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Crude protein and condensed tannins in lamb diets: productivity, metabolic status and gastrointestinal immune and antioxidant response

Jonathan Pelegrin Valls

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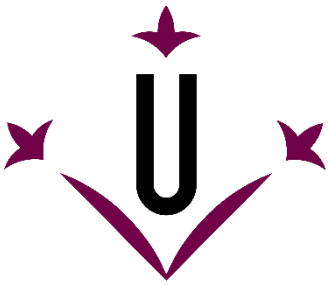

Universitat de Lleida

DOCTORAL THESIS

Crude protein and condensed tannins in lamb diets: productivity, metabolic status and gastrointestinal immune and antioxidant response

Jonathan Pelegrin Valls

Memoria presentada para optar al grado de Doctor por la Universitat de Lleida

Programa de Doctorado en Ciencia y Tecnología Agraria y Alimentaria

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Universitat de Lleida

Javier Álvarez Rodríguez i **Beatriz Serrano Pérez**, Doctors per la Universidad de Zaragoza i Professors Agregats al Departament de Ciència Animal de la Universitat de Lleida,

Javier Álvarez Rodríguez and Beatriz Serrano Pérez, Doctors by the University of Zaragoza and Lecturers at Animal Science Department of the University of Lleida,

CERTIFIQUEN/*CERTIFY*:

Que la memòria titulada "**Proteína bruta y taninos condensados en la dieta del cordero: productividad, estado metabólico y respuesta inmunitaria y antioxidante gastrointestinal"**, elaborada per **Jonathan Pelegrin Valls**, ha estat realitzada sota la direcció dels directors i reuneix les condicions exigides a la legislació vigent per optar al grau de Doctor en Ciència i Tecnologia Agrària i Alimentària per la Universitat de Lleida.

The thesis entitled "Crude protein and condensed tannins in lambs diet: Productivity, metabolic status, and gastrointestinal immune and antioxidant response," authored by Jonathan Pelegrin Valls, has been conducted under the guidance of the advisors and fulfills all the conditions required by the current legislation to qualify for the Doctorate degree in Agricultural and Food Science and Technology from the University of Lleida.

I per a què consti, signem el present,
Signed by,

This thesis has been developed as part of a Research Project funded by the Ministry of Science and Innovation of Spain and the European Regional Development Funds (Grant number: INIA RTA2017-00008-C1 and C2). The PhD student Jonathan Pelegrin Valls received an early-stage research grant by the Generalitat de Catalunya-European Social Funds (2019 FI_B 00376).

"Pot ser que ho doni tot.
I no ho aconsegueixi.
Que em quedi sense forces.
Que tot i així, segueixi."
Oques Grasses

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Sempre he pensat que no hi ha camins difícils sinó llocs i moments plens d'aprenentatges. Per això, considero que el coneixement és una font infinita d'enriquiment personal i, probablement, per aquest motiu em vaig aventurar a fer la tesi doctoral. Malgrat tot, aquests gairebé 4 anys no els he fet sol i ara ha arribat el moment de dedicar un espai d'aquest treball a totes aquelles persones que m'han acompanyat:

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I a totes les persones, que en menor o major mesura, m'han acompanyat en aquest projecte.

RESUM

Aquesta tesi doctoral es va plantejar per a avaluar els efectes de la reducció de la proteïna bruta i la inclusió de polpa de garrofa (com a font de tanins condensats) en la dieta del corder d'engreix intensiu, així com per entendre l'impacte de la trepadella fresca (també font de tanins condensats) en la dieta de les ovelles sobre la salut digestiva de corders lletons. Per aquest motiu, es van plantejar tres assajos en els quals es va estudiar la productivitat, el metabolisme nutricional i la resposta immunitària i antioxidant gastrointestinal.

En primer lloc, es va estudiar la reducció d'un 2% de la proteïna bruta a la fase de creixement (20% vs. 18%) i d'engreix (19% vs. 17%) dels corders, observant que aquesta disminució no va interferir en els resultats productius ni en el metabolisme proteic. Tanmateix, tampoc va afectar a l'estat oxidatiu o al balanç de la resposta antioxidant i immunitària intestinal, millorant la digestibilitat aparent de la matèria orgànica i del fòsfor. L'avaluació del mostreig a diferents hores del dia va reflectir que no existeix interacció entre el nivell de proteïna bruta i l'hora de mostreig sobre la digestibilitat de la matèria orgànica, de la proteïna bruta o del fòsfor, ni sobre la urea o la creatinina plasmàtica. No obstant això, durant la fase de creixement s'aprecien diferències d'estimació en la digestibilitat de la proteïna a les 8:00 a.m.

En segon lloc, es van incloure 150 g/kg i 300 g/kg de polpa de garrofa en la dieta del corder reflectint que aquest ingredient no va afectar a la productivitat dels animals ni al metabolisme energètic o proteic, encara que el nivell més alt d'inclusió va disminuir la digestibilitat dels nutrients i va incrementar l'índex de conversió. D'altra banda, la inclusió de polpa de garrofa va beneficiar la salut ruminal en reduir la queratinització, incrementar el gruix dels estrats vius de les papil·les del rumen i augmentar els enzims eliminadors de radicals lliures. Així mateix, aquest ingredient va mostrar un efecte coccidiostàtic en reduir l'excreció d'oocists fecals i regular la resposta immunitària-antioxidant gastrointestinal. No obstant això, en una situació d'estrès tèrmic, la inclusió de polpa de garrofa no tindria suficient influència per a generar un estat antioxidant d'adaptació positiva. En aquest sentit, l'època càlida va afectar negativament a la productivitat, a l'estat metabòlic (proteïnes totals i ferro), als paràmetres histològics del rumen i a la infecció per coccidis. La resposta antiinflamatòria i antioxidant va augmentar en els corders de l'estació càlida, la qual cosa, va demostrar una modulació dels efectes proinflamatoris i oxidatius associats a l'estrès tèrmic.

En tercer lloc, la inclusió de trepadella en la dieta de les mares dels corders lletons va promoure la reducció d'oocists de coccidis en la femta, però sense reflectir efectes negatius a l'anàlisi histopatològic del jejú i l'ili. L'activitat dels tanins condensats es va associar a una disminució de la resposta immunitària i d'enzims antioxidants en el jejú, probablement per la seva capacitat de neutralitzar radicals lliures, mentre que a l'ili va augmentar l'expressió d'enzims antioxidants involucrats en la degradació del peròxid d'hidrogen.

RESUMEN

Esta tesis doctoral se planteó para evaluar los efectos de la reducción de la proteína bruta y la inclusión de pulpa de algarroba (como fuente de taninos condensados) en la dieta del cordero ligero de cebo intensivo, así como entender el impacto de la esparceta fresca (también fuente de taninos condesados) en la dieta de las ovejas sobre la salud digestiva de corderos lechales. Para ello, se plantearon tres ensayos en los que se estudió la productividad, el metabolismo nutricional y, la respuesta inmunitaria y antioxidante gastrointestinal.

En primer lugar, se estudió la reducción de un 2% de la proteína bruta en la fase de crecimiento (20% vs. 18%) y engorde (19% vs. 17%) de los corderos, observando que esa disminución no interfirió en los resultados productivos ni en el metabolismo proteico. Además, tampoco afectó al balance de la respuesta antioxidante e inmunitaria intestinal, mejorando la digestibilidad aparente de la materia orgánica y del fósforo. La evaluación del muestreo a diferentes horas del día reflejó que no existe interacción entre el nivel de proteína bruta y la hora de muestreo sobre la digestibilidad de la materia orgánica, de la proteína bruta o del fósforo, ni sobre la urea o la creatinina plasmática. Sin embargo, durante la fase de crecimiento se aprecian diferencias de estimación en la digestibilidad de la proteína a las 8:00 a.m.

En segundo lugar, se incluyeron 150 g/kg y 300g/kg de pulpa de algarroba en la dieta del cordero reflejando que este ingrediente no afectó a la productividad de los animales ni en el metabolismo energético y proteico, aunque el nivel más alto de inclusión disminuyó la digestibilidad de los nutrientes e incrementó el índice de conversión. Por otra parte, la inclusión de pulpa de algarroba benefició la salud ruminal al reducir la queratinización, incrementar el grosor de los estratos vivos de las papilas del rumen y aumentar las enzimas eliminadoras de radicales libres. Asimismo, este ingrediente mostró un efecto coccidiostático al reducir la excreción de ooquistes fecales y regular la respuesta inmunitaria-antioxidante gastrointestinal. Sin embargo, en una situación de estrés térmico, la inclusión de pulpa de algarroba no tendría suficiente influencia para generar un estado antioxidante de adaptación positiva. En este sentido, la época cálida afectó negativamente a la productividad, el estado metabólico (proteínas totales y hierro), los parámetros histológicos del rumen y la infección por coccidios. La respuesta antinflamatoria y antioxidante aumentó en los corderos de la estación cálida, lo que demostró una modulación de los efectos proinflamatorios y oxidativos asociados al estrés térmico.

