R., Casasús, I., and Lobón, S.** Effect of proanthocyanindins of sainfoin on secondary compounds in milk and suckling lamb meat. Poster: 71$^{th}$ Annual Meeting of the European Federation of Animal Science. 22-24 May 2019. Publication: Book of Abstracts: 175

**Lobón, S., Baila, C., Blanco, M., Casasús, I., Bertolín, J.R., and Joy, M.** Effect of condensed tannins of sainfoin on the fatty acid profile of ewe's milk and lamb's meat. Oral Communication. 71$^{th}$ Annual Meeting of the European Federation of Animal Science. 22-24 May 2019. Publication: Book of Abstracts: 175

**Pelegrin-Valls, J.; Álvarez-Rodríguez, J.; Aquilue, B.; Martín-Alonso, M.J.; Baila, C.; Lobon, S.; Joy, M., and Serrano-Pérez, B.** Effect of maternal dietary condensed tannins on coccidiosis and gut immunity in suckling lambs. Poster: 71th Annual Meeting of the European Federation of Animal Science. 22-24 May 2019. Publication: Book of Abstracts: 338

**Baila, C., Lobón, S., Blanco, M., Casasús, I., Ripoll, G. and Joy, M.** Inclusión de esparceta en la dieta de ovejas en lactación: efectos sobre los rendimientos, la canal y la carne del cordero lechal. Comunicación oral. XIX Jornadas sobre Producción Animal- Asociación Interprofesional para el Desarrollo Agrario (AIDA). Zaragoza, España. 1 y 2 de junio de 2021.

**Baila, C., Lobón, S., and Joy, J.** Inclusion of condensed tannins from sainfoin (*Onobrychis viciifolia*) in the rearing of light lambs. Poster. 1st Webinar of the Mediterranean Pasture and Forage. FAO-CIHEAM sub-network. 3 June 2021. Online.

**Lobón, S., Baila, C., Blanco, M., Casasús, I., and Joy, M.** Condensed tannins from Sainfoin (*Onobrychis viciifolia*): effects on *in vitro* ruminal parameters. Poster. 72th Annual Meeting of the European Federation of Animal Science. Davos, Switzerland. 30 August-3 September 2021. Book of Abstracts: 200.

**Baila, C., Joy, M., Blanco, M., Casasús, I., Ripoll, G., and Lobón, S.** Inclusion of sainfoin in the fattening concentrate: performance and carcass traits of light lambs. Oral Communication. 72th Annual Meeting of the European Federation of Animal Science. Davos, Switzerland. 30 August-3 September 2021. Book of Abstracts: 513.

**Baila, C., Joy, M., Casasús, I., Blanco, M., Bertolín, J. R., and Lobón, S.** Inclusion of sainfoin in lamb concentrate on blood metabolites and oxidation status. Poster, 73th Annual Meeting of the European Federation of Animal Science. Porto, Portugal. 2-5 September 2022. Book of Abstracts: 625.

**Baila, C., Joy, M., Blanco, M., Casasús, I., Ripoll, G., Bertolín, J. R., and Lobón, S.** Inclusion of sainfoin in the fattening concentrate: meat quality of light lambs. Oral Communication. 73th Annual Meeting of the European Federation of Animal Science. Porto, Portugal. 2-5 September 2022. Book of Abstracts: 622.

**Baila, C., Lobón, S., Bertolín, J.R., Alves, S.P., Bessa, R.J.B., Blanco, M., Casasús, I. y Joy, M.** Cambios en la biohidrogenación ruminal producidos por la inclusión de esparceta en el pienso de cebo de corderos. Comunicación oral. XX Jornadas sobre Producción Animal- Asociación Interprofesional para el Desarrollo Agrario (AIDA). Zaragoza, España. 13 y 14 de junio de 2023.

# Agradecimientos

**AGRADECIMIENTOS INSTITUCIONALES**

La presente tesis doctoral se encuentra englobada dentro del proyecto nacional "Optimización del uso de recursos proteicos mediterráneos en la cría de corderos ligeros" (INIA RTA2017-00080-C02-01), cuyo investigador principal fue la Dra, Margalida Joy Torrens y que se llevó a cabo de forma conjunta con la Universitat de Lleida (UdL). Gracias a la Agencia Estatal de Investigación y al Ministerio de Ciencia, Innovación y Universidades por hacer posible ese proyecto y por otorgarme la subvención destinada a la formación y contratación de personal investigador para la formación de doctores (PRE2018-086670) de la que he podido disfrutar durante los 4 años que ha durado esta etapa.

Gracias al Centro de Investigación y Tecnología Agroalimentaria del Gobierno de Aragón (CITA-Aragón), lugar en el que se ha llevado a cabo la presente Tesis Doctoral, por sus instalaciones, recursos y personal.

Gracias también a la Universidad de Zaragoza, en particular al Programa de Doctorado en Producción Animal, al que pertenece esta tesis. En particular, a Antonio de Vega, por ser mi tutor académico.

Por último, gracias al Servicio de Experimentación Animal (SAEA) de la Facultad de Veterinaria de Zaragoza por dejarnos usar el liofilizador cuando ha sido necesario.

**AGRADECIMIENTOS PERSONALES**

De lo primero que me di cuenta cuando pensaba en escribir los agradecimientos de la tesis es de lo mucho que me alegra tener a tanta gente a la que dedicarle unas palabras.

En primer lugar, a Marga y a Sandra por acompañarme durante el camino y trabajar duro durante estos años. Por confiar en mí y guiarme cuando lo necesitaba. Han sido muchas las horas de trabajo y de convivencia y me siento muy orgullosa del resultado. Habéis contribuido a que crezca en mí la pasión por la investigación.

Gràcies a la meua família, petita però preciosa. Als abuelos, que estarien molt orgullosos. A l'abuela Rosa per molts anys de companyia. A l'abuelo José Alberto per les seues històries, el seu amor per la cultura i per fer-me la seua secretària. A l'abuela

Conxa, per ser una de les millors persones que coneixeré al llarg de la meua vida, per la passió per la cuina i per desprendre amor i paciència (gràcies per les espelmes). Als meus pares, per deixar-me ser lliure per pensar i decidir sempre per mi mateixa, per ser un exemple de valors i fer-me créixer envoltada de llibres, cine i art. Mamà, cada vegada m'importa menys ser cada dia més pareguda a tu. Papà, gràcies per ensenyar-me a pensar i raonar amb calma i a "apretar les dents" per esforçar-me en el que faig. A Sara, per ser la meua millor amiga i el millor regal que m'ha donat la vida. Viure de la teua mà és preciòs. Als tios, per ser com els meus pares, estar presents sempre i ser persones meravelloses. A la tia Amparo, per la seua entrega i dedicació en tot el que fa. Al tio Fermín per transmetre'm la seua passió per la natura i per escoltar sempre. A Núria, por ser mi segunda hermana y haber crecido a mi lado, por muchos viajes (y los que quedan), confesiones y amor.

Al Tossal per donar-me la llibertat que necessitava per créixer. Per hores i hores a la tumba, al Mas Blanc, a la curva i al xiri. A les meues amigues María, Alba, Paula i Andrea, per demostrar-me que estimar-se és lo únic necessari per mantindre una relació d'amistat a quilòmetres de distància i per fer-me sentir que res ha canviat cada vegada que torno a Castelló. Sin sentiros tan cerca hubiera sido todo más complicado. María, volia dedicar-te una línea per a deixar escrit que em sento molt orgullosa de tenir-te. A les amistats que perduren amb el pas de les dècades i a les que estic molt agraïda: Laura, Ramón, Alma, María, Edu…

A todo el personal de la Casa Amarilla, por haberme hecho sentir arropada en todo momento. En especial a Isabel y Mireia por compartir su tiempo, correcciones y opiniones. Gracias también a Javi Álvarez y Dani Villalba por resolver las dudas y aportar su conocimiento. Al Laboratorio de Digestibilidad por su inmenso trabajo en esta tesis y por hacerme sentir en casa día tras día al cruzar vuestra puerta. A Angelines por hacer de madre y recibirme siempre con una sonrisa ("¡La chiquica!"). A Andrés por su bonita amistad y por ser una persona increíble. A Juanra por ser mi principal apoyo desde el primer día hasta el último, por alimentar mi pasión por el laboratorio (¡hasta por la química!), por confiar en mí pese a que siempre he sido un poco "manos de árbol" y por muchos consejos cuando andaba algo perdida. "Tú eres un soplo de aire fresco allá donde vas. No lo pierdas." A todo el personal del CITA, especialmente al personal de

campo, que, en algún momento dado, haya trabajado con muestras y/o animales de esta tesis. A Anabel y Arancha por su trabajo en los ensayos y en las muestras. A todos los becarios. En primer lugar, a Pablo, que me recibió con los brazos abiertos desde el primer día y me introdujo en el maravilloso mundo del "Sector Hostil". Eres una gran persona. Gracias por seguir presente pese a haberte ido lejos. A Agustí per la seva essència, per escoltar com ningú i per experiències multiaventura d'excursió i escalada combinades amb calimotxos compartits on sempre surts guanyant. A Kenza por su gran dulzura y su atención durante todos estos años. A Karina, por muchos momentos, incluyendo trabajo, cafés y hasta bailes. A Alejandro por bailoteos, risas y apoyo moral. A Enrique, por desistir ante mi "insistencia invasiva" y dejarse conocer. Has hecho que estos últimos años en el trabajo (y fuera de él) hayan sido infinitamente mejores. A las nuevas juventudes de becarios. A Alicia, siempre tan fresca, directa y alegre y con quien conecté desde el principio. A Olaia y Leire por aportar mucho en poco tiempo. Sois muy bonicas.

Special thanks to Rui and Susana for hosting me in Lisbon, teaching me so much in such a short time and contributing to my personal and professional growth.

Muchas gracias también a Aurora, que es lo mejor que me he llevado de mi estancia en Lisboa. Poder compartir contigo nuestro sprint final ha sido genial.

Thanks to Dr. Giuseppe Luciano and Dr. José M.B. Santos-Silva for the international review of this PhD.

A Adri, por convertirse en un pilar principal desde el mismo día que nos conocimos y no soltarme la mano desde entonces. La vida a tu lado está llena de luz, árboles, nubes, montañas, pájaros, música, fotos, series y cine.

Por último, a Cala, por ser un antídoto diario y compensar mi histeria, nervios y preocupaciones con una lista innumerable de cosas que no cabe aquí. Por haber compartido unos años maravillosos y por hacer de esta etapa un camino precioso.