En tercer lugar, la inclusión de esparceta en la dieta de las madres de los corderos lechales promovió la reducción de ooquistes de coccidios en las heces, pero sin reflejar efectos negativos en los análisis histopatológicos del yeyuno e íleon. La actividad de los taninos condensados se asoció a una disminución de la respuesta inmunitaria y enzimas antioxidantes en el yeyuno, posiblemente por su capacidad de neutralizar radicales libres, mientras que en el íleon aumentó la expresión de enzimas antioxidantes involucradas en la degradación del peróxido de hidrógeno.

ABSTRACT

This doctoral thesis was designed to evaluate the effects of crude protein reduction and the inclusion of carob pulp (as a source of condensed tannins) in the diet of intensive fattening light lambs, as well as to understand the impact of fresh sainfoin (also a source of condensed tannins) in the diet of ewes on the gastrointestinal health of suckling lambs. For this purpose, three trials were conducted to study the productivity, the nutritional metabolism, and the immune and antioxidant responses in the gastrointestinal tract.

Firstly, a 2% reduction of crude protein in the growing (20% vs. 18%) and finishing (19% vs. 17%) phases of lambs were studied, observing that this reduction did not affect the productive performance or protein metabolism. Furthermore, it did not affect the intestinal antioxidant and immune response balance, and improved the apparent digestibility of organic matter and phosphorus. Evaluation of sampling at different times of the day did not show interaction between crude protein level and sampling time on digestibility of organic matter, crude protein or phosphorus, nor in plasma urea or creatinine. However, during the growing phase, differences between diets in protein digestibility at 08:00 a.m. were found.

Secondly, an inclusion of 150 g/kg and 300 g/kg of carob pulp in the lamb did not impact on animal productivity, energy and protein metabolism, although the highest level of inclusion decreased nutrient digestibility and increased the feed conversion ratio. On the other hand, the inclusion of carob pulp improved rumen health by reducing keratinisation, increasing the thickness of the living layers of the rumen papillae and increasing free radical scavenging enzymes. In addition, there was a coccidiostatic effect by reducing faecal oocyst excretion and regulating the gastrointestinal immune-antioxidant response. However, under heat stress, the inclusion of carob pulp would not have enough influence to generate a positive adaptive antioxidant status. In this regard, the warm season had a negative impact on productivity, metabolic status (total protein and iron), rumen histological parameters and coccidial infection. The anti-inflammatory and antioxidant response was increased in warm season lambs, which demonstrated a modulation of the pro-inflammatory and oxidative effects associated to heat stress.

Thirdly, the inclusion of sainfoin in dams' diets promoted a reduction of coccidial oocysts in faeces, but had no negative effects on histopathological analyses of the jejunum and ileum. Condensed tannin activity was associated to decreased immune response and antioxidant enzymes in the jejunum, probably because of its ability to scavenge free radicals, whereas in the ileum it increased antioxidant enzyme expression involved in hydrogen peroxide degradation.

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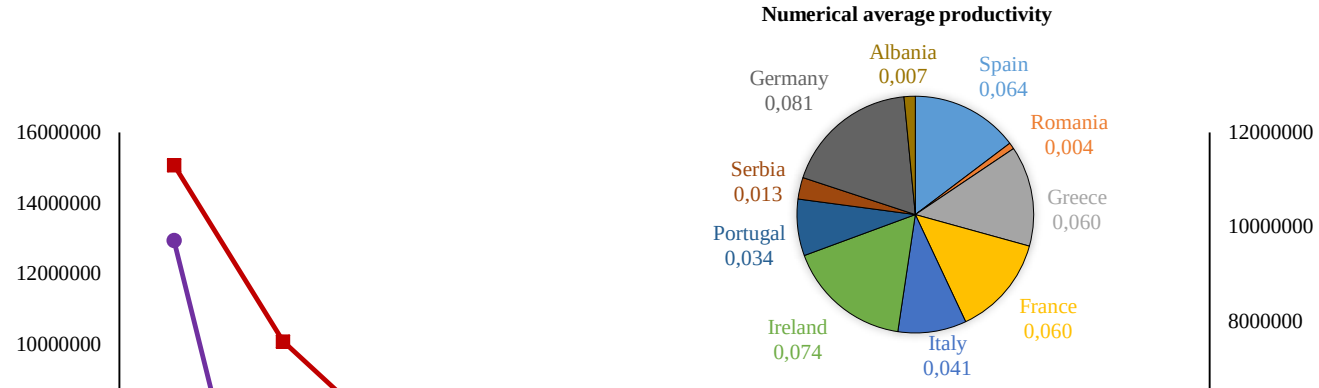
I. INTRODUCTION

1. Main sheep meat production systems in Spain.

Spain is the largest sheep producer in Europe, accounting for 25% of the total EU sheep population and 15,081,347 animals in 2021 (Figure 1). At territorial level, the Autonomous Communities with the largest number of sheep are Extremadura, Castilla y León, Castilla-La Mancha, Andalusia and Aragón (Eurostat, 2021; MAPA, 2021).



	Spain	Romania	Greece	France	Italy	Ireland	Portugal	Serbia	Germany	Albania
No. of animals	15081347	10087400	7253000	6994630	6728350	3991180	2237970	1695000	1508100	1480450
No. of slaughters	9711250	385790	4352710	4200090	2757910	2971810	756910	219350	1215400	99200

Figure 1. Top 10 European countries of sheep meat production, slaughterings of the selected countries and the average numerical productivity (ratio of number of slaughterings to number of animals) (Eurostat, 2021).

The sheep sector has an important role in rural development, ecosystem conservation and employment generation due to its great adaptability in disadvantaged areas. For this reason, sheep farming in Europe has a multitude production systems and feeding models (Ruiz et al., 2022), largely because of their interest in meat and milk production. In Spain meat lamb production is carried out using different feeding systems, but the most common are those for suckling and light lambs. Suckling lambs remain with their mothers feeding almost exclusively on maternal or artificial milk until they reach 9-12 kg body weight (BW) and producing carcasses of between 5-7 kg (Bello et al., 2015). In contrast, light lambs are mainly reared in two stages: the growing phase from weaning (10-12 kg BW) to 16-18 kg BW and the finishing phase, where the animals reach a target BW of approximately 21-25 kg, based on sex and breed conformation (Bello et al., 2015; Ruiz et al., 2022).

Fattening lambs under intensive systems is one of the most common methods used in Spanish meat

production. After weaning, the animals are fed with highly enriched diets based on concentrate and cereal straw which promote fast growth rates (González et al., 2016). These systems favour the commercial feedlots, where lambs from different farms are kept together, which generates various physiological changes that can compromise the immune system of lambs. Animal feedlots, weaning and feed changes, among other stressors, causes important economic losses in the sector due to the development of parasites such as coccidia (Carrau et al., 2018; González et al., 2016).

On the other hand, the physiological stage of the lamb influences the development of the gastrointestinal tract. For example, the transition from monogastric to ruminant phase is one of the most important developmental events for young ruminants. This is mainly because the morphological transition from preruminant to ruminant animal occurs gradually within the first 4 weeks of life. However, the rumen and reticulum only become completely functional at 8 weeks of age (Abdelsattar et al., 2021; Bush et al., 2019). Besides rumen development, there are also responses to dietary changes on the intestinal epithelium, immunity and metabolism (Baldwin et al., 2004; Celi et al., 2019). Therefore, these processes could be closely related as, firstly, the gastrointestinal tract protects the animal from toxicity, pathogens and environmental stressors, and secondly, it promotes the absorption of nutrients (Jiang et al., 2023; Steele et al., 2016). In this regard, in contrast to a forage diet, feeding high levels of concentrates leads to a lower pH and ruminal acidosis (Asín et al., 2021; Commun et al., 2009). This acidosis may be observed acutely or subacutely, being the subacute acidosis one of the most common nutritional metabolic disorders in small ruminants. The negative effects on production are seen by reduced feed intake and the appearance of diarrhoea, among other clinical signs (Asín et al., 2021; Han et al., 2021).

The feeding on intensive systems is the major part of the economic cost in livestock farms, which is why the price of ingredients should be carefully analysed. In 2022, feed for fattening lambs cost 0.39 €/kg on average (MAPA, 2023). According to RENGRATI (2022), this resulted in feed costs of around 2.40 €/kg of BW. On the other hand, the income from sales of suckling and light lambs on slaughter ranged between 3 €/kg of BW and 3.7 €/kg of BW, respectively (RENGRATI, 2022). This scenario requires an adequate control of the combination of ingredients and the nutrient profile of the concentrates to optimize the economic performance and lambs health.

2. Crude protein in light lambs.

In order to ensure an adequate body growth and development of lambs, it is essential to take into account crude protein (CP) requirements (INRA, 2018; Herath et al., 2021). Breed, sex, production phase and slaughter weight have an influence on the ingredients of the concentrates required to supply the nutrient requirements of lambs. Indeed, in Spain the CP content of feedstuffs varies between 15% and 21% (on dry matter) (Bello et al., 2015). Likewise, this wide range of concentration is related to growing and finishing phases, whose nutritional requirements are linked to the age of the animals.

Concentrate formulation manufacturers choose high concentrations of CP to assure the nutrient requirements and production performances of all breeds of lamb. However, some studies have found that feed conversion ratios remain stable with 13 to 18% CP concentrations (Bernard et al., 2019; Karim et al., 2003). This suggests that feed may contain a CP excess which, in part, has a negative impact on the production costs of farms and, indirectly, on environmental pollution. It is estimated that between 30% and 50% of the nitrogen intake might volatilise, especially through the loss of ammonia from excretions (Cole et al., 2006). Hence, there is a threshold above which lambs are unable to digest, transform and deposit ingested nitrogen in the muscle, which results in its excretion via urine and faeces.

To reduce the environmental impact of intensive production systems, animal nutritionists must understand how changes in formulations affect lamb performance and metabolism. Although BW and feed conversion ratio are useful parameters to understand the effect of variations in diets formulation, the analysis of blood metabolites and nutrient digestibility in faeces could help to assess dietary CP content more efficiently (INRA, 2018; Kohn et al., 2005; Zewdie, 2018). However, the circadian rhythm of some metabolites, such as urea, is regulated by an exogenous way depending on the input provided through feeding. Feeding strategies could change the timing of intake and, consequently, the nutrient assimilation and metabolite production (Nikkhah, 2011; Nikkhah, 2013). In conclusion, to understand the pattern and time effect of nutrient metabolism is critical to improve animal physiology and nutrition (Li et al., 2021).

In addition to the economic and environmental benefits of reducing CP in lamb diets, analysing how nutritional changes impact on the immune system of small ruminants is clearly another key point to understand the role of diet on immunity (Jaiswal et al., 2020). In other words, proinflammatory and anti-inflammatory cytokine production is closely related to nutrition (Montout et al., 2021; Trabi et al., 2020). For instance, changes in diet, malnutrition or parasitosis are precursors of the inflammatory response and, therefore, in order to respond optimally to immune challenges, the mechanisms that trigger the inflammatory response must be understood (Calder, 2013).

Cytokines are activated by sentinel cell signalling with the aim of regulating the immune response (Kany et al., 2019; Tizard, 2018). Thereby, proinflammatory cytokines would trigger immune activation and cytokines such as tumour necrosis factor α (TNF-α) or interferon γ (IFN-γ) would be synthesised and released.

When this occurs, transforming growth factor β1 (TGF-β1) and interleukin 10 (IL10) are mobilised as regulatory mechanisms of inflammation (Calder et al., 2009). Furthermore, another physiological mechanism that the gastrointestinal environment uses to maintain proper intestinal homeostasis is the Toll-like receptors (TLRs). Within this inflammatory regulatory group, TLR2 and TLR4 may be expressed in the rumen due to an increased growth rate of bacterial population induced by a high-concentrate diet (Liu et al., 2015; Malmuthuge et al., 2012).

3. Inclusion of legumes in lamb diets.

In European, the inclusion of legumes in animal feed is attracting a great deal of interest because of their sustainable approach and because they play a fundamental role in local agrosystems. Furthermore, through the appropriate production of these foods, biodiversity can be restored and soil fertility improved, as well as contributing to a 5-7-fold reduction in greenhouse gas emissions, as they can capture carbon from soils and/or fix atmospheric nitrogen (Beal et al., 2023; Rubio and Molina, 2016; Stagnari et al., 2017). While it is true that Spain has cultivated and consumed legumes since ancient times, their cultivation declined in favour of other crops that were technologically more profitable for the farmer (González-Bernal and Rubiales, 2016; Olmedilla-Alonso et al., 2010). However, leguminous plants, compared to gramineae, have flowers that promote the establishment of pollinating insect populations, which is of vital importance for the maintenance of plant multiplication (Potts et al., 2009). For all these reasons, and because the sheep sector is interconnected with the surrounding territory, the inclusion of alternative ingredients in sheep feed can be crucial to stimulate the circular economy and mitigate the current environmental crisis (Correddu et al., 2020; Ferreira et al., 2021; Pulina et al., 2018).

Legumes and their by-products are rich sources of bioactive components that are useful for maintaining the productivity, metabolism and health of small ruminants (Correddu et al., 2020; Singh et al., 2016). For example, carob pulp (*Ceratonia siliqua* L.) is a by-product from a legume traditionally cultivated in the Mediterranean area with condensed tannins (CT) in its structure (Basharat et al., 2023). Although carob pulp is not a good source of protein, carob is a tree with a high capacity to tolerate drought and sequester carbon to improve soil fertility (Batlle and Tous, 1997). However, the inclusion of large amounts of carob pulp in animal feed can reduce voluntary feed intake due to its astringent effect, generating a negative impact on ruminant performance (Correddu et al., 2020; Frutos et al., 2004). However, CT-rich ingredients may also have beneficial health properties such as antioxidant and anti-inflammatory capacity and the activation of genes that act as regulatory pathways of oxidative stress (Rodríguez-Solana et al., 2021; Soldado et al., 2021).

Sainfoin (*Onobrychis viciifolia*) is another legume with great agronomic, environmental, nutritional and nutraceutical attributes (Mora-Ortiz and Smith, 2018). Furthermore, it has a multitude of benefits for livestock as it is a good source of protein, does not cause tympanism, is palatable, contains CT and can help mitigate methane emissions and emissions from ammonia volatilisation (Kelln et al., 2020; Mora-Ortiz and Smith,

2018; Mueller-Harvey et al., 2019; Nawab et al., 2020). However, although CT prevent ruminal meteorism, they are considered anti-nutritional due to their ability to bind to proteins, enzymes and even microbial cells, thus disrupting ruminal digestion (Kelln et al., 2020). This conflict between the benefits and disadvantages of using sainfoin in ruminant diets raises questions about the physiological mechanisms regulating the digestive response. In addition, it should be added that suckling lambs are fed on maternal milk, so it would be necessary to know whether feeding sainfoin to dams is able to show positive effects on lambs. The inclusion of sainfoin in the diet of ewes leads to variations in milk production and composition (Baila et al., 2022a; Mueller-Harvey et al., 2019), but little literature has studied the effects of the transfer of metabolites such as polyphenols through milk. In fact, the immune effects of CT on dairy lambs remain unknown.

Most of the studies on legume use focus on animal productivity, gut health and environmental implications (Atiba et al., 2020; Baila et al., 2022b; Nawab et al., 2020). However, exploring the gene expression of the defence system when CTs are ingested may help to clarify the immunomodulatory impact of these components. As CT are key to antioxidant activity in ruminants (Soldado et al., 2021), it is evident that there is a mechanism acting at the level of the gastrointestinal tract that can promote the activation of antioxidant enzyme pathways. Among the defence mechanisms that are activated by polyphenolic compounds there are enzymes that regulate oxidative stress situations and modulate protein expression in the balance of antioxidant defence (Lee et al., 2017; Patra et al., 2019; Soldado et al., 2021).

The enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) use activation pathways through which they attempt to minimise the impact of reactive oxygen species (Ighodaro and Akinloye, 2017; Snyder and Silva, 2021). For example, superoxide radical (*O2), generated from endogenous processes or exogenous sources, is catalysed through SOD into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). In contrast, the CAT enzyme, which is present in most oxygen-using tissues, degrades and reduces H_2O_2 to water and O_2. Finally, GPX acts at the intracellular level by degrading H_2O_2 into water, as well as degrading lipid peroxides generated in cells (Ighodaro and Akinloye, 2017). Alongside these antioxidant enzymes, gene expression of the transcription factor (NRF2) plays an important homeostatic role, being inhibited when the organism is without oxidative stress and activated when stress is generated (Königsberg, 2007; Shi et al., 2020). Moreover, both peroxisome proliferator-activated receptor γ (PPARG) and nuclear factor kappa β (NF-kB) act as transcriptional regulators of inflammatory and oxidative processes in ruminants (Bhatt and Ghosh, 2014; Bionaz et al., 2013). PPARG regulates the expression of genes involved in different metabolic pathways, being a gene with a complex activity that can vary depending on the target tissue and physiological context. Besides having anti-inflammatory effects and modulating the immune response, it also modulates energy metabolism, regulating lipid synthesis and its accumulation in adipose tissue (Bionaz et al., 2013; Houseknecht et al., 2002; Lehrke and Lazar, 2005). Finally, like PPARG, NF-kB regulates its activity by a highly complex signalling pathway involving several metabolic processes which include in inflammation, as it promotes proinflammatory cytokines such as TNF-α by mediating their

production or, in animal antioxidant response, by triggers the expression of antioxidant enzymes such as SOD, CAT and GPX (Surai et al., 2021; Surai and Earle-Payne, 2022).

4. Global warming implications in ovine sector.

The planet is currently facing climate change with direct and indirect effects on animal and human populations. These include global warming affecting livestock performance and the production of protein sources such as meat and milk (Cheng et al., 2022). Although livestock is considered one of the drivers of climate change, it also generates up to 33% of the protein consumed worldwide (Thornton, 2010). Furthermore, the use of CT-rich dietary alternatives improves the energy use of feed and can therefore neutralise the production of greenhouse gases (Thompson and Rowntree, 2020). It is therefore essential to develop as many adaptation measures as necessary to support more sustainable animal production. In this regard, advances in feeding strategies could moderate the effects of global warming (Rojas-Downing et al., 2017), and the use of local feed sources that are highly adapted to the territory would reduce the risks associated with rising temperatures, such as the alteration of feed stocks (Cheng et al., 2022). The circular economy in feed and the ability of small ruminants to recycle food resources are presented as optimal strategies to fight environmental changes (Prado and del Prado, 2020; Velasco-Muñoz et al., 2021).