# References

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

**Aerts, R. J., Barry, T. N., and McNabb, W. C. 1999**. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. *Agric Ecosyst Environ*. 75:1–12. doi:10.1016/S0167-8809(99)00062-6

**Akkari, H., Ben Salem, H., Gharbi, M., Abidi, S., and Darghouth, M. A. 2008.** Feeding *Acacia cyanophylla Lindl.* foliage to Barbarine lambs with or without PEG: Effect on the excretion of gastro-intestinal nematode eggs. *Anim Feed Sci Technol.* 147:182–192. doi :10.1016/j.anifeedsci.2007.09.017

**Aldai, N., de Renobales, M., Barron, L. J. R., and 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. doi:10.1002/ejlt.201370029

**Álvarez-Rodríguez, J., Sanz, A., Ripoll-Bosch, R., and Joy, M. 2010.** Do alfalfa grazing and lactation length affect the digestive tract fill of light lambs? *Small Rumin Res.* 94, 109–116. doi:10.1016/j.smallrumres.2010.07.009

**Álvarez-Rodríguez, J., Monleón, E., Sanz, A., Badiola, J. J., and Joy, M. 2012.** Rumen fermentation and histology in light lambs as affected by forage supply and lactation length. *Res Vet Sci.* 92:247–253. doi:10.1016/j.rvsc.2011.03.010.

**Álvarez-Rodríguez, J., Ripoll, G., Lobón, S., Sanz, A., Blanco, M., and Joy, M. 2018.** Alfalfa but not milk in lamb's diet improves meat fatty acid profile and α-tocopherol content. *Food Res. Int.* 107:708–716. doi:10.1016/j.foodres.2018.03.007.

**Álvarez-Rodríguez, J, Villalba, D., Molina, E., Serrano-Pérez, B., Bertolín, J. R., and Joy, M. 2020**. Do dietary condensed tannins affect the growth performance, fatty acid composition and lamb meat colour? *ITEA.* 116:116–130. doi:10.12706/itea.2019.024

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

**Alves, S. P., and 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. doi:10.1016/j.chroma.2009.04.079.

**Alves, S. P., Santos-Silva, J., Cabrita, A. R. J., Fonseca, A. J. M., and 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. doi:10.1371/journal.pone.0058386.

**Alves, S. P., and 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. doi:10.1007/s11745-014-3897-4.

**Alves, S. P., Francisco, A., Costa, M., Santos-Silva, J., and 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. doi:10.1016/j.anifeedsci.2017.01.018.

**Alves, S. P., Mendonça, S. H., Silva, J. L., and Bessa, R. J. B. 2018.** Nannochloropsis oceanica, a novel natural source of rumen-protected eicosapentaenoic acid (EPA) for ruminants. *Sci Rep*. 8. doi:10.1038/s41598-018-28576-7.

**Alves, S. P., Vahmani, P., Mapiye, C., McAllister, T. A., Bessa R. J. B., and Dugan, M. E. 2021.** *Trans*-10 18:1 in ruminant meats: A review. *Lipids.* 56:539–562. doi:10.1002/lipd.12324

**Amaral, A. B., da Silva, M. V., and da S. Lannes, S. C. 2018.** Lipid oxidation in meat: mechanisms and protective factors–a review. *Food Sci Technol.* 38:1–15. doi:10.1590/fst.32518

**AOAC. 2000.** Official Methods of Analysis. 17th Edition. Association of Official Analytical Chemist, editor. Arlington, VA, USA.

**AOCS. 2005.** Rapid Determination of Oil/Fat Utilizing High Temperature Solvent Extraction. Approved Procedure Am 5-04.

**Arroyo-López, C., Manolaraki, F., Saratsis, A., Saratsi, K., Stefanakis, A., Skampardonis, V., Voutzourakis, N., Hoste, H., and Sotiraki, S. 2014.** Anthelmintic effect of carob pods and sainfoin hay when fed to lambs after experimental trickle infections with *Haemonchus contortus* and *Trichostrongylus colubriformis*. *Parasite.* 21:71. doi:10.1051/parasite/2014074

**Assefa, A. D., Choi, S., Lee, J.-E., Sung, J.-S., Hur, O.-S., Ro, N.-Y., Lee, H.-S., Jang, S.-W., and Rhee, J.-H. 2019.** Identification and quantification of selected metabolites in differently pigmented leaves of lettuce (*Lactuca sativa L.*) cultivars harvested at mature and bolting stages. *BMC Chem*. 13:56. doi:10.1186/s13065-019-0570-2.

**Aufrère, J., Dudilieu, M., and Poncet, C. 2008.** *In vivo* and *in situ* measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Animal*. 2:1331–1339. doi:10.1017/S1751731108002450

**Azuhnwi, B. N., Boller, B., Dohme-Meier, F., Hess, H. D., Kreuzer, M., Stringano, E., and Mueller-Harvey, I. 2013.** Exploring variation in proanthocyanidin composition and content of sainfoin (*Onobrychis viciifolia*). *J Sci Food Agric*. 93:2102–2109. doi:10.1002/jsfa.6119

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

**Baila, C., Lobón, S., Blanco, M., Casasús, I., Ripoll, G., and Joy, M. 2022b.** Sainfoin in the Dams' Diet as a Source of Proanthocyanidins: Effect on the Growth, Carcass and Meat Quality of Their Suckling Lambs. *Animals*. 12:408. doi:10.3390/ani12040408.

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

**Banakar, P. S., Anand Kumar, N., Shashank, C. G., and Lakhani, N. 2018.** Physically effective fibre in ruminant nutrition: A review. *J Pharmacogn Phytochem.* 7:303–308.

**Bauchart, D. 1993.** Lipid Absorption and Transport in Ruminants. *J Dairy Sci.* 76:3864–3881. doi:10.3168/jds.S0022-0302(93)77728-0.

**Bauman, D. E., Mellenberger, R. W., and Derrig, R. G. 1973.** Fatty Acid Synthesis in Sheep Mammary Tissue. *J Dairy Sci*. 56:1312–1318. doi:10.3168/jds.S0022-0302(73)85352-4.

**Ben Salem, H., Nefzaoui, A., Ben Salem, L., and Tisserand, J. L. 1999.** Intake, digestibility, urinary excretion of purine derivatives and growth by sheep given fresh, air-dried or polyethylene glycol-treated foliage of *Acacia cyanophylla Lindl*. *Anim Feed Sci Technol*. 78:297–311. doi:10.1016/S0377-8401(98)00277-6

**Benchaar, C., McAllister, T. A., and Chouinard, P. Y. 2008.** Digestion, Ruminal Fermentation, Ciliate Protozoal Populations, and Milk Production from Dairy Cows Fed Cinnamaldehyde, Quebracho Condensed Tannin, or Yucca schidigera Saponin Extracts 1. *J Dairy Sci*. 91:4765–4777. doi:10.3168/jds.2008-1338.

**Bernués, A., Ripoll, G., and Panea, B. 2012.** Consumer segmentation based on convenience orientation and attitudes towards quality attributes of lamb meat. *Food Qual Prefer*. 26:211–220. doi:10.1016/j.foodqual.2012.04.008.

**Bertolín, J. R., Joy, M., and Blanco, M. 2019.** Malondialdehyde determination in raw and processed meat products by UPLC-DAD and UPLC-FLD. *Food Chem*. 298:125009. doi:10.1016/j.foodchem.2019.125009.

**Bessa, R. J. B., Portugal, P. V, Mendes, I. A., and Santos-Silva, J. 2005.** Effect of lipid supplementation on growth performance, carcass and meat quality and fatty acid composition of intramuscular lipids of lambs fed dehydrated lucerne or concentrate. *Livest Prod Sci.* 96:185–194. doi:10.1016/j.livprodsci.2005.01.017

**Bessa, R. J. B., Alves, S. P., and Santos-Silva, J. 2015.** Constraints and potentials for the nutritional modulation of the fatty acid composition of ruminant meat. *Eur J Lipid Sci Technol.* 117:1325–1344. doi:10.1002/ejlt.201400468.

**Black, A. D., Laidlaw, A. S., Moot, D. J., and O'Kiely, P. 2009.** Comparative growth and management of white and red clovers. *Irish J. Agric. Food Res.* 149–166.

**Blanco, C., Bodas, R., Prieto, N., Andrés, S., López, S., and Giráldez, F. J. 2014.** Concentrate plus ground barley straw pellets can replace conventional feeding systems for light fattening lambs. *Small Rumin Res.* 116, 137–143. doi:10.1016/j.smallrumres.2013.11.008.

**Blanco, C., Giráldez, F. J., Prieto, N., Benavides, J., Wattegedera, S., Morán, L., Andrés, S., and Bodas, R. 2015.** Total mixed ration pellets for light fattening lambs: Effects on animal health. *Animal*. 9:258–266. doi:10.1017/S1751731114002249.

**Blanco, M., Ripoll, G., Casasús, I., Bertolín, J. R., and Joy, M. 2019.** Carotenoids and tocopherol in plasma and subcutaneous fat colour to trace forage-feeding in growing steers. *Livest Sci.* 219:104–110. doi:10.1016/j.livsci.2018.12.003.

**Blanco, M., Ripoll, G., Lobón, S., Bertolín, J. R., Casasús, I., and Joy, M. 2021.** The inclusion of pea in concentrates had minor effects on the meat quality of light lambs. *Animals*. 11. doi:10.3390/ani11082385.

**Bocquier, F., Barillet, F., Guillouet, P., and Jacquin, M. 1993.** Prediction of energetic content of ewe's milk from different chemical analyses: proposal for a standard milk for dairy ewes. *Ann. Zootech.* 42 (1):57–66. doi:10.1051/animres:19930106

**Bonanno, A., Di Miceli, G., Di Grigoli, A., Frenda, A. S., Tornambè, G., Giambalvo, D., and Amato, G. 2011.** Effects of feeding green forage of sulla (*Hedysarum coronarium L*.) on lamb growth and carcass and meat quality. *Animal*. 5:148–154. doi:10.1017/S1751731110001576.

**Borreani, G., Peiretti, P., and Tabacco, E. 2003.** Evolution of yield and quality of sainfoin (*Onobrychis viciifolia Scop*.) in the spring growth cycle. *Agronomie*. 23:193–201. doi:10.1051/agro:2002082.

**Borton, R. J., Loerch, S. C., Mcclure, K. E., and Wulf, D. M. 2005.** Comparison of characteristics of lambs fed concentrate or grazed on ryegrass to traditional or heavy slaughter weights. I. Production, carcass, and organoleptic characteristics. *J Anim Sci*. 83:679–685. doi:10.2527/2005.833679x.

**Brainard, D. H. 2003.** Color appearance and color difference specification. In S. K. Shevell (Ed.). The science of color (pp.340). (2nd ed.). Oxford; Elsevier.

**Bravo-Lamas, L., Barron, L. J. R., Kramer, J. K. G., Etaio, I., and 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. doi:10.1016/j.meatsci.2016.02.043.

**Brunet, S., Aufrere, J., El Babili, F., Fouraste, I., and Hoste, H. 2007.** The kinetics of exsheathment of infective nematode larvae is disturbed in the presence of a tannin-rich plant extract (sainfoin) both *in vitro* and *in vivo*. *Parasitology*. 134:1253–1262. doi:10.1017/S0031182007002533

**Buccioni, A., Decandia, M., Minieri, S., Molle, G., and Cabiddu, A. 2012**Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. *Anim Feed Sci Technol*. 174:1–25. doi:10.1016/j.anifeedsci.2012.02.009.

**Buccioni, A., Pauselli, M., Viti, C., Minieri, S., Pallara, G., Roscini, V., Rapaccini, S., Marinucci, M. T., Lupi, P., and 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. doi:10.3168/jds.2014-8651.