Legumes are a useful alternative for increasing protein content in ruminant diets and for the adaptation of the possible global shortage of protein sources in Europe (Prado and del Prado, 2020). Furthermore, the adjustment of protein concentration in lamb diets is also a determinant factor in reducing environmental pollution through nitrogen and ammonia losses (Abbasi et al., 2017). Probably, these two key points in animal feeding are factors that can improve environmental conditions for society. However, on a global level, the increase in environmental temperature also presents a detrimental scenario for small ruminants (Al-Dawood, 2017). In this regard, heat stress eventually leads to a pathological condition which directly affects animal productivity (Cheng et al., 2022).

Heat stress is one of the most influential effects on animal production. Generally, this occurs when temperatures rise above the animals' ability to dissipate heat that would keep them in a normal homeothermic status (Daramola et al., 2012). In sheep, the thermoneutrality is between 5 and 25°C depending on environmental and individual factors (Schütz, 2022), or when the temperature-humidity index (THI) is < 22.2 (Marai et al., 2001). Thus, global warming is the most important medium- and long-term challenge for small ruminant husbandry (Al-Dawood, 2017). This is in part because heat stress plays a major role in aspects such as productivity, growth, reproduction and behaviour, as well as on natural immunity, being a critical threshold between susceptibility to disease and death (Al-Dawood, 2017; Cheng et al., 2022; Escarcha et al., 2018; Joy et al., 2020). Furthermore, high temperatures induce changes in blood components such as protein synthesis and have consequences on biological pathways by activating the production of cellular reactive oxygen species (Abdelnour et al., 2019). In this regard, it has been suggested that besides the activation of this

oxidative stress pathway, heat stress could be increasing inflammatory signalling in the gastrointestinal tract as it is hypersensitive to these thermal changes (Abdelnour et al., 2019).

Feeding strategy management may represent an option to minimise the effects of heat stress on the tissues of the gastrointestinal tract. The epithelial tissue acts as a barrier between the external environment of the intestinal lumen and the animal organism itself, but it is also a space where heat dysbiosis can be generated, containing pathogens, antigens or food molecules, so it is not unusual for the digestive tract to express a multitude of genes associated with the immune response (Bush et al., 2019; Jiang et al., 2023). Therefore, readjusting protein levels and/or using ingredients with antioxidant capacity in the diet of lambs could act positively on nutrient digestive utilisation and post-absorption metabolism, reflecting the thermal resilience strategies of the ruminant (Abdelnour et al., 2019; Chauhan et al., 2014; Ellamie et al., 2020).

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II. OBJECTIVES

The main objective of this thesis was to study the restrictive effect of crude protein in light lambs' diets during the growing and finishing phases, as well as its circadian effect. Furthermore, the impact of the inclusion of legumes rich in condensed tannins was evaluated, on the one hand, in light lambs fed with carob pulp and, on the other hand, through the inclusion of sainfoin in dams' diets of suckling lambs.

To reach these general objectives, the following specific objectives were developed:

a) To evaluate the effects of reduce crude protein from 20-19% to 18-17% at growing-finishing phases, respectively, in light lambs on productive efficiency, blood metabolites related to protein and oxidative status and apparent nutrient digestibility. Furthermore, to evaluate oxidative status and expression of proinflammatory and regulatory cytokines in rumen and ileum tissues.

b) To study the viability of using a single faecal or blood sample to test the apparent digestibility of nutrients and blood metabolites related to the protein status of growing and finishing lambs fed different levels of crude protein concentration.

c) To evaluate the effects of carob pulp inclusion level (up to 30%) in concentrate pellets on animal performance and behaviour, total nutrient digestibility in the digestive tract and blood metabolic markers in light lambs of 15-25 kg body weight.

d) To analyse the effects of condensed tannins from carob pulp and rearing time on blood metabolism, coccidiosis and the relationship with immune and antioxidant homeostasis.

e) To investigate the effects of condensed tannins from fresh sainfoin administered to dams (using polyethylene glycol-PEG as a blocking agent in a control treatment) on selected markers of intestinal health and homeostasis in their suckling male lambs. To determine the effects on blood fructosamine, excretion of coccidial oocysts in faeces and expression of immune and antioxidant markers in jejunal and ileal tissues of suckling lambs at slaughter.

III. CHAPTER 1

Effect of dietary crude protein on productive efficiency, nutrient digestibility, blood metabolites and gastrointestinal immune markers in light lambs.

Abstract: This study hypothesized that reducing the level of crude protein (CP) in lambs′ feed may improve nutrient utilization and did not negatively affect their productive efficiency, blood metabolites, oxidative status (OS) or intestinal immune barrier function. A total of 120 weaned male Ripollesa lambs (45–60 days old and 15.0 ± 1.5 kg of body weight) were used. Four feed concentrates were formulated for two different phases (growing and finishing): CP20/19 group (20% and 19% of CP on dry matter basis, for each phase, respectively) and CP18/17 group (18% and 17% of CP on dry matter basis, for each phase, respectively). Lambs were randomly assigned to feeding treatments by balancing initial body weight between groups. The reduction of dietary CP level did not impair their growth performance parameters, while it did improve the apparent digestibility of organic matter. Furthermore, the lambs of the CP18/17 group showed lower plasma urea levels with no effect on OS (malondialdehyde levels) or gastrointestinal immunity markers (gene expression of interleukin 10 (*IL10*), tumor necrosis factor-α (*TNFA*) and transforming growth factor-β1 (*TGFB*)).

1. Introduction

Spain is the second largest producer of sheep meat in Europe, after the United Kingdom (Eurostat, 2019). In most Spanish farms, finishing lambs are raised until 20–25 kg of body weight (BW) by means of small framed breeds and intensive feeding systems based on concentrate and straw ad libitum. Dietary crude protein (CP) requirements are normally attained by including vegetable protein concentrates, such as soybean and rapeseed meal in feed, but a CP excess may increase the feed costs and the excreted nitrogen to the environment. Optimizing the use of feed ingredients in the lamb diet could improve the production efficiency, reduce feed costs and contribute to the mitigation of environmental emissions of ammonia (NH_3) and nitrous oxide (N_2O) (Cardenas et al., 2007; Hirstov et al., 2013), which may improve the fertilizer value of manure.

Currently, the Spanish feed industry formulates lamb concentrates containing between 15% and 21% of CP on a dry matter basis (DM) (Bello et al., 2016). In general, the recommended dietary CP for local Spanish sheep breeds ranges from 19 to 21% of CP on DM basis in starter feed (up to 14–16 kg BW) and 16% to 20% of CP on DM basis in growing-finishing diets, depending on the growth potential of the breed (autochthonous or improved crossbreds) and the energy density of the feed (Ferret et al., 2008). In contrast, in early-maturing improved breeds, dietary CP requirements on a DM basis would range from 11.7% to 12.8% during finishing until heavy weights (NRC, 2007). More recent studies have estimated that the dietary CP requirements would range from 16.2% to 15.1% on a DM basis in growing-finishing lambs between 15 and 25 kg of BW, respectively (INRA, 2018).

In addition, it is necessary to understand how the reduction of dietary CP affects the balance between the immune defense and tolerance mechanisms of the gastrointestinal tract. Low protein diets have been associated with states of oxidative stress that form free radicals and promote pro-inflammatory cytokines (Darmon et al., 1993). Consequently, the intestinal mucosa may be affected and develop an immune imbalance. In this case, anti-inflammatory cytokines would act as a tolerance mechanism (Flynn et al., 2008). An animal suffering from oxidative stress develops high levels of malondialdehyde (MDA) as a product of lipid peroxidation, which can be assessed in blood plasma as an indicator of oxidative status (OS) (Castillo et al., 2006).

This study evaluated the effects of the reduction of dietary CP in lambs on the productive efficiency, selected blood metabolites associated with protein (urea and creatinine) and oxidative (MDA) status and apparent nutrient digestibility. Furthermore, the OS and the expression of pro-inflammatory and regulatory cytokines in ruminal and ileal tissue were assessed.

2. Materials and methods

The animals were handled and slaughtered in accordance with the Spanish Animal Protection Regulations RD 53/2013, which complies with European Union Directive 2010/63 with regard to the protection of animals used for experimental and other scientific purposes. The lambs were raised in

commercial conditions following the Council Directive 98/58/EC concerning the protection of animals kept for farming purposes.

2.1. Animals, diets and experimental design

The experiment was conducted between January and May 2018 in the experimental facilities Nial of BonÀrea Agrupa, located in Guissona (Lleida, Catalonia, Spain, 41°46′32.2" N, 1°16′33.2" E; 484 m above sea level), where the lambs were kept in loose-housed sheds. The average monthly temperature for January, February, April and May was 5.3, 3.0, 11.7 and 14.4 °C, respectively.