**Butter, N. L., Dawson, J. M., Wakelin, D., and Buttery, P. J. 2000.** Effect of dietary tannin and protein concentration on nematode infection (*Trichostrongylus colubriformis*) in lambs. *J Agric Sci*. 134:89–99. doi:10.1017/S0021859699007315

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

**Calabrò, S., Tudisco, R., Balestrieri, A., Piccolo, G., Infascelli, F., and Cutrignelli, M. I. 2009.** Fermentation characteristics of different grain legumes cultivars with the *in vitro* gas production technique. *Ital J Anim Sci.* 8:280. doi:10.4081/ijas.2009.s2.280

**Calvete, C., R. Calavia, L. M. Ferrer, J. J. Ramos, D. Lacasta, and J. Uriarte. 2012.** Management and environmental factors related to benzimidazole resistance in sheep nematodes in Northeast Spain. *Vet Parasitol.* 184:193–203. doi:10.1016/j.vetpar.2011.08.020.

**Calvete, C., González, J. M., Ferrer, L. M., Ramos, J. J., Lacasta, D., Delgado, I., and Uriarte, J. 2020.** Assessment of targeted selective treatment criteria to control subclinical gastrointestinal nematode infections on sheep farms. *Vet Parasitol.* 277:109018. doi:10.1016/j.vetpar.2019.109018.

**Campidonico, L., Toral, P. G., Priolo, A., Luciano, G., Valenti, B., Hervás, G., Frutos P., Copani, G., Ginane, C., and 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. doi:10.2527/jas.2015-9922.

**Cannas, A., Pes, A., Mancuso, R., Vodret B., and Nudda, A. 1998.** Effect of dietary energy and protein concentration on the concentration of milk urea nitrogen in dairy ewes. *J Dairy Sci.* 81:499–508. doi:10.3168/jds.S0022-0302(98)75602-4

**Carrasco, S., Panea, B., Ripoll, G., Sanz, A., and Joy, M. 2009.** Influence of feeding systems on cortisol levels, fat colour and instrumental meat quality in light lambs. *Meat Sci*. 83:50–56. doi:10.1016/j.meatsci.2009.03.014.

**Cavallarin, L., Antoniazzi, S., Borreani, G., and Tabacco, E. 2005.** Effects of wilting and mechanical conditioning on proteolysis in sainfoin (*Onobrychis viciifolia Scop*) wilted herbage and silage. *J Sci Food Agric.* 85:831–838. doi:10.1002/jsfa.2022

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

**Chapagain, T., and Riseman, A. 2015.** Nitrogen and carbon transformations, water use efficiency and ecosystem productivity in monocultures and wheat-bean intercropping systems. *Nutr Cycl Agroecosyst*. 101:107–121. doi:10.1007/s10705-014-9647-4.

**Chauveau-Duriot, B., Doreau, M., Noziere, P., and Graulet, B. 2010.** Simultaneous quantification of carotenoids, retinol, and tocopherols in forages, bovine plasma, and milk: validation of a novel UPLC method. *Anal Bioanal Chem*. 397:777–790. doi:10.1007/s00216-010-3594-y

**Cherry, N. M., Bullinger, M., Lambert, B. D., Muir, J. P., Whitney, T. W., Miller, J. E., and Sawyer, J. T. 2014.** Feeding panicled tick-clover to growing goats reduces *Haemonchus contortus* infection without negative effects on growth. *J. Appl. Anim. Nutr.* 2, e15. doi:10.1017/jan.2014.9

**Chilliard, Y., Ferlay, A., Mansbridge, R. M., and Doreau, M. 2000.** Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. *Ann Zootech.* 49:181–205. doi:10.1051/animres:2000117

**Christie, W. W. 1981.** The effects of diet and other factors on the lipid composition of ruminant tissues and milk. In: Lipid Metabolism in Ruminant Animals. Elsevier. p. 193–226. doi:10.1016/B978-0-08-023789-3.50009-X

**Commission Regulation (EC) Nº 1107/96** of 12 June 1996 on the registration of geographical indication and designation of origin under the procedure laid down in Article 17 of Council Regulation (EEC) Nº 2081/92. *O J. Eur. Union.* 148:0001–0010.

**Conte, G., Dimauro, C., Serra, A., Macciotta N. P., and Mele, M. 2018.** A canonical discriminant analysis to study the association between milk fatty acids of ruminal origin and milk fat depression in dairy cows. *J Dairy Sci*. 101:6497–6510. doi:10.3168/jds.2017-13941.

**Copani, G., Niderkorn, V., Anglard, F., Quereuil, A., and Ginane, C. 2016.** Silages containing bioactive forage legumes: a promising protein-rich feed source for growing lambs. *Grass Forage Sci.* 71:622–631. doi:10.1111/gfs.12225

**Costa, M., Alves, S. P., Cabo, Â., Guerreiro, O., Stilwell, G., Dentinho, M. T., and 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. doi:10.1002/jsfa.7777.

**Delgadillo-Puga, C., Cuchillo-Hilario, M., León-Ortiz, L., Ramírez-Rodríguez, A., Cabiddu A., Navarro-Ocaña A., Morales-Romero A. M., Medina-Campos O. N., and Pedraza-Chaverri, J. 2019.** Goats' feeding supplementation with Acacia farnesiana pods and their relationship with milk composition: Fatty acids, polyphenols, and antioxidant activity. *Animals*. 9:515. doi:10.3390/ani9080515

**Delgado, I., Andrés, C., Muñoz, and F. 2008.** Effect of the environmental conditions on different morphological and agronomical characteristics of sainfoin. *Options Méditerranéennes*. 79:199–202.

**Dey, A., Dutta, N., Sharma, K., Pattanaik, A.K., 2008.** Effect of dietary inclusion of *Ficus infectoria* leaves as a protectant of proteins on the performance of lambs. *Small Rumin Res*. 75:105–114. doi:10.1016/j.smallrumres.2007.06.013

**Dıaz, M. T., Velasco, S., Caneque, V., Lauzurica, S., De Huidobro, F. R., Pérez C., González, J., and Manzanares, C. 2002.** Use of concentrate or pasture for fattening lambs and its effect on carcass and meat quality. *Small Rumin Res*. 43:257–268. doi:10.1016/S0921-4488(02)00016-0

**Doney, J. M., Peart, J. N., Smith, W. F., and Louda, F. 1979.** A consideration of the techniques for estimation of milk yield by suckled sheep and a comparison of estimates obtained by two methods in relation to the effect of breed, level of production and stage of lactation. *J Agric Sci.* 92:123–132. doi:10.1017/S0021859600060573.

**EEC 461/93. 1993.** Community Scale for the Classification of Carcasses of Light Lambs. Directorate-General for Agriculture and Rural Development (European Commission): Brussels, Belgium. Available from: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2002:0295:FIN:EN:PDF

**Eggleston, H. S., Buendia, L., Miwa, K., Ngara, T., and Tanabe, K. 2006.** IPCC guidelines for national greenhouse gas inventories.

**Farrer, M., Fulcer, G., Albers, C.J., Neil, H.A.W., Adams, P.C., and Alberti, K.G.M.M. 1995.** Patients undergoing coronary artery bypass graft surgery are at high risk of impaired glucose tolerance and diabetes mellitus during the first postoperative year. *Metabolism* 44:1016–1027. doi:10.1016/0026-0495(95)90099-3

**FEDNA. 2019.** Tablas FEDNA de composición y valor nutritivo de alimentos para la fabricación de piensos compuestos. Madrid: Fundación Española para el Desarrollo de la Nutrición Animal.http://www.fundacionfedna.org/tablas-fedna-composicion-alimentos-valor-nutritivo.

**Feeny, P. 1976.** Plant apparency and chemical defense. In: Wallace, J.W., Mansell, R.L. (eds) Biochemical Interaction Between Plants and Insects. Recent Advances in Phytochemistry, vol 10. Springer, Boston, MA. p.1-40. doi:10.1007/978-1-4684-2646-5_1

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

**Fimbres, H., Kawas, J. R., Hernández-Vidal, G., Picón-Rubio, J. F., and Lu, C. D. 2002.** Nutrient intake, digestibility, mastication and ruminal fermentation of lambs fed finishing ration with various forage levels. *Small Rumin Res*. 43:275–281. doi:10.1016/S0921-4488(02)00013-5.

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

**Frutos, P., Hervás, G., Natalello A., Luciano G., Fondevila, M., Priolo, A., and 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. doi:10.1016/j.anifeedsci.2020.114623.

**Fu, H., Xie, B., Ma, S., Zhu, X., Fan, G., and Pan, S. 2011.** Evaluation of antioxidant activities of principal carotenoids available in water spinach (*Ipomoea aquatica*). *J. Food Compos. Anal.* 24:288–297. doi:10.1016/j.jfca.2010.08.007

**Fusaro, I., Giammarco, M., Chincarini, M., Odintsov Vaintrub, M., Palmonari, A., Mammi, L. M. E., Formigoni, A., Di Giuseppe, L., and Vignola, G. 2020.** Effect of ewe diet on milk and muscle fatty acid composition of suckling lambs of the protected geographical origin abbacchio romano. *Animals*. 10:25. doi:10.3390/ani10010025.

**Gallardo, B., Gómez-Cortés, P., Mantecón, A. R., Juárez, M., Manso, T., and De La Fuente, M. A. 2014.** Effects of olive and fish oil Ca soaps in ewe diets on milk fat and muscle and subcutaneous tissue fatty-acid profiles of suckling lambs. *Animal*. 8:1178–1190. doi:10.1017/S1751731114000238

**Galles, K., Ham, J., Westover, E., Stratton, J., Wagner, J., Engle, T., and Bryant, T. C. 2011.** Influence of reduced nitrogen diets on ammonia emissions from cattle feedlot pens. *Atmosphere*. 2:655–670. doi:10.3390/atmos2040655

**Gargouri, A., Caja, G., Casals, R., and Mezghani, I. 2006.**Lactational evaluation of effects of calcium soap of fatty acids on dairy ewes. *Small Rumin Res*. 66:1–10. doi:10.1016/j.smallrumres.2006.03.004

**Gea, A., Stringano, E., Brown, R. H., and Mueller-Harvey, I. 2011.** *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *J Agric Food Chem.* 59:495–503. doi:10.1021/jf103609p.

**Giller, K., Sinz, S., Messadene-Chelali J., and Marquardt, S. 2021.** Maternal and direct dietary polyphenol supplementation affect growth, carcass and meat quality of sheep and goats. *Animal*. 15:100333. doi:10.1016/j.animal.2021.100333

**Giner-Chavez, B. I. 1996.** Condensed tannins in tropical forages. Thesis. Cornell University, Ithaca, NY.

**Girard, M., Dohme-Meier, F., Silacci, P., Ampuero Kragten, S., Kreuzer, M., and Bee, G. 2016.** Forage legumes rich in condensed tannins may increase n-3 fatty acid levels and sensory quality of lamb meat. *J Sci Food Agric*. 96:1923–1933. doi:10.1002/jsfa.7298.

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

**Gómez-Cortés, P., Guerra-Rivas, C., Gallardo, B., Lavín, P., Mantecón, A. R., De la Fuente, M. A., and Manso, T. 2018.** Grape pomace in ewes diet: Effects on meat quality and the fatty acid profile of their suckling lambs. *Food Res Int*. 113:36–42. doi:10.1016/j.foodres.2018.06.052

**Grabber, J. H., Zeller, W. E., and Mueller-Harvey I. 2013.** Acetone enhances the direct analysis of procyanidin-and prodelphinidin-based condensed tannins in Lotus species by the butanol–HCl–iron assay. *J Agric Food Chem*. 61:2669–2678. doi:10.1021/jf304158m

**Gravador, R. S., Luciano, G., Jongberg, S., Bognanno, M., Scerra, M., Andersen, M. L., Lund, M. N., and Priolo, A. 2015.** Fatty acids and oxidative stability of meat from lambs fed carob-containing diets. *Food Chem*. 182:27–34. doi:10.1016/j.foodchem.2015.02.094

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

**Griinari, J. M., and Bauman, D. E. 1999.** Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. Advances in conjugated linoleic acid research. 1:180–200.

**Gross, G. G. 1999.** G.G. Gross, Comprehensive Natural Product Chemistry, B.M. Pinto and S. Fraser, (Ed.), p. 799, Elsevier, Amsterdam, Vol. 3.

**Grosse Brinkhaus, A. G., Bee, G., Silacci, P., Kreuzer, M., and Dohme-Meier, F. 2016.** Effect of exchanging *Onobrychis viciifolia* and *Lotus corniculatus* for *Medicago sativa* on ruminal fermentation and nitrogen turnover in dairy cows. *J Dairy Sci*. 99:4384–4397. doi:10.3168/jds.2015-9911.

**Gruffat, D., Durand, D., Rivaroli, D., Do Prado, I. N., and Prache, S. 2020.** Comparison of muscle fatty acid composition and lipid stability in lambs stall-fed or pasture-fed alfalfa with or without sainfoin pellet supplementation. *Animal*. 14:1093–1101. doi:10.1017/S1751731119002507.

**Guyader, J., Janzen, H. H., Kroebel, R., and Beauchemin, K. A. 2016.** Forage use to improve environmental sustainability of ruminant production. *J Anim Sci.* 94:3147–3158. doi:10.2527/jas.2015-0141

**Hagerman, A. E., and Butler, L. G. 1981.** The specificity of proanthocyanidin-protein interactions. *J Biol Chem*. 256:4494–4497. doi:10.1016/S0021-9258(19)69462-7

**Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W., and Riechel, T. L. 1998.** High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J Agric Food Chem*. 46:1887–1892. doi:10.1021/jf970975b

**Harnly, J., Picklo, M. J., Kalscheur, K. F., Magnuson, A., Bukowski, M. R., Fukagawa, N. K., and Finley, J. W. 2022.** Deriving information from complex data sets: Impact of forage on fatty acids in cow milk. *J Food Compos Anal.* 107:104179. doi:10.1016/j.jfca.2021.104179.

**Hasanuzzaman, M., Araújo, S., and Gill, S. S. 2020.** The Plant Family Fabaceae. Springer Singapore.

**Hatew, B., Hayot Carbonero, C., Stringano, E., Sales, L. F., Smith, L. M. J., Mueller-Harvey I., Hendriks, W. H., and 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. doi:10.1111/gfs.12125

**Hatfield, P. G., Hopkins, J. A., Ramsey, S., and Gilmore, A. 1998.** Effects of level of protein and type of molasses on digesta kinetics and blood metabolites in sheep. *Small Rumin Res,* 28:161-170. doi:10.1016/S0921-4488(97)00085-0

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

**Hegarty, R. S., McFarlane, J. R., Banks, R., and Harden, S. 2006.** Association of plasma metabolites and hormones with the growth and composition of lambs as affected by nutrition and sire genetics. *Aust J Agric Res*. 57:683–690. doi:10.1071/AR05260

**Hennessy, A. A., Ross, R. P., Devery, R., and Stanton C. 2011**. The health promoting properties of the conjugated isomers of α-linolenic acid. *Lipids*. 46:105–119. doi:10.1007/s11745-010-3501-5

**Hervás, G., Pérez, V., Giráldez, F. J., Mantecón, A. R., Almar, M. M., and Frutos, P. 2003.** Intoxication of sheep with quebracho tannin extract. *J Comp Pathol*. 129:44–54. doi:10.1016/S0021-9975(02)00168-8

**Hoste, H., Torres-Acosta, J. F. J., Sandoval-Castro, C. A., Mueller-Harvey, I., Sotiraki, S., Louvandini, H., Thamsborg, S. M., and Terrill, T. H. 2015.** Tannin containing legumes as a model for nutraceuticals against digestive parasites in livestock. *Vet Parasitol.* 212:5–17. doi:10.1016/j.vetpar.2015.06.026

**Huyen, N. T., Verstegen, M. W. A., Hendriks, W. H., and Pellikaan W. F. 2020.** Sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations reduces ruminal biohydrogenation and increases transfer efficiencies of unsaturated fatty acids from feed to milk. *Anim Nutr*. 6:333–341. doi:10.1016/j.aninu.2020.05.001.

**Huyen, N. T., Verstegen, M. W. A., Hendriks, W. H., and Pellikaan W. F. 2020.** Antioxidant mechanism of grape procyanidins in muscle tissues: Redox interactions with endogenous ascorbic acid and α-tocopherol. *Food Chem*. 134:1767–1774. doi:10.1016/j.foodchem.2012.03.072.

**IPCC, 2022.** Climate Change 2022: Impacts, Adaptation and Vulnerability. Cambridge, UK and New York.

**Jenkins, T. C. 1993.** Advances in ruminant lipid metabolism. *J Dairy Sci.* 76:3851–3863. doi:10.3168/jds.S0022-0302(93)77727-9.

**Jenkins, T. C., Wallace, R. J., Moate, P. J., and Mosley, E. E. 2008.** Board-Invited Review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J Anim Sci*. 86:397–412. doi:10.2527/jas.2007-0588.

**Jerónimo, E., Alves, S. P., Dentinho, M. T. P., Martins, S. V., Prates, J. A. M., Vasta, V., Santos-Silva J., and Bessa, R. J. B. 2010.** Effect of grape seed extract, *Cistus ladanifer L*., and Vegetable oil supplementation on fatty acid composition of abomasal digesta and intramuscular fat of lambs*. J Agric Food Chem.* 58:10710–10721. doi:10.1021/jf1021626.

**Jerónimo, E., Alves, S. P., Alfaia, C. M., Prates J. A. M., Santos-Silva, J., and Bessa, R. J. B. 2011.** Biohydrogenation intermediates are differentially deposited between polar and neutral intramuscular lipids of lambs. *Eur J Lipid Sci Technol*. 113:924–934. doi:10.1002/ejlt.201000398.

**Jiménez-Escrig, A., Dragsted, L. O., Daneshvar, B., Pulido, R., and Saura-Calixto, F. 2003**. *In vitro* antioxidant activities of edible artichoke (*Cynara scolymus L*.) and effect on biomarkers of antioxidants in rats. *J Agric Food Chem*. 51:5540–5545. doi:10.1021/jf030047e.

**Jöbstl, E., O'Connel, J. l, Fairclough, J. P. A., and Williamson, M. P. 2004.** Molecular Model for Astringency Produced by Polyphenol/Protein Interactions. *Biomacromolecules*. 5:942–949. doi:10.1021/bm0345110.

**Johansen, M., Lund, P., and Weisbjerg, M. R. 2018.** Feed intake and milk production in dairy cows fed different grass and legume species: A meta-analysis. Animal. 12:66–75. doi:10.1017/S1751731117001215.

**Jones, W. T., Broadhurst, R. B., and Lyttleton J. W. 1976.** The condensed tannins of pasture legume species. *Phytochemistry*. 15:1407–1409. doi:10.1016/S0031-9422(00)97128-0

**Jonker, A., and Yu, P. 2017.** The occurrence, biosynthesis, and molecular structure of proanthocyanidins and their effects on legume forage protein precipitation, digestion and absorption in the ruminant digestive tract. *Int J Mol Sci*. 18:1105. doi:10.3390/ijms18051105

**Jordán, M. J., Moñino, M. I., Martínez, C., Lafuente, A., and Sotomayor, J. A. 2010.** Introduction of distillate rosemary leaves into the diet of the Murciano-Granadina goat: Transfer of polyphenolic compounds to goats' milk and the plasma of suckling goat kids. *J Agric Food Chem*. 58:8265–8270. doi:10.1021/jf100921z

**Joy, M., Ripoll, G., and Delfa, R. 2008.** Effects of feeding system on carcass and non-carcass composition of Churra Tensina light lambs. *Small Rumin Res.* 78:123–133. doi:10.1016/j.smallrumres.2008.05.011.

**Joy, M., Sanz, A., Ripoll, G., Panea, B., Ripoll-Bosch, R., Blasco, I., and Álvarez-Rodríguez, J. 2012.** Does forage type (grazing vs. hay) fed to ewes before and after lambing affect suckling lambs performance, meat quality and consumer purchase intention? *Small Rumin Res.* 104:1–9. doi:10.1016/j.smallrumres.2011.09.048.

**Julkunen-Tiitto, R. 1985.** Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *J Agric Food Chem*. 33:213–217. doi:10.1021/jf00062a013

**Kafantaris, I., Kotsampasi, B., Christodoulou, V., Makri, S., Stagos, D., Gerasopoulos, K., Petrotos, K., Goulas, P., and Kouretas, D. 2018.** Effects of dietary grape pomace supplementation on performance, carcass traits and meat quality of lambs. *In Vivo*. 32:807–812. doi:10.21873/invivo.11311

**Kaneko, J. J., Harvey, J. W., and Bruss, M. L. 2008.** Clinical biochemistry of domestic animals. Academic Press, Amsterdam, The Netherlands.

**Karnezos, T. P., and Matches, A. G. 1993.** Fatty acid composition of dock-fat from lambs grazing alfalfa, sainfoin, wheatgrass. *J Food Qual*. 16:409–415. doi:10.1111/j.1745-4557.1993.tb00267.x

**Kingston-Smith, A. H., Edwards, J. E., Huws, S. A., Kim E. J., and Abberton, M. 2010.** Plant-based strategies towards minimising 'livestock's long shadow.' Proceedings of the Nutrition Society. 69:613–620. doi:10.1017/S0029665110001953

**Kishino, S., Takeuchi, M., Park, S. B., Hirata, A., Kitamura, N., Kunisawa, J., Kiyono, H., Iwamoto, R., Isobe, Y., Arita, M., Arai, H., Ueda, K., Shima, J., Takahashi, S., Yokozeki K., Shimizu, S., and Ogawa, J. 2013.** Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. *Proc Natl Acad Sci U S A.* 110:17808–17813. doi:10.1073/pnas.1312937110.