A total of 120 weaned male breed Ripollesa lambs of 45–60 days old and 15.0 ± 1.5 kg of BW were used. The study was replicated in two equal batches of 60 lambs each: batch 1, from January to February (6 weeks) and batch 2, from April to May (6 weeks). The lambs of both batches were housed in 12 pens (5 animals/pen; 1.04 m^2 per animal) and were distributed in homogeneous groups according to their initial BW.

The lambs were submitted to a two-phase feeding program: growing (14 to 19 kg BW), which lasted 21 days in both batches, and finishing (19 to 25 kg BW), which lasted 21 ± 2 days in batch 1 and 18 ± 5 days in batch 2. Four diets with different levels of CP were formulated and supplied in two treatments: the CP20/19 group (n = 60), which was fed with 20% and 19% of CP on DM basis in the growing and finishing phase, respectively; and the CP18/17 group (n = 60), which was fed with 18% and 17% of CP on DM basis in the growing and finishing phase, respectively. Each pen was randomly assigned to one of the diets, taking into account that each treatment had the same initial BW and had half of the pens with small (14.0 ± 1.2 kg BW) and large (16.0 ± 1.1 kg BW) framed animals.

All diets were isoenergetic (1 UFC/kg) and they were formulated with the same ingredients and additives in the same manufacturing lot. Only the percentage of inclusion of vegetable protein concentrates was modified (Table 1). The growing phase diet included coccidiostatic (decoquinate). The feed presentation was granulated with a pellet diameter of 3.5 mm and the granulation temperature was 60 °C.

In the growing and finishing phase, in all pens, ad libitum water and cereal straw was offered, the nutritional value of which was: 90.5% of DM, 4.3% ash, 3.6% CP, 68.3% neutral-detergent fiber (NDF), 41.1% acid-detergent fiber (ADF) and 0.07% phosphorus (P).

Table 1. Ingredients and chemical composition of the experimental diets.

	Growing (14–19 kg of BW)		Finishing (19–25 kg of BW)	
	20% CP	18% CP	19% CP	17% CP
Ingredients				
Wheat	29.9	30.0	29.9	29.9
Barley	20.5	21.8	23.1	25.5
Maize	20.5	21.9	23.3	23.6
Soybean meal 47	16.0	13.3	10.7	7.9
Dry maize distillery grains	6.0	6.0	6.0	6.0
European rapeseed meal	3.0	3.0	3.0	3.0
Calcium carbonate	2.3	2.3	2.4	2.4
Salt	0.5	0.5	0.5	0.5
Ammonium chloride	0.5	0.5	0.5	0.5
Vitamin-mineral premix *	0.3	0.3	0.3	0.3
Oil/surfactant premix	0.2	0.2	0.2	0.2
Analyzed Chemical Composition				
Dry matter (DM, % on fresh matter)	88.6	89.2	90.9	90.4
Ash (% on DM basis)	5.6	5.9	5.4	5.5
Crude protein (% on DM basis)	20.4	18.3	19.1	17.4
Ether extract (% on DM basis)	2.87	2.67	2.81	2.69
Starch (% on DM basis)	49.2	49.9	52.2	55.9
Neutral-detergent fiber (% on DM basis)	14.5	13.6	14.2	13.8
Acid-detergent fiber (% on DM basis)	5.3	4.8	4.6	4.6
Phosphorus (% on DM basis)	0.42	0.45	0.42	0.42

* Ingredients of vitamin-mineral premix contained in each kilogram of feed: Vitamin A, 9900 UI; Vitamin D_3, 2250 UI; Vitamin E, 300 UI; Vitamin B1, 1 mg; Zn, 77.7 mg; Mn, 35.6 mg; Cu, 2.2 mg; Co, 0.96 mg; I, 0.62 mg; Se, 0.20 mg; BHT (E321), 62.5 mg and Propyl gallate (E310), 7.5 mg.

2.2. Feed intakes and growth performance

The offered concentrate and straw were recorded daily, and the refused quantities of both ingredients were recorded once weekly on a pen basis (6 replicates/group × 2 batches). Lambs were individually weighed once a week to calculate the average daily gain (ADG, g/day). The feed conversion rate (FCR, g/g) was calculated as the ratio between average daily concentrate intake and ADG. On the slaughter day, the lambs had water ad libitum and were fasted for 18–20 h. Pre-slaughter BW and carcass weight (kg) of half the lambs in each group (6 replicates/group × 2 batches) were recorded to calculate the carcass yield (%).

2.3. Blood samples

Blood samples were collected in vacuum tubes with EDTA (5 mL) (BD Vacutainer®, Becton, Dickinson and Company, Plymouth, UK) from the jugular vein of 2 randomly selected animals per pen (6 replicates/group × 2 batches). The samples were taken at 8:00 a.m. in the last week of each feeding phase. The same lambs were sampled during the growing and finishing phases. After extraction, the samples were centrifuged in situ (3000× *g* for 10 min) to obtain the plasma and stored at −20 °C until analysis.

Plasma concentrations of urea (mg/dL) and creatinine (mg/dL) were determined as indicators of protein metabolism. Both metabolites were determined with an automatic analyzer (GernonStar, RAL/TRANSASIA, Dabhel, India). Reagents were provided by the analyzer manufacturer. For urea quantification, the kinetic method based on the enzyme urease was used to catalyze the hydrolysis of urea into ammonia and carbon dioxide. The test had a measurement range between 2 and 350 mg/dL. The mean intra- and inter-assay coefficients of variation of the test were 2.8% and 2.7%, respectively. The creatinine as final by-product of the muscular metabolism, that originates from the creatine, was quantified by means of the enzymatic method. The creatinine measurement range was 0.03 to 50 mg/dL. The mean intra- and inter-assay coefficients of variation of the test were 3.1% and 5.1%, respectively.

Plasma samples in lambs were treated to determinate the total MDA (TMDA) as a result of the quantification of free MDA (FMDA) and protein-bound (PBMDA) separately following the procedure of Yonny et al. (2016). Proteins of plasma were precipitated with trichloroacetic acid and separated from the supernatant by centrifugation. The FMDA was determined in the supernatant while the PBMDA was determined in the pellet, in both cases after the reaction of this MDA with 2-thiobarbituric acid (TBA) in acid medium (with trichloroacetic acid) and high temperatures (≈100 °C) to form the adduct MDA-TBA_2 as indicated in Yonny et al. (2016). After this sample treatment, plasma concentrations of MDA as an oxidative biomarker (µM/L) was determined by liquid chromatography using an ACQUITY UPLC H-Class liquid chromatograph (Waters, Milford, Massachusetts, USA) equipped with a silica-based bonded phase column (Acquity UPLC HSS PFP, 100 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). The quantification of MDA was by fluorescence detection at $\lambda_{excitation}$ = 530 nm and $\lambda_{emission}$ = 550 nm (Bertolin et al., 2019). To quantify the MDA, an external linear curve calibration between 0.02 and 40 µM was used. More details of the chromatographic conditions used are described in Bertolin et al. (2019).

2.4. Feces, concentrate and straw samples

Feces samples (approximately 50 g) were collected at 8:00 a.m. by rectal stimulation at the end of each phase (growing and finishing) in at least 3 lambs from each pen to make a pool of feces (6 replicates/group × 2 batches). Concentrate samples, straw and feces were collected to estimate the apparent nutrient digestibility coefficients of organic matter (OM), CP and P. All samples were kept at −20 °C until analysis. After thawing, the samples were weighed and dried in a forced air stove at 60 °C for 72 h. The DM of the samples was determined by the weight difference between the fresh matter and the DM. After drying, the samples were mill-ground and stored in watertight plastic bags until analysis.

2.5. Calculation of apparent digestibility, estimation of fecal nitrogen volatilization and estimation of urine urea nitrogen

The total digestive tract apparent digestibility of CP, OM and P was calculated using the nutrient-to-marker ratio in the diet and feces, as follows:

$$\text{Apparent digestibility coefficient (\%)} = 100 - [100 \times (\text{Marker}_{diet}/\text{Marker}_{feces}) \times (Z_{feces}/Z_{diet})]$$

where Z_{feces} and Z_{diet} are the nutrient concentrations (%) in the feces and in the diet, respectively. The Marker_{feces} and Marker_{diet} are the concentrations (%) of acid insoluble ash (AIA) in the feces and in the diet, respectively. The Z_{diet} and Marker_{diet} were calculated by considering the amount of concentrate and straw consumed per pen.

The apparent volatilization of fecal nitrogen (N) was estimated from the change in the N:P proportions of diet and feces following the equation used by Todd et al. (2005):

$$\text{N volatilization (\% of intake)} = (\text{N:P diet} - \text{N:P feces})/(\text{N:P diet})$$

Urea nitrogen excreted in urine was estimated from blood urea concentrations according to the equation described in Institut National de la Recherche Agronomique (INRA, 2018):

$$\text{Urine ureic nitrogen (g N/d/kg of BW)} = (0.078 + 1.06 * \text{urea N})/100$$

where urea N (mg/mL) is the nitrogenous fraction of plasma urea concentrations.

2.6. Chemical analysis of food and feces

Feed and fecal DM (index no. 934.01), ash (index no. 942.05), ethereal extract (index no. 920.39) and starch (index no. 996.11) contents were determined according to AOAC methods (AOAC, 2019). NDF and ADF were analyzed with an Ankom200/220 (Ankom, 1998) following the sequential procedure described by Van Soest et al. (1991). The NDF of the concentrates was analyzed, including a stable amylase at high temperatures (aNDF), and all values of NDF and ADF were corrected by discounting their ash content. The CP content of the feed (N × 6.25) was determined following the DUMAS procedure, using a nitrogen and protein analyzer (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain). Phosphorus was determined by ultraviolet-visible spectroscopy (ICP-OES, HORIBA Jobin Yvon, Activa family, with AS-500 Autosampler, HORIBA Scientific, Madrid, Spain). Apparent digestibility of nutrients was estimated by AIA technique, which was determined following the procedure described in Álvarez-Rodríguez et al. (2017). All samples were analyzed in duplicate.

2.7. Histology and RT-qPCR analysis

Immediately after slaughter, ruminal and ileal tissues were collected for histology and quantitative real-time PCR (qPCR) analysis from four lambs from CP20/19 group and four lambs from CP18/17 group. For histological examination, 5-cm^2 samples from the cranial dorsal sac were taken, rinsed with phosphate-buffered saline solution (PBS) and fixed in a 10% formalin solution. For cytokine gene expression, several portions of ruminal tissue from the cranial dorsal sac and a 2-cm segment of the ileum proximal to ileocecal valve were divided, rinsed with PBS, incubated in RNAlater (Invitrogen, Madrid, Spain) and stored at −80 °C.

2.7.1. Histological and morphometric analysis

Formalin-fixed tissue samples were trimmed and processed according to standard histological procedures, and sections were stained with hematoxylin-eosin. From each sample, two sections with 5 or more papillae were examined. Slides were examined with a Motic BA310E microscope and digital pictures were taken at 40× magnification with a digital camera (Moticam 1080) to determine the variations of the epithelial keratinization degree (Scocco et al., 2013). Thickness of epithelium and keratin layer was measured at 10 different sites in each picture using the image processing and analysis software (Motic Images Plus 3.0 ML, Kowloon, Hong Kong). Epithelial keratinization degree was calculated as the proportion that represented the keratin layer respecting the thickness of epithelium.

2.7.2. Cytokine gene expression

Total RNA was extracted from 100 mg of ruminal and ileal tissues according to the method of Chomczynski and Sacchi (1987). Concentrations of RNA were determined spectrophotometrically. Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Complementary DNA was synthesized from 1 µg of total RNA in the presence of random primers using the RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

Messenger RNA expression was determined by qPCR for three target genes—interleukin 10 (*IL10*), tumor necrosis factor-α (*TNFA*) and transforming growth factor-β1 (*TGFB*). The genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) were used as housekeeping genes. Sequences of primers for *GAPDH* and *ACTB* have been described elsewhere (Puech et al., 2015). Primers for *IL10*, *TNFA*, *TGFB* were designed with the Primer3Plus tool and synthesized by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany) (Table 2). To avoid genomic contamination, all primers were designed to span an intron. For each gene, a standard curve was generated by amplifying serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplification was

conducted using the SYBR green method of the ABI PRISM 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) under the conditions specified by the manufacturer: an initial activation and denaturation step of 10 min at 95 °C followed by 40 cycles consisting of 10 s at 95 °C and 1 min at 60 °C. PCR reactions were run using 3 μL of 30-fold diluted cDNA as template in a total volume of 8 μL containing 1 × Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) as reported elsewhere (Serrano-Pérez et al., 2016). Each measurement was carried out in triplicate and the average used to calculate the relative gene amount. Data were normalized and analyzed by the $2^{-\Delta\Delta Ct}$ method using the mean Ct value obtained for the two reference genes and the Ct values for each cytokine primer (Schmittgen and Livak, 2008). The relative expression value was set to 1 for the CP20/19 group.

Table 2. Primer Sequences for *IL10*, *TGFB*, *TNFA*, *GAPDH* and *ACTB* used for quantitative Real-Time PCR.

Gene	Forward and Reverse primer (5′–3′)	bp	Access. No.	E (%)	n M	Source
GAPDH	F: ATCTCGCTCCTGGAAGATG R: TCGGAGTGAACGGATTCG	200	NM_001190390.1	1.90	600 300	Puech et al., 2015
ACTB	F: CTGGACTTCGAGCAGGAGAT R: GATGTCGACGTCACACTTC	194	NM_001009784	1.94	600	Puech et al., 2015
IL10	F: TTAAGGGTTACCTGGGTTGC R: TTCACGTGCTCCTTGATGTC	109	NM_001009327.1	1.96	200	Primer3plus
TGFB	F: TTGACGTCACTGGAGTTGTG R: CGTTGATGTCCACTTGAAGC	120	NM_001009400.2	2.04	200	Primer3plus
TNFA	F: CAAATAACAAGCCGGTAGCC R: TGGTTGTCTTTCAGCTCCAC	118	NM_001024860.1	1.96	200	Primer3plus

Note: F, Forward; R, Reverse; bp, amplified product length in base pairs; E (%), Efficiency; nM, Optimal primer concentration

2.8. Statistical analysis

For statistical analysis, individual data from 3 lambs in the CP18/17 group (one from batch 1 and two from batch 2) were removed due to death or pathological growth retardation. The data were analyzed with the statistical package JMP Pro13 (SAS Institute Inc. Cary, NC, USA). The growth and carcass performance, blood metabolites and nutrient digestibility data of individual animals were analyzed through mixed models with repeated measurements that included the treatment and the block (batch) as fixed effects, and the pen as a random effect. The feed intake, FCR and within-pen coefficient of variation of BW data of pens were analyzed with simple least squares models that included the same fixed effects as the mixed models. In all the evaluated variables, the pen was considered as the replicate (n = 12). The single interaction between the two fixed effects did not affect any parameter and was removed from the final model. The Student's t-test or one-way ANOVA test were used to compare relative cytokine gene expression and epithelial keratinization degree for fixed effects and their interactions. Least square means and their standard error are described. The separation of means was carried out with Tukey′s test. The level of significance was set at 0.05, but tendencies were commented on if the level of significance was below 0.10.

3. Results

The effects of experimental diets on the productive performance of lambs is shown in Tables 3−5. The initial and final BW of the growing and finishing phases, as well as the ADG, were similar for both phases and experimental groups (Table 3; $p > 0.05$).

Table 3. Effect of experimental diets on body weight (BW) and average daily gain (ADG).

Item	CP20/19	CP18/17	SE	*p*-Value
Growing (14 to 19 kg)				
Initial BW (kg)	15.0	15.0	0.15	0.97
Within-pen coefficient of variation of BW (%)	5.8	6.5	0.73	0.49
ADG (g)	235	234	13.0	0.92
Finishing (19 to 25 kg)				
Initial BW (kg)	19.8	19.7	0.28	0.75
Within-pen coefficient of variation of BW (%)	9.6	10.0	1.09	0.83
ADG (g)	254	269	8.3	0.23
Slaughter BW (kg)	24.5	24.8	0.24	0.40
Within-pen coefficient of variation of BW (%)	8.6	8.0	0.85	0.60

Note: CP20/19 group was supplied 20% of dietary CP on DM and 19% of CP on DM in growing and finishing phases, respectively, whereas CP18/17 group was supplied 18% of dietary CP on DM and 17% of CP on DM in growing and finishing phases, respectively. SE = Standard error.

No significant differences were observed between the CP20/19 and CP18/17 groups in the average daily concentrate and straw intake during the growing and finishing phases (Table 4; $p > 0.05$).

The different levels of CP used in the CP20/19 and CP18/17 groups did not affect the FCR of the growing phase (Table 4; $p > 0.05$). However, the FCR of the finishing phase showed a tendency to be higher in the CP20/19 group compared to the CP18/17 group (Table 4). The overall FCR for the CP20/19 and CP18/17 treatments were 3.34 and 3.17 g/g ± 0.09 ($p = 0.21$), respectively.

The dietary CP level of the experimental diets did not affect the carcass weight and carcass dressing (Table 5; $p > 0.05$).

Table 4. Effect of experimental diets on average daily concentrate and straw intake and feed conversion rate (FCR).

Item	CP20/19	CP18/17	SE	*p*-Value
Growing (14 to 19 kg)				
Concentrate intake (g/day)	715	710	22	0.89
Straw intake (g/day)	108	107	6	0.97
FCR (g/g)	3.11	3.06	0.13	0.82
Finishing (19 to 25 kg)				
Concentrate intake (g/day)	878	854	24	0.48
Straw intake (g/day)	125	125	8	0.95
FCR (g/g)	3.50^{x}	3.21^{y}	0.11	0.07

Note: CP20/19 group was supplied 20% of dietary CP on DM and 19% of CP on DM in growing and finishing phases, respectively, whereas CP18/17 group was supplied 18% of dietary CP on DM and 17% of CP on DM in growing and finishing phases, respectively. The FCR was calculated as the ratio between average daily concentrate intake and ADG. $^{x, y}$ = $p < 0.10$; SE = Standard error.