**Komáromyová, M., Petrič, D., Kucková, K., Batťányi, D., Babják, M., Dolinská, M. U., Königová, A., Barčák, D., Dvorožňáková, E., and Čobanová, K. 2022.** Impact of Sainfoin (*Onobrychis viciifolia*) Pellets on Parasitological Status, Antibody Responses, and Antioxidant Parameters in Lambs Infected with Haemonchus contortus. *Pathogens*. 11:301. doi:10.3390/pathogens11030301

**Kramer, J. K. G., Fellner, V., Dugan, M. E. R., Sauer, F. D., Mossoba, M. M., and 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. doi:10.1007/s11745-997-0156-3.

**Kraus, T. E. C., Yu, Z., Preston, C. M., Dahlgren, R. A., and Zasoski, R. J. 2003.** Linking chemical reactivity and protein precipitation to structural characteristics of foliar tannins. *J Chem Ecol.* 29:703–730. doi:10.1023/A:1022876804925

**Lamy, E., Rawel, H., Schweigert, F. J., Silva, F. C. E., Ferreira, A., Costa, A. R., Antunes, C., Antunes, Almeida, A. M., Coelho, A. V., and Sales-Baptista, E. 2011.** The effect of tannins on mediterranean ruminant ingestive behavior: The role of the oral cavity. *Molecules*. 16:2766–2784. doi:10.3390/molecules16042766.

**Landau, S., Friedman, S., Devash, L., and Mabjeesh, S. J. 2002.** Polyethylene glycol, determined by near-infrared reflectance spectroscopy, as a marker of fecal output in goats. *J Agric Food Chem.* 50:1374–1378. doi:10.1021/jf011346b

**Lead, C. 2020.** Climate and environmental change in the mediterranean basin–current situation and risks for the future. Union for the Mediterranean, Plan Bleu; UNEP/MAP: Marseille, France.

**Leal, L. N., Jordán, M. J., Bello, J. M., Otal, J., den Hartog, L. A., Hendriks, W. H., and Martín-Tereso, J. 2019.** Dietary supplementation of 11 different plant extracts on the antioxidant

capacity of blood and selected tissues in lightweight lambs. *J Sci Food Agric.* 99:4296–4303. doi:10.1002/jsfa.9662.

**Ledgard, S. F., and Steele, K. W. 1992.** Biological nitrogen fixation in mixed legume/grass pastures. *Plant Soil*. 141:137–153. doi:10.1007/BF00011314.

**Lee, M. R. F., Tweed, J. K. S., Kim, E. J., and Scollan, N. D. 2012.** Beef, chicken and lamb fatty acid analysis — a simplified direct bimethylation procedure using freeze-dried material. *Meat Sci*. 92:863–866. doi:10.1016/j.meatsci.2012.06.013.

**Leparmarai, P. T., Sinz, S., Kunz, C., Liesegang, A., Ortmann, S., Kreuzer, M., and Marquardt, S. 2019.** Transfer of total phenols from a grapeseed-supplemented diet to dairy sheep and goat milk, and effects on performance and milk quality. *J Anim Sci*. 97:1840–1851.

**Leroy, J.L., Vanholder, T., Van Knegsel, A.T., Garcia-Ispierto, I., and Bols, P.E. 2008**. Nutrient prioritization in dairy cows early postpartum: mismatch between metabolism and fertility? *Reprod Domest Anim*. 43:96–103. doi:10.1111/j.1439-0531.2008.01148.x

**Li, Y., Iwaasa, A. D., Wang, Y., Jin, L., Han, G., and Zhao, M. 2014.** Condensed tannins concentration of selected prairie legume forages as affected by phenological stages during two consecutive growth seasons in western Canada. *Can J Plant Sci*. 94:817–826. doi:10.4141/cjps2013-234

**Lobón, S., Sanz, A., Blanco, M., and Joy, M. 2016.** Effect of the type of forage (Grazing vs. Hay) and the inclusion of condensed tannins in ewe's diet on milk quality and suckling lamb's growth. In: CIHEAM. p: 269.

**Lobón, S., Blanco, M., Sanz, A., Ripoll, G., Bertolín, J. R., and Joy, M. 2017a.** 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. doi:10.2527/jas2017.1595.

**Lobón, S., Sanz, A., Blanco, M., and Joy, M. 2017b.** The type of forage and condensed tannins in dams' diet: Influence on meat shelf life of their suckling lambs. *Small Rumin Res.* 154:115–122. doi:10.1016/j.smallrumres.2017.08.005

**Lobón, S., Blanco, M., Sanz, A., Ripoll, G., and Joy, M. 2019a.** Effects of feeding strategies during lactation and the inclusion of quebracho in the fattening on performance and carcass traits in light lambs. *J Sci Food Agric*. 99:457–463. doi:10.1002/jsfa.9207.

**Lobón, S., Joy, M., Sanz, A., Álvarez-Rodríguez, J., and Blanco, M. 2019b**. The fatty acid composition of ewe milk or suckling lamb meat can be used to discriminate between ewes fed different diets. *Anim Prod Sci*. 59:1108–1118. doi:10.1071/AN18082

**Lobón, S., Joy, M., Casasús, I., Rufino-Moya, P. J., and Blanco, M. 2020.** Field pea can be included in fattening concentrate without deleterious effects on the digestibility and performance of lambs. *Animals*. 10. doi:10.3390/ani10020243.

**López-Andrés, P., Luciano, G., Vasta, V., Gibson, T. M., Biondi, L., Priolo, A., and Mueller-Harvey, I. 2013.** Dietary quebracho tannins are not absorbed, but increase the antioxidant capacity of liver and plasma in sheep. *Br J Nutr.* 110:632–639. doi:10.1017/S0007114512005703

**López-Andrés, P., Luciano, G., Vasta, V., Gibson, T. M., Scerra, M., Biondi, L., Priolo, A., and Mueller-Harvey, I. 2014.** Antioxidant effects of ryegrass phenolics in lamb liver and plasma. *Animal*. 8:51–57. doi:10.1017/S1751731113001821

**Lourenço, M., Van Ranst, G., Vlaeminck, B., De Smet, S., and Fievez, V. 2008.** Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. *Anim Feed Sci Technol.* 145:418–437. doi:10.1016/j.anifeedsci.2007.05.043.

**Luciano, G., Monahan, F. J., Vasta, V., Biondi, L., Lanza, M., and Priolo, A. 2009.** Dietary tannins improve lamb meat colour stability. *Meat Sci.* 81:120–125. doi:10.1016/j.meatsci.2008.07.006

**Luciano, G., Vasta, V., Monahan, F. J., López-Andrés, P., Biondi, L., Lanza M., and Priolo, A. 2011.** Antioxidant status, colour stability and myoglobin resistance to oxidation of *longissimus dorsi* muscle from lambs fed a tannin-containing diet. *Food Chem.* 124:1036–1042. doi:10.1016/j.foodchem.2010.07.070

**Maia, M. R. G., Chaudhary, L. C., Figueres, L., and Wallace R. J. 2007.** Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie Van Leeuwenhoek*. 91:303–314. doi:10.1007/s10482-006-9118-2.

**Maillard, M.-N., and Berset, C. 1995.** Evolution of Antioxidant Activity during Kilning: Role of Insoluble Bound Phenolic Acids of Barley and Malt. *J Agric Food Chem*. 43:1789–1793. doi:10.1021/jf00055a008.

**Makkar, H. P. S. 2003.** Quantification of tannins in tree and shrub foliages. A Laboratory Manual Food and Agriculture Organization of the United Nations/International Atomic Energy (FAO/IAEA), p: 49–53.

**Malau-Aduli, A. E. O., Siebert, B. D., Bottema, C. D. K., and Pitchford, W. S. 1997.** A comparison of the fatty acid composition of triacylglycerols in adipose tissue from Limousin and Jersey cattle. *Aust J Agric Res*. 48:715–722. doi:10.1071/A96083.

**Malisch, C. S., Lüscher, A., Baert, N., Engström, M. T., Studer, B., Fryganas, C., Suter, D., Mueller-Harvey I., and Salminen, J.-P. 2015.** Large variability of proanthocyanidin content and composition in sainfoin (*Onobrychis viciifolia*). *J Agric Food Chem.* 63:10234–10242. doi:10.1021/acs.jafc.5b04946

**Mangan, J. L., Vetter, R. L., Jordan, D. J., and Wright, P. C. 1976.** The effect of the condensed tannins of sainfoin (*Onobrychis viciaefolia*) on the release of soluble leaf protein into the food bolus of cattle [proceedings]. *Proc Nutr Soc*. 35:95A-97A.

**Manolaraki, F. 2011.** Propriétés anthelminthiques du sainfoin (*Onobrychis viciifolia*): Analyse des facteurs de variations et du rôle des composés phénoliques impliqués. Thesis. 491 Dissertation, INP, Université de Toulouse, Toulouse, France.

**MAPA. 2020.** Ministerio de Agricultura, Pesca y Alimentación. Anuario de estadísticas agrarias. https://www.mapa.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2020/default.aspx?parte=3&capitulo=07&grupo=5&seccion=11.

**MAPA. 2021.** Ministerio de Agricultura, Pesca y Alimentación. Caracterización del sector ovino y caprino en España. https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/caracterizacionovinoycaprinocarnedatos2021_tcm30-579776.pdf

**Martin, K. M., McGilchrist, P., Thompson, J. M., and Gardner, G. E. 2011**. Progeny of high muscling sires have reduced muscle response to adrenaline in sheep. *Animal*. 5:1060–1070. doi:10.1017/S1751731110002764.

**McDonald, M., Mila, I., and Scalbert, A. 1996.** Precipitation of metal ions by plant polyphenols: Optimal conditions and origin of precipitation. *J Agric Food Chem*. 44:599–606. doi:10.1021/jf950459q.

**McMahon, L. R., McAllister, T. A., Berg, B. P., Majak, W., Acharya, S. N., Popp, J. D., Coulman B. E., Wang, Y., and Cheng K.-J. 2000.** A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Can J Plant Sci.* 80:469–485. doi:10.4141/P99-050

**McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. O. 2001.** Microbial interactions with tannins: nutritional consequences for ruminants. *Anim Feed Sci Technol*. 91:83–93. doi:10.1016/S0377-8401(01)00232-2

**Mertens, D. R. 1983.** Using neutral detergent fiber to formulate dairy rations and estimate the net energy content of forages. Cornell Nutr. Conf. Cornell University, Ithaca, NY. pp 60--68.

**Min, B. R., Barry, T. N., Attwood, G. T., and McNabb, W. C. 2003.** The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Anim Feed Sci Technol.* 106:3–19. doi:10.1016/S0377-8401(03)00041-5.

**Min, B. R., Hart, S. P., Miller, D., Tomita, G. M., Loetz, E., and Sahlu, T. 2005.** The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. *Vet Parasitol*. 130:105–113. doi:10.1016/j.vetpar.2005.03.011.