Table 5. Effect of experimental diets on slaughter weight, carcass weight and carcass dressing.

Item	CP20/19	CP18/17	SE	*p*-Value
Slaughter weight (kg)	24.8	24.9	0.27	0.41
Carcass weight (kg)	11.8	11.7	0.19	0.43
Carcass dressing (%)	47.8	47.0	0.69	0.47

Note: CP20/19 group was supplied 20% of dietary CP on DM and 19% of CP on DM in growing and finishing phases, respectively, whereas CP18/17 group was supplied 18% of dietary CP on DM and 17% of CP on DM in growing and finishing phases, respectively. These carcass variables take into account half of the experiment animals (60 out of 120) that were allotted in 24 pens (12 replicates per dietary treatment). SE = Standard error.

Blood urea concentration was higher in the CP20/19 group than in the CP18/17 group in both growing and finishing phases (Table 6; $p < 0.05$). Blood creatinine did not differ between groups in the growing phase, but it tended to be higher in the CP18/17 group of the finishing phase than in the CP20/19 group ($p = 0.07$). The U/C ratio was higher in the CP20/19 group than in the CP18/17, both in growing and finishing phases ($p < 0.01$). The plasma concentrations of free MDA, bound to proteins and total MDA, as biomarker aldehyde reflecting OS, were similar between the experimental diets in both feeding phases (Table 6; $p > 0.05$).

Table 6. Effect of experimental diets on metabolites related to the protein nutritional status and on the oxidative status of the lambs.

Item	CP20/19	CP18/17	SE	*p*-Value
Growing (14 to 19 kg)				
Urea (mg/dL)	41 [a]	32.3 [b]	1.19	0.0004
Creatinine (mg/dL)	0.84	0.84	0.02	0.93
Rate U/C	48.8 [a]	38.8 [b]	1.59	0.0012
FMDA (µM/L)	0.52	0.54	0.01	0.27
PBMDA (µM/L)	6.77	6.85	0.21	0.78
TMDA (µM/L)	7.30	7.40	0.21	0.73
Finishing (19 to 25 kg)				
Urea (mg/dL)	32.5 [a]	28 [b]	1.45	0.05
Creatinine (mg/dL)	0.79 [x]	0.88 [y]	0.03	0.07
Rate U/C	40.4 [a]	29.5 [b]	1.56	0.0023
FMDA (µM/L)	0.66	0.68	0.05	0.85
PBMDA (µM/L)	6.39	6.74	0.31	0.44
TMDA (µM/L)	7.06	7.43	0.32	0.43

Note: CP20/19 group was supplied 20% of dietary CP on DM and 19% of CP on DM in growing and finishing phases, respectively, whereas CP18/17 group was supplied 18% of dietary CP on DM and 17% of CP on DM in growing and finishing phases, respectively. FMDA = Free Malondialdehyde; PBMDA = Protein-Bound Malondialdehyde; TMDA = Total Malondialdehyde; [a, b] = $p < 0.05$; [x, y] = $p < 0.10$; SE = Standard error.

The dietary AIA content was 0.86 ± 0.05% and 0.80 ± 0.07% for CP20/19 and CP18/17 groups, respectively, that, in both cases, consumed a 13:87 forage to concentrate ratio. The percentage of DM in feces, as well as the apparent digestibility of CP in the growing and finishing phases did not differ between the CP20/19 and CP18/17 groups (Table 7; $p > 0.05$). The apparent digestibility of OM was lower in the CP20/19 than in the CP18/17 group during the growing and finishing phases ($p < 0.05$). On the other hand, the digestibility of P in the growing phase was not different between the CP20/19 and CP18/17 groups ($p > 0.05$). However, during the finishing phase, the apparent digestibility of P was lower in the CP20/19 than in the CP18/17 group (Table 7; $p = 0.02$).

Table 7. Effect of experimental diets on dry matter (DM) of feces, apparent digestibility of organic matter (OM), crude protein (CP) and phosphorus (P), nitrogen-phosphorus rate (N:P) of diet, N:P of feces, volatilization of nitrogen (N) and estimated ureic N.

Item	CP20/19	CP18/17	SE	*p*-Value
Growing (14 to 19 kg)				
DM of feces (DM; %)	32.9	32.7	0.72	0.91
Digestibility of OM (%)	67.7 [a]	71.4 [b]	1.21	0.04
Digestibility of CP (%)	59.1	61.4	2.15	0.46
Digestibility of P (%)	37.7	39.5	4.37	0.77
N:P of diet	7.89 [a]	6.85 [b]	0.003	0.0001
N:P of feces	4.03	4.11	0.25	0.83
Fecal N volatilization (% of intake)	48.8 [x]	39.9 [y]	3.30	0.07
Estimated ureic N (g/N/d/kg)	0.28 [a]	0.24 [b]	0.005	0.0004
Finishing (19 to 25 kg)				
DM of feces (%)	32.6	33.3	0.91	0.58
Digestibility of OM (%)	64.1 [a]	69.4 [b]	1.64	0.03
Digestibility of CP (%)	55.1	60.1	2.43	0.17
Digestibility of P (%)	21.2 [a]	37.9 [b]	4.71	0.02
N:P of diet	7.14 [a]	6.84 [b]	0.002	0.0001
N:P of feces	3.69	4.18	0.22	0.13
Fecal N volatilization (% of intake)	48.2 [a]	38.8 [b]	3.21	0.049
Estimated ureic N (g/N/d/kg)	0.24 [x]	0.22 [y]	0.007	0.050

Note: CP20/19 group was supplied 20% of dietary CP on DM and 19% of CP on DM in growing and finishing phases, respectively, whereas CP18/17 group was supplied 18% of dietary CP on DM and 17% of CP on DM in growing and finishing phases, respectively. [a, b] = $p < 0.05$; [x, y] = $p < 0.10$; SE = Standard error.

The dietary N:P ratio showed differences between the CP20/19 and CP18/17 groups in both growing and finishing phases (Table 7; $p < 0.05$). Conversely, the reduction in dietary CP did not affect the N:P ratio of feces in any of the phases studied ($p > 0.05$). The estimated fecal volatilization of N tended to be higher in the growing phase ($p = 0.07$), and it was indeed significantly higher in the finishing phase ($p < 0.05$) in the CP20/19 compared to the CP18/17 group (Table 7). During the growing phase, the estimated ureic N in urine was different between the CP20/19 and CP18/17 groups (Table 7; $p < 0.05$). During the finishing phase, the estimated ureic N in urine tended to be higher in the CP20/19 than in the CP18/17 group (Table 7; $p = 0.05$).

The reduction of dietary CP did not affect the rumen epithelial keratinization degree (32.0 vs. 33.8 ± 2.9%, in CP20/19 and CP18/17 groups, respectively, $p > 0.05$).

No effects of reduction of dietary CP were observed on cytokine expression (Figure 1). The CP18/17 group showed similar mRNA expression of *TNFA*, *IL10* and *TGFB* in ileal and ruminal tissues, as compared to that of the CP20/19 group ($p > 0.05$).

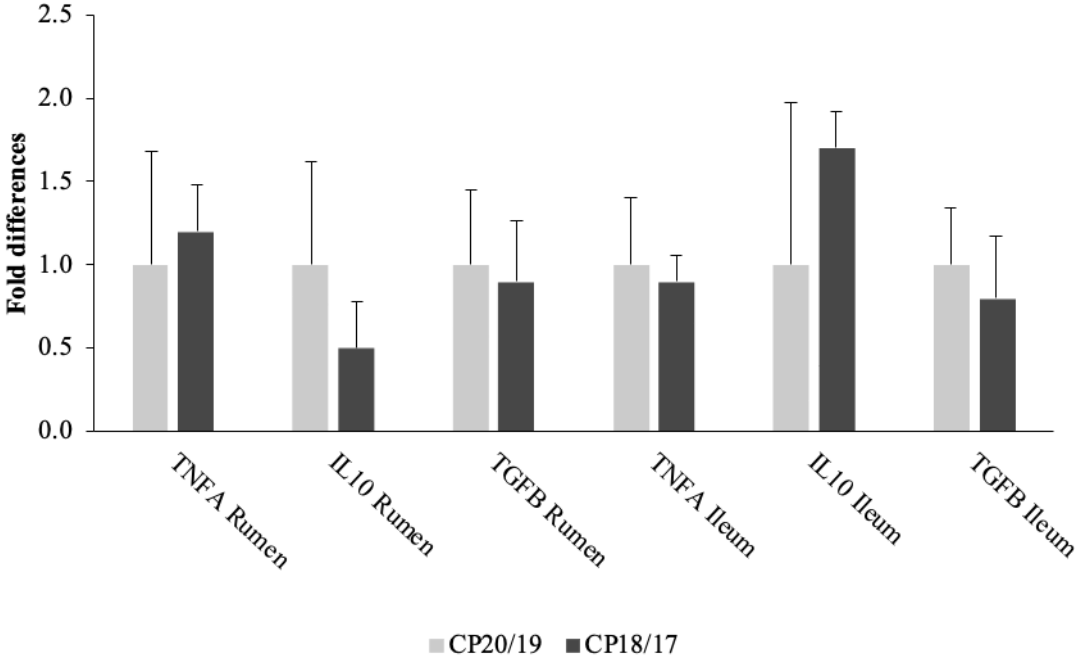

Figure 1. Relative fold differences in TNFA, IL10 and TGFB gene expression according to the reduction of dietary CP. The CP20/19 group was designated as calibrator to compare expression levels. Bars represent least square mean values ± SE (CP20/19 vs. CP18/17; $p > 0.05$).