**Molan, A. L., Waghorn, G. C., and McNabb, W. C. 2002.** Effect of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis in vitro*. *Vet Rec*. 150:65–69. doi:10.1136/vr.150.3.65

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

**Morrissey, P. A., Sheehy, P. J. A., Galvin, K., Kerry, J. P., and Buckley, D. J. 1998.** Lipid stability in meat and meat products. *Meat Sci*. 49:S73–S86. doi:10.1016/S0309-1740(98)90039-0

**Mottet, A., de Haan, C., Falcucci, A., Tempio, G., Opio, C., and Gerber, P. 2017.** Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Glob Food Sec*. 14:1–8.doi:10.1016/j.gfs.2017.01.001

**Mueller-Harvey, I., and McAllan, A. B. 1992.** Tannins: their biochemistry and nutritional properties. *Advances in plant cell biochemistry and biotechnology*. 1:151–217.

**Mueller-Harvey, I. 1999.** Tannins: their nature and biological significance. In: J. C. Caygill and I. Mueller-Harvey, editors. Secondary plant products: antinutritional and beneficial actions in animal feeding. Nottingham University Press, Nottingham, UK. p. 17–39.

**Mueller-Harvey, I., Bee, G., Dohme-Meier, F., Hoste, H., Karonen, M., Kölliker, R., Lüscher, A., Niderkorn, V., Pellikaan W. F., and Salminen, J.-P. 2019.** Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration, and diet composition. *Crop Sci*. 59:861–885. doi:10.2135/cropsci2017.06.0369.

**Naczk, M., Oickle, D., Pink D., and Shahidi, F. 1996.** Protein precipitating capacity of crude canola tannins: effect of pH, tannin, and protein concentrations. *J Agric Food Chem*. 44:2144–2148. doi:10.1021/jf960165k

**Natalello, A., Luciano, G., Morbidini, L., Valenti, B., Pauselli, M., Frutos, P., Biondi, L., Rufino-Moya, P. J., Lanza M., and Priolo, A. 2019.** Effect of Feeding Pomegranate Byproduct on Fatty Acid Composition of Ruminal Digesta, Liver, and Muscle in Lambs*. J Agric Food Chem*. 67:4472–4482. doi:10.1021/acs.jafc.9b00307.

**Niderkorn, V., Barbier, E., Macheboeuf, D., Torrent, A., Mueller-Harvey, I., and 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. doi:10.1016/j.anifeedsci.2019.114357.

**Niezen, J. H., Waghorn, T. S., Charleston, W. A. G., and Waghorn, G. C. 1995.** Growth and gastrointestinal nematode parasitism in lambs grazing either lucerne (*Medicago sativa*) or sulla (*Hedysarum coronarium*) which contains condensed tannins. *J Agric Sci.* 125:281–289. doi:10.1017/S0021859600084422

**Novobilský, A., Mueller-Harvey, I., and Thamsborg, S. M. 2011.** Condensed tannins act against cattle nematodes. *Vet Parasitol*. 182:213–220. doi:10.1016/j.vetpar.2011.06.003.

**Novobilský, A., Stringano, E., Hayot Carbonero, C., Smith, L. M. J., Enemark, H. L., Mueller-Harvey, I., and Thamsborg, S. M. 2013.** *In vitro* effects of extracts and purified tannins of sainfoin (*Onobrychis viciifolia*) against two cattle nematodes. *Vet Parasitol*. 196:532–537. doi:10.1016/j.vetpar.2013.03.024.

**Ntambi, J. M., and Miyazaki, M. 2004.** Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog Lipid Res*. 43:91–104. doi:10.1016/S0163-7827(03)00039-0.

**Nudda, A., Atzori, A. S., Correddu, F., Battacone, G., Lunesu, M. F., Cannas A., and Pulina, G. 2020.** Effects of nutrition on main components of sheep milk. *Small Rumin Res*. 184:106015. doi:10.1016/j.smallrumres.2019.11.001.

**Palmquist, D. L., St-Pierre, N., and Mcclure, K. E. 2004.** Tissue fatty acid profiles can be used to quantify endogenous rumenic acid synthesis in lambs*. J. Nutr.* 134:2407–2414.

2407–2414.**Palmquist, D. L. 2006.** Milk fat: Origin of fatty acids and influence of nutritional factors thereon. *Advanced dairy chemistry.* 2:43–92. doi:10.1007/0-387-28813-9_2

**Parodi, P. W. 2016.** Dietary guidelines for saturated fatty acids are not supported by the evidence. *Int Dairy J.* 52:115–123. doi:10.1016/j.idairyj.2015.08.007

**Pascual, A., Pineda-Quiroga C., Goiri I., Atxaerandio R., Ruiz R., and García-Rodríguez, A. 2019.** Effects of feeding UFA-rich cold-pressed oilseed cakes and sainfoin on dairy ewes' milk fatty acid profile and curd sensory properties. *Small Rumin Res*. 175:96–103. doi:10.1016/j.smallrumres.2019.04.009.

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

**Peng, K., Shirley, D. C., Xu, Z., Huang, Q., McAllister, T. A., Chaves, A. V., Acharya S., Liu, C., Wang, S., and Wang, Y. 2016.** Effect of purple prairie clover (*Dalea purpurea Vent*.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood

metabolites and ruminal fermentation in lambs fed total mixed rations. *Anim Feed Sci Technol*. 222:100–110. doi:10.1016/j.anifeedsci.2016.10.012

**Piluzza, G., Sulas, L., and Bullitta, S. 2014.** Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. *Grass Forage Sci.* 69:32–48. doi:10.1111/gfs.12053.

**Piñeiro-Vázquez, A. T., Canul-Solís, J. R., Alayón-Gamboa, J. A., Chay-Canul, A. J., Ayala-Burgos, A. J., Aguilar-Pérez, C. F., Solorio-Sánchez F. J., and Ku-Vera, J. C. 2015.** Potential of condensed tannins for the reduction of emissions of enteric methane and their effect on ruminant productivity. *Arch Med Vet*. 47:263–272. doi:10.4067/S0301-732X2015000300002

**Ponnampalam, E.N., Plozza, T., Kerr, M.G., Linden, N., Mitchell, M., Bekhit, A.E.-D.A., Jacobs, J.L., and Hopkins, D.L. 2017.** Interaction of diet and long ageing period on lipid oxidation and colour stability of lamb meat. *Meat Sci*. 129:43–49. doi:10.1016/j.meatsci.2017.02.008

**Porter, L. J. 1992.** Structure and Chemical Properties of the Condensed Tannins. In: R. W. Hemingway and P. E. Laks, editors. Plant Polyphenols: Synthesis, Properties, Significance. Springer US, Boston, MA. p. 245–258. doi:10.1007/978-1-4615-3476-1_14

**Prache, S., Aurousseau, B., Thériez, M., and Renerre, M. 1990.** Discoloration of sheep carcass subcutaneous fat. *INRA Prod. Anim*. 1990, 3, 275–285. doi:10.20870/productions-animales.1990.3.4.4386

**Prache, S., and Theriez, M. 1999.** Traceability of lamb production systems: carotenoids in plasma and adipose tissue. *Anim Sci*. 69:29–36. doi:10.1017/s1357729800051067.

**Priolo, A., Waghorn, G. C., Lanza, M., Biondi, L., and Pennisi, P. 2000.** Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: effects on lamb growth performance and meat quality. *J Anim Sci*. 78:810–816. doi:10.2527/2000.784810x

**Priolo, A., Micol, D., Agabriel, J., Prache, S., and Dransfield, E. 2002a.** Effect of grass or concentrate feeding systems on lamb carcass and meat quality. *Meat Sci.* 62:179–185. doi:10.1016/S0309-1740(01)00244-3.

**Priolo, A., Lanza, M., Bella, M., Pennisi, P., Fasone, V., Biondi, L. 2002b.** Reducing the impact of condensed tannins in a diet based on carob pulp using two levels of polyethylene glycol: Lamb growth, digestion and meat quality. *Anim Res.* 51:305–313. https://doi.org/10.1051/animres:2002026

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

**Priolo, A., Vasta, V., Fasone, V., Lanza, C. M., Scerra, M., Biondi L., Bella, M., and Whittington, F. M. 2009.** Meat odour and flavour and indoles concentration in ruminal fluid and adipose tissue of lambs fed green herbage or concentrates with or without tannins. *Animal*. 3:454–460. doi:10.1017/S1751731108003662

**Provenza, F. D., Burritt, E. A., Perevolotsky, A., and Silanikove, N. 2000.** Self-regulation of intake of polyethylene glycol by sheep fed diets varying in tannin concentrations. *J Anim Sci.* 78:1206–1212. doi:10.2527/2000.7851206x.

**Provorov, N. A., and Tikhonovich, I. A. 2003**. Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. *Genet Resour Crop Evol.* 50:89–99. doi:10.1023/A:1022957429160.

**Quijada, J., Drake, C., Gaudin, E., El-Korso, R., Hoste H., and Mueller-Harvey, I. 2018.** Condensed tannin changes along the digestive tract in lambs fed with sainfoin pellets or hazelnut skins. *J Agric Food Chem.* 66:2136–2142. doi:10.1021/acs.jafc.7b05538

**Ramos, J. J., Verde, M. T., Marca, M. C., and Fernandez, A. 1994**. Clinical chemical values and variations in Rasa Aragonesa ewes and lambs. *Small Rumin Res*, 13:133-139. doi:10.1016/0921-4488(94)90088-4

**Ramsay, A., Drake, C., Grosse Brinkhaus, A., Girard M., Copani, G., Dohme-Meier, F., Bee, G., Niderkorn, V., Mueller-Harvey, and I. 2015.** Sodium hydroxide enhances extractability and analysis of proanthocyanidins in ensiled sainfoin (*Onobrychis viciifolia*). *J Agric Food Chem*. 63:9471–9479. doi:10.1021/acs.jafc.5b04106

**Rana, M. S., Tyagi, A., Hossain, S. A., and Tyagi, A. K. 2012.** Effect of tanniniferous *Terminalia chebula* extract on rumen biohydrogenation,$\Delta^9$-desaturase activity, CLA content and fatty acid composition in *longissimus dorsi* muscle of kids. *Meat Sci.* 90:558–563. doi:10.1016/j.meatsci.2011.09.016

**Ran-Ressler, R. R., Khailova, L., Arganbright, K. M., Adkins-Rieck, C. K., Jouni, Z. E., Koren, O., Ley, R. E., Brenna, J. T., and Dvorak, B. 2011.** Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a neonatal rat model. *PLoS One.* 6. doi:10.1371/journal.pone.0029032.

**Reed, J. D. 1995**. Nutritional toxicology of tannins and related polyphenols in forage legumes. *J Anim Sci.* 73:1516–1528. doi:10.2527/1995.7351516x.

**Regos, I., and Treutter, D. 2010**. Optimization of a high-performance liquid chromatography method for the analysis of complex polyphenol mixtures and application for sainfoin extracts (*Onobrychis viciifolia*). *J Chromatogr A.* 1217:6169–6177. doi:10.1016/j.chroma.2010.07.075

**Ripoll, G., Joy, M., Muñoz, F., and Albertí, P. 2008**. Meat and fat colour as a tool to trace grass-feeding systems in light lamb production. *Meat Sci.* 80:239–248. doi:10.1016/j.meatsci.2007.11.025.

**Ripoll, G., Albertí, P., and Joy, M. 2012.** Influence of alfalfa grazing-based feeding systems on carcass fat colour and meat quality of light lambs. *Meat Sci*. 90:457–464. doi:10.1016/j.meatsci.2011.09.007.

**Ripoll, G., González-Calvo, L., Molino, F., Calvo, J. H., and Joy, M. 2013.** Effects of finishing period length with vitamin E supplementation and alfalfa grazing on carcass color and the evolution of meat color and the lipid oxidation of light lambs. *Meat Sci*. 93:906–913. doi:10.1016/j.meatsci.2012.09.017.

**Ripoll-Bosch, R. 2013.** Diagnóstico de los sistemas ganaderos ovinos en áreas desfavorecidas: caracterización productiva de la raza ojinegra de Teruel, análisis integrado de sostenibilidad y evaluación de la huella de carbono. Thesis. Universidad de Zaragoza, Zaragoza, Spain.

**Ripoll-Bosch, R., Joy, M., Sanz, A., Blasco, I., Ripoll, G., and Álvarez-Rodríguez, J. 2014.** Effect of concentrate supplementation and prolificacy on the productive and economic

performance of autochthonous sheep breeds fed forage-based diets. *Span J Agric Res*, 2014, 12 (4):1099-1104. doi:10.5424/sjar/2014124-6321

**Rivaroli, D., Prunier, A., Meteau, K., do Prado, I. N., and Prache, S. 2019**. Tannin-rich sainfoin pellet supplementation reduces fat volatile indoles content and delays digestive parasitism in lambs grazing alfalfa. *Animal*. 13:1883–1890. doi:10.1017/S1751731118003543.

**Robbins, C. T., Hagerman, A. E., Austin, P. J., McArthur, C., and Hanley, T. A. 1991.** Variation in mammalian physiological responses to a condensed tannin and its ecological implications. *J Mammal*. 72:480–486. doi:10.2307/1382130

**Rochette, P., and Janzen, H. H. 2005.** Towards a revised coefficient for estimating N 2 O emissions from legumes. *Nutr Cycl Agroecosyst*. 73:171–179. doi:10.1007/s10705-005-0357-9

**Rochon, J. J., Doyle, C. J., Greef, J. M., Hopkins, A., Molle, G., Sitzia, M., Scholefield, D., and Smith, C. J. 2004**. Grazing legumes in Europe: A review of their status, management, benefits, research needs and future prospects. *Grass Forage Sci.* 59:197–214. doi:10.1111/j.1365-2494.2004.00423.x.

**Rufino-Moya, P. J., Blanco, M., Bertolín, J. R., and Joy, M. 2019a.** 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. doi:10.1016/j.anifeedsci.2019.02.005

**Rufino-Moya, P. J., Blanco, M., Bertolín, J. R., and Joy, M. 2019b.** 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. doi:10.3390/ani9050197

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

**Rufino-Moya, P. J., Joy, M., Lobón, S., Bertolín, J. R., and Blanco, M. 2020.** Carotenoids and liposoluble vitamins in the plasma and tissues of light lambs given different maternal feedings and fattening concentrates. *Animals*. 10:1813. doi:10.3390/ani10101813

**Rufino-Moya, P. J., Bertolín, J. R., Blanco, M., Lobón, S., and Joy, M. 2022.** Fatty acid profile, secondary compounds and antioxidant activities in the fresh forage, hay and silage of sainfoin (*Onobrychis viciifolia*) and sulla (*Hedysarum coronarium*). *J Sci Food Agric.* 102:4736–4743. doi:10.1002/jsfa.11834.

**Russel, A. J. F., Doney, J. M., and Gunn, R. G. 1969**. Subjective assessment of body fat in live sheep. *J Agric Sci*. 72:451–454. doi:10.1017/s0021859600024874.

**Sackmann, J. R., Duckett, S. K., Gillis, M. H., Realini, C. E., Parks, A. H., and 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. *J Anim Sci.* 81:3174-3181. doi:10.2527/2003.81123174x

**Salminen, J., and Karonen, M. 2011.** Chemical ecology of tannins and other phenolics: we need a change in approach. *Funct Ecol.* 25:325–338. doi:10.1111/j.1365-2435.2010.01826.x

**Sánchez-Rangel, J. C., Benavides, J., Heredia, J. B., Cisneros-Zevallos, L., and Jacobo-Velázquez, D. A. 2013.** The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Anal Methods*. 5:5990–5999. doi:10.1039/C3AY41125G

**Santos, N. W., Santos, G. T. D., Silva-Kazama, D. C., Grande, P. A., Pintro, P. M., de Marchi, F. E., Jobim, C. C., and Petit, H. V. 2014.** Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape residue silage. *Livest Sci*. 159:37–45. doi:10.1016/j.livsci.2013.11.015.

**Santos-Silva, J., Bessa, R. J. B., and Santos-Silva, F. 2002.** Effect of genotype, feeding system and slaughter weight on the quality of light lambs: II. Fatty acid composition of meat. *Livest Prod Sci.* 77:187–194. doi:10.1016/S0301-6226(02)00059-3

**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., and 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. doi:10.1016/J.MEATSCI.2023.109098.

**Sañudo, C., Santolaria, M. P., Maria, G., Osorio, M., and Sierra, I. 1996.** Influence of carcass weight on instrumental and sensory lamb meat quality in intensive production systems. *Meat Sci.* 42:195–202. doi:10.1016/0309-1740(95)00026-7

**Sañudo, C., Sánchez, A., and Alfonso, M. 1998.** Small ruminant production systems and factors affecting lamb meat quality. *Meat Sci.* 49:S29–S64. doi:10.1016/S0309-1740(98)90037-7

**Sañudo, C., Alfonso, M., San Julián, R., Thorkelsson, G., Valdimarsdottir, T., Zygoyiannis, D., Stamataris, C., Piasentier, E., Mills, C., Berge, P., Dransfield, E., Nute, G. R., Enser, M., and Fisher, A. V. 2007**. Regional variation in the hedonic evaluation of lamb meat from diverse production systems by consumers in six European countries. *Meat Sci*. 75:610–621. doi:10.1016/j.meatsci.2006.09.009.

**Sanz, A., Álvarez-Rodríguez, J., Cascarosa, L., Ripoll, G., Carrasco, S., Revilla, R., and Joy, M. 2008**. Carcass characteristics of the commercial types of suckling lamb, light lamb and castrated lamb of Churra Tensina breed. *ITEA*. 104:42–57.

**Saratsis, A., Regos, I., Tzanidakis, N., Voutzourakis, N., Stefanakis, A., Treuter, D., Joachim, A., and Sotiraki, S. 2012.** *In vivo* and *in vitro* efficacy of sainfoin (*Onobrychis viciifolia*) against *Eimeria spp* in lambs. *Vet Parasitol*. 188:1–9. doi:10.1016/j.vetpar.2012.03.014

**Saratsis, A., Voutzourakis, N., Theodosiou, T., Stefanakis, A., and Sotiraki, S. 2016.** The effect of sainfoin (*Onobrychis viciifolia*) and carob pods (*Ceratonia siliqua*) feeding regimes on the control of lamb coccidiosis. *Parasitol Res*. 115:2233–2242. doi:10.1007/s00436-016-4966-9

**Scalbert, A. 1991.** Antimicrobial properties of tannins. *Phytochemistry*. 30:3875–3883. doi:10.1016/0031-9422(91)83426-L.

**Scalbert, A., and Williamson, G. 2000.** Dietary intake and bioavailability of polyphenols. *J Nutr.* 130:2073S-2085S. doi:10.1093/jn/130.8.2073S

**Schofield, P., Mbugua, D. M., and Pell, A. N. 2001.** Analysis of condensed tannins: a review. *Anim Feed Sci Technol.* 91:21–40. doi:10.1016/S0377-8401(01)00228-0.

**Schreurs, N. M., Lane, G. A., Tavendale, M. H., Barry, T. N., and McNabb, W. C. 2008**. Pastoral flavour in meat products from ruminants fed fresh forages and its amelioration by forage condensed tannins. *Anim Feed Sci Technol*. 146:193–221. doi:10.1016/j.anifeedsci.2008.03.002.

**Scollan, N. D., Hocquette, J. F. Nuernberg, K., Dannenberger, D., Richardson, I., and Moloney, A. 2006**. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Sci.* 74:17–33. doi:10.1016/j.meatsci.2006.05.002.

**Scollan, N. D., Dannenberger, D., Nuernberg, K., Richardson, I., MacKintosh, S., Hocquette, J. F., and Moloney, A. P. 2014.** Enhancing the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Sci*. 97:384–394. doi:10.1016/j.meatsci.2014.02.015.

**Scollan, N. D., Price, E. M., Morgan, S. A., Huws, S. A., and Shingfield, K. J. 2017.** Can we improve the nutritional quality of meat? *Proc Nutr Soc*. 76:603–618. doi:10.1017/S0029665117001112

**Seoni, E., Battacone, G., Silacci, P., Kragten, S. A., Chelali, J. M., Dohme-Meier, F., and Bee, G. 2018**. Effect of condensed tannins from Birdsfoot trefoil and dietary protein level on growth performance, carcass composition and meat quality of ram lambs. *Small Rumin Res*. 169:118–126. doi:10.1016/j.smallrumres.2018.07.021

**Shingfield, K. J., Reynolds, C. K., Hervás, G., Griinari, J. M., Grandison, A. S., and Beever, D. E. 2006.** Examination of the persistency of milk fatty acid composition responses to fish oil and sunflower oil in the diet of dairy cows. *J Dairy Sci*. 89:714–732. doi:10.3168/jds.S0022-0302(06)72134-8.

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

**Shingfield, K. J., Bonnet, M., and Scollan, N. D. 2013.** Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal*. 7:132–162. doi:10.1017/S1751731112001681

**Shrivastava, V. S., and Talapatra, S. K. 1962.** Pasture studies in Uttar Pradesh II. Use of some natural indicators to determine the plane of nutrition of a grazing animal. *Ind J Dairy Sci*. 15:154–160.

**Silanikove, N., Perevolotsky, A., and Provenza, F. D. 2001.** Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. *Anim Feed Sci Technol.* 91:69–81. doi:10.1016/S0377-8401(01)00234-6

**Silanikove, N., Landau, S., Or, D, Kababya, D., Bruckental, I., and Nitsan, Z. 2006.** Analytical approach and effects of condensed tannins in carob pods (*Ceratonia siliqua*) on feed intake, digestive and metabolic responses of kids. *Livest Sci.* 99:29–38. doi:10.1016/j.livprodsci.2005.05.018

**Smith, A. H., Zoetendal, E., and Mackie, R. I. 2005.** Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microb Ecol*. 50:197–205. doi:10.1007/s00248-004-0180-x

**Smith, S. B., and Crouse, J. D. 1984.** Relative contributions of acetate, lactate and glucose to lipogenesis in bovine intramuscular and subcutaneous adipose tissue. *J Nutr.* 114:792–800. doi:10.1093/jn/114.4.792

**Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., and Bahorun, T. 2005**. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat Res-Fund Mol M.* 579:200–213. doi:10.1016/j.mrfmmm.2005.03.023

**Stewart, M., Webster, J. R., Verkerk, G. A., Schaefer, A. L., Colyn, J. J., and Stafford, K. J. 2007**. Non-invasive measurement of stress in dairy cows using infrared thermography. *Physiol Behav.* 92:520-525. doi:10.1016/j.physbeh.2007.04.034

**Stienezen, M., Waghorn, G. C., and Douglas, G. B. 1996.** Digestibility and effects of condensed tannins on digestion of sulla (Hedysarum coronarium) when fed to sheep. *New Zealand J Agric Res.* 39:215–221. doi:10.1080/00288233.1996.9513180

**Stringano, E., Hayot Carbonero, C., Smith, L. M. J., Brown, R. H., and Mueller-Harvey, I. 2012.** Proanthocyanidin diversity in the EU "HealthyHay" sainfoin (*Onobrychis viciifolia*) germplasm collection. *Phytochemistry*. 77:197–208. doi:10.1016/j.phytochem.2012.01.013.

**Sukhija, P. S., and Palmquist, D. L. 1988.** Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J Agric Food Chem.* 36:1202–1206. doi:10.1021/jf00084a019.

**Terrill, T. H., Rowan, A. M., Douglas, G. B., and Barry, T. N. 1992.** Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *J Sci Food Agric*. 58:321–329. doi:10.1002/jsfa.2740580306.

**Theodoridou, K., Aufrère, J., Andueza, D., Pourrat, J., Le Morvan, A., Stringano, E., Mueller-Harvey, I., and Baumont, R. 2010.** Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. *Anim Feed Sci Technol*. 160:23–38. doi:10.1016/j.anifeedsci.2010.06.007

**Toral, P. G., Angulo, G. H., Missaoui, H., Llorente, S. A., García, F. J. G., Jellali, S., and Frutos, P. 2016**. Effects of a tannin-rich legume (*Onobrychis viciifolia*) on *in vitro* ruminal biohydrogenation and fermentation. *Span J Agric Res*, 14, e0602. doi:10.5424/sjar/2016141-8989.

**Toral, P. G., Monahan, F. J., Hervás, G., Frutos, P., and Moloney, A. P. 2018. Modulating** ruminal lipid metabolism to improve the fatty acid composition of meat and milk. Challenges and opportunities. *Animal*. 12:s272–s281. doi:10.1017/S1751731118001994

**Tricon, S., Burdge, G. C., Kew, S., Banerjee, T., Russell, J. J., Jones, E. L., Grimble, R. F., Williams, C. M., Yaqoob, P., and Calder, P. C. 2004.** Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. *Am J Clin Nutr*. 80:614-620. doi:10.1093/ajcn/80.3.614

**Ulbricht, T. L. V, and Southgate, D. A. T. 1991.** Coronary heart disease: seven dietary factors. *The lancet.* 338:985–992. doi:10.1016/0140-6736(91)91846-M.

**Vahmani, P., Mapiye, C., Prieto, N., Rolland, D. C., McAllister, T. A., Aalhus, J. L., and Dugan, M. E. R. 2015.** The scope for manipulating the polyunsaturated fatty acid content of beef: A review. *J Anim Sci Biotechnol.* 6. doi:10.1186/s40104-015-0026-z.

**Vahmani, P., Ponnampalam, E. N., Kraft, J., Mapiye, C., Bermingham, E. N., Watkins, P. J., Proctor, S. D., and Dugan, M. E. R. 2020.** Bioactivity and health effects of ruminant meat lipids. Invited Review. *Meat Sci.* 165. doi:10.1016/j.meatsci.2020.108114. doi:10.1016/j.meatsci.2020.108114

**Valderrábano, J., Calvete, C., and Uriarte, J. 2010.** Effect of feeding bioactive forages on infection and subsequent development of Haemonchus contortus in lamb faeces. *Vet Parasitol.* 172:89–94. doi:10.1016/j.vetpar.2010.04.018

**Valenti, B., Natalello, A., Vasta, V., Campidonico, L., Roscini, V., Mattioli, S., Pauselli, M., Priolo, A., Lanza, M., and Lucian, G. 2019**. Effect of different dietary tannin extracts on lamb growth performances and meat oxidative stability: comparison between mimosa, chestnut and tara. *Animal*. 13:435–443. doi:10.1017/S1751731118001556

**Van Ackeren, C., Steingaß, H., Hartung, K., Funk, R., and Drochner, W. 2009.** Effect of roughage level in a total mixed ration on feed intake, ruminal fermentation patterns and chewing activity of early-weaned calves with ad libitum access to grass hay. *Anim Feed Sci Technol*. 153:48–59. doi:10.1016/j.anifeedsci.2009.05.009.

**Van Soest, P. J., Robertson, J. B., and Lewis, B. A. 1991**. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. 74:3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2.

**Van Soest, P. J. 1994**. Nutritional Ecology of the Ruminant. 2nd Edition. Cornell University Press, Ed. Ithaca, NY, USA.

**Vasta, V., Nudda, A., Cannas, A., Lanza, M., and Priolo, A. 2008**. Alternative feed resources and their effects on the quality of meat and milk from small ruminants. *Anim Feed Sci Technol*. 147:223–246. doi:10.1016/j.anifeedsci.2007.09.020

**Vasta, V., Mele, M., Serra, A., Scerra, M., Luciano, G., Lanza, M., and Priolo, A. 2009a**. Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins. *J Anim Sci*. 87:2674–2684. doi:10.2527/jas.2008-1761.

**Vasta, V., Priolo, A., Scerra, M., Hallett, K. G., Wood, J. D., and Doran, O. 2009b**. 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. doi:10.1016/j.meatsci.2009.02.007

**Vasta, V., Makkar, H. P. S., Mele, M., and Priolo, A. 2009c.** Ruminal biohydrogenation as affected by tannins *in vitro*. *Br J Nutr.* 102:82–92. doi:10.1017/S0007114508137898

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

**Vasta, V., and Bessa, R. J. B. 2012.** Manipulating ruminal biohydrogenation by the use of plants bioactive compounds. In: Dietary Phytochemicals and Microbes. Vol. 9789400739260. Springer Editors, Netherlands. p. 263–284. doi:10.1007/978-94-007-3926-0_9

**Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C., and 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. doi:10.3168/JDS.2018-14985.

**Vázquez, C. V., Rojas, M. G. V., Ramírez, C. A., Chávez-Servín, J. L., García-Gasca, T., Martínez, R. A. F., García, O. P., Rosado, J. L., López-Sabater, C. M., and Castellote, A. I. 2015**. Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin–Ciocalteu method. *Food Chem*. 176:480–486. doi:10.1016/j.foodchem.2014.12.050

**Vieira, C., Guerra-Rivas, C., Martínez, B., Rubio, B., and Manso, T. 2022**. Effects of grape pomace supplementation on the diet of lactating ewes as compared to vitamin E on the meat shelf life of suckling lambs. *Meat Sci*. 184:108666. doi:10.1016/j.meatsci.2021.108666

**Villalba, J. J., Provenza, F. D., Hall, J. O., and Lisonbee, L. D. 2010.** Selection of tannins by sheep in response to gastrointestinal nematode infection. *J Anim Sci*. 88:2189–2198. doi:10.2527/jas.2009-2272

**Vlaeminck, B., Fievez, V. A. R. J., Fonseca, J. M., and Dewhurst, R. J. 2006.** Factors affecting odd-and branched-chain fatty acids in milk: A review. *Anim Feed Sci Technol.* 131:389–417. doi:10.1016/j.anifeedsci.2006.06.017.

**Waghorn, G. 1996.** Condensed tannins and nutrient absorption from the small intestine. In: Proc of the 1996 Canadian Society of Animal Science Annual Meeting, Lethbridge, Canada (Rode LM, ed.). p. 175–194.

**Wang, Y., Douglas, G. B., Waghorn, G. C., Barry, T. N., and Foote, A. G. 1996.** Effect of condensed tannins in Lotus corniculatus upon lactation performance in ewes. *J Agric Sci.* 126:353–362. doi:10.1017/S0021859600074918

**Waghorn, G. C., Douglas, G. B., Niezen, J. H., McNabb, W. C., and Foote, A. G. 1998.** Forages with condensed tannins-their management and nutritive value for ruminants. *Proceedings of the New Zealand Grassland Association.* 60:89–98. doi:10.33584/jnzg.1998.60.2315

**Waghorn, G. C., and Clark, D. A. 2006**. Greenhouse gas mitigation opportunities with immediate application to pastoral grazing for ruminants. *Int Congr Ser*. 1293:107–110. doi:10.1016/j.ics.2006.02.025.

**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. doi:10.1016/j.anifeedsci.2007.09.013.

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

**Werne, S., Perler, E., Maurer, V., Probst, J. K., Hoste, H., Drewek, A., and Heckendorn, F. 2013.** Effect of sainfoin (*Onobrychis viciifolia*) and faba bean (*Vicia faba*) on the periparturient rise in ewes infected with gastrointestinal nematodes. *Small Rumin Res*. 113:454–460. doi:10.1016/j.smallrumres.2013.03.022.

**Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., Garnett, T., Tilman, D., DeClerck, F., Wood, A. Jonell, M., Clark, M., Gordon, L. J., Fanzo, J., Hawkes, C., Zurayk, R., Rivera, J. A., De Vries, W., Majele Sibanda, L., Afshin, A., Chaudhary, A., Herrero,**

**M., Agustina, R., Branca, F., Lartey, A., Fan, S., Crona, B., Fox, E., Bignet, E., Troell, M., Lindahl, M., Singh, S., Cornell, S. E., Srinath Reddy, K., Narain, S., Nishtar, S., and Murray, C. J. L. 2019.** Food in the Anthropocene: the EAT–Lancet Commission on healthy diets from sustainable food systems. *The Lancet.* 393:447–492. doi:10.1016/S0140-6736(18)31788-4.

**Wolfe, R. M., Terrill, T. H., and Muir, J. P. 2008**. Drying method and origin of standard affect condensed tannin (CT) concentrations in perennial herbaceous legumes using simplified butanol-HCl CT analysis. *J Sci Food Agric*. 88:1060–1067. doi:10.1002/jsfa.3188

**Woods, V. B., and 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. doi:10.1016/j.livsci.2009.07.002.

**Zeller, W. E., Sullivan, M. L., Mueller-Harvey, I., Grabber, J. H., Ramsay, A., Drake, C., and Brown, R. H. 2015.** Protein precipitation behavior of condensed tannins from Lotus pedunculatus and Trifolium repens with different mean degrees of polymerization. *J Agric Food Chem.* 63:1160–1168. doi:10.1021/jf504715p

**Zeller, W. E. 2019.** Activity, purification, and analysis of condensed tannins: Current state of affairs and future endeavors. *Crop Sci*. 59:886–904. doi:10.2135/cropsci2018.05.0323

**Zhao, J., Pang, Y., and Dixon, R. A. 2010.** The mysteries of proanthocyanidin transport and polymerization. *Plant Physiol*. 153:437–443. doi:10.1104/pp.110.155432