4. Discussion

This work was designed to assess the effects of tailoring dietary CP supply to the requirements of intensively-fed light lambs from a Spanish local sheep breed (Ripollesa), that are generally early maturing and small frame in size (Esteban, 2003). Therefore, the growth performance of this breed did not respond to the nutrient supply in the same manner as selected breeds whose protein requirements have been fitted through prediction equation models in several rationing systems (for instance, INRA or National Research Council).

Lambs fed CP18/17 CP had a similar ADG and final BW compared to lambs fed the CP20/19 diet, which had 2% greater dietary CP (20% vs. 18% CP and 19% vs. 17% CP on a DM basis, in growing and finishing phases, respectively). The concentrate and straw intake were not affected by dietary CP either. Similar results were obtained by Purroy et al. (1993) in light lambs (15 to 25 kg of BW) from a close local sheep breed (Rasa Aragonesa) fed 15% and 18% dietary CP on a DM basis. However, they observed that feed intake was reduced when dietary CP decreased to 12% on DM basis. Low dietary CP strategies (up to 11% CP on DM basis) have been also used in heavy lamb production (<44 kg of final BW) without detrimental effects on growth performance compared to standard feed (16% dietary CP on a DM basis) (Hajji et al., 2016), thereby improving farm profitability.

Despite no overall differences being detected, during the finishing phase there was a tendency for greater FCR in the CP20/19 group than in the CP18/17 group. This may be related to a disruption in nitrogen-energy balance in the rumen that could have impaired microbial protein synthesis (Zhou et al., 2019), and consequently, the greater dietary CP in the CP20/19 group was not counterbalanced with an increased growth performance. The last INRA (2018) feeding system for ruminants estimates that growing-finishing lambs from 15 to 25 of BW, with 250 g of ADG, require between 110 and 103 g metabolizable protein PDI (protein digestible in the small intestine)/kg DM intake. This represents a dietary CP supply between 16.2% and 15.1% on a DM basis, respectively (INRA, 2018). Recently, it has been observed that selected Romane lambs (with ADG > 320 g/day) can respond to increases up to 20% of dietary CP (on a DM basis) by improving their apparent CP digestibility and growth performance, although their FCR is similar to lambs fed 15% CP (Bernard et al., 2019). Thus, our findings suggest that FCR remains steady when the dietary CP is kept within the range of 13% to 18% on a DM basis, in agreement with studies performed in other breeds (Bernard et al., 2019; Karim and Santra, 2003; Purroy et al., 1993).

The dietary CP requirements may be also affected by the dietary energy content. In order to seize maximal growth potential from improved Merino crossbreeds, some authors suggest increasing the protein/energy ratio up to 140 g PDI/net energy supply value (UFV) (López et al., 2015). However, the current Spanish nutritional guidelines for finishing lambs recommend dietary supplies ranging from 105 to 125 g PDI/UFV for light lambs and improved crossbred lambs, respectively (Ferret et al., 2008). In this study, this ratio ranged from 138 to 130 g PDI/UFV for the CP20/19 group and from 127 to 120 g PDI/UFV in the CP18/17 group, during growing and finishing phases, respectively. Indeed, the response to nutrient supply would be dependent on the genetic growth potential and the health status of the lambs. Therefore, it is not expected that the current autochthonous breeds raised in Spain respond to a much greater dietary CP supply.

Neither the BW at slaughter nor carcass dressing proportion differed between dietary CP groups. These results are in agreement with studies performed in heavy lambs (32 to 44 kg of BW) that were fed 11% and 16% CP diets (on DM basis) (Hajji et al., 2016).

Blood urea is considered to be an indicator of ingested (dietary) or mobilized (body) protein, while circulating creatinine highlights the creatine degradation which is involved in muscle energy metabolism (Bilancio et al., 2014). During the growing and finishing phases, the circulating urea concentrations were greater in the CP20/19 group compared to the CP18/17 group. Blood urea is a hepatic by-product of protein breakdown resulting from dietary CP supply and body protein utilization (Kaneko et al., 1997). This explains the greater circulating urea in the CP20/19 group compared to the CP18/17 group lambs. However, circulating creatinine, which may be a skeletal muscle biomarker (Patel et al., 2013), did not differ between treatments during the growing phase but tended to be lower in the CP20/19 group than in the CP18/17 group. This may suggest certain differences between the groups in daily muscle turnover. The observed blood urea concentration was in line with Mahmoud (2013), who concluded that lambs fed 17% dietary CP showed

greater blood urea than those receiving 14% and 11% dietary CP (on DM basis). A reduction of dietary CP also triggers lower ruminal NH_3-N concentration and CH_4 production (Haro et al., 2019).

The dietary CP reduction did not affect fecal consistency, as the dry matter content of feces was similar across groups. However, the apparent digestibility of organic matter was lower in the CP20/19 group than in the CP18/17 group, both in growing and finishing phases. Previous studies evidenced a curvilinear relationship between dietary CP and the apparent digestibility of OM, with maximum outcomes at 18% CP (on a DM basis) but lower values above and below this point (Kaddad et al., 2001; Kaya et al., 2009). The apparent digestibility of CP was not affected by dietary CP reduction during the growing and finishing phases. According to Haddad et al. (2001), a decrease in the apparent digestibility of CP was observed when dietary CP was set at 10%.

Excessive dietary CP increases environmental load by ruminants (Todd et al., 2005), since between 30% and 50% of N intake may be affected by volatilization, especially through NH_3 loss (Cole et al., 2006). The estimated fecal N volatilization tended to be higher in the CP20/19 group than in their CP18/17 counterparts. Similar results were obtained when using the same predictive equation of Cole et al. (2006) in beef steers fed different dietary CP levels (11.5 vs. 13% of CP). Therefore, nitrogen supply, as some other minerals as P, may be matched to requirements to reduce their excretion in manure (Hristov et al., 2013). In this study, the dietary N:P ratio was greater in the CP20/19 group than in the CP18/17 group both in growing and finishing phases. However, the differences in the estimated fecal N volatilization were more marked in the finishing than in the growing phase.

Blood urea nitrogen may be used to estimate the excreted urinary nitrogen, which may be maintained between 0.20 and 0.30 g N/day/kg of BW to meet protein requirements and reduce nitrogen excretion (INRA, 2018). In this study, the estimated urinary nitrogen excreted based on INRA (2018) was 0.28 and 0.23 g N/day/kg of BW for CP20/19 group, and 0.23 and 0.21 g N/day/kg of BW for CP18/17 group, in growing and finishing phases, respectively. This calculation suggests that lambs fed CP18/17 CP were more efficient in dietary CP use. The reduction of dietary CP seems a feasible strategy to mitigate the nitrogen gas emissions, as both NH_3 (an environmental acidifier) and N_2O (a potent warming gas aroused from the NH_4 nitrification–denitrification process) may be reduced (Zhou et al., 2019).

Characterization of blood OS is required to understand nutrient metabolism when nutrition is challenged and how this may influence the growth performance. In this regard, the blood OS may be considered a metabolic marker in animal health (Celi and Gabai, 2015). Severe protein restriction has been associated with oxidative stress status (Darmon et al., 1993), leading to the production of free radicals and pro-inflammatory cytokines, which may damage the ruminal and intestinal tissue. Thus, it is expected that blood MDA, a lipid oxidation end product, increases in OS condition (Yonny et al., 2016). Nevertheless, in this study, there were no differences in this blood compound between groups in any phase. This may suggest that the evaluated dietary CP reduction did not trigger either a blood oxidative stress or dysfunctional pro-inflammatory

responses in the gastrointestinal tract. Immune function in ileal and ruminal tissues was not compromised and no differences were observed in proinflammatory and anti-inflammatory cytokine expression as a consequence of a 2% protein reduction. On the other hand, increased parakeratosis has been also observed in lambs in response to diets with concentrate (19.9% CP on DM) vs. grazing alfalfa (24.7% CP on DM) (Van Soest et al., 1991). However, as noted for keratin layer in ruminal epithelium, ruminal histomorphogenesis remained unaltered in this study. Hence, management practices related to improving protein content in light lamb diets seemed here not to affect the interplay between nutrition, metabolism and immune function.

5. Conclusions

In conclusion, a 2% reduction of dietary CP in intensively-raised light lambs did not impair their growth performance, reduced blood urea without affecting their oxidative status or proinflammatory and anti-inflammatory cytokine gene expression, and improved the apparent digestibility of organic matter and phosphorus.

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IV. CHAPTER 2

Minimum effects of sampling time on the apparent digestibiliy of nutrients and blood protein catabolites in light lambs.

Abstract: This experiment aimed to evaluate the effects of sampling time on organic matter (OM), crude protein (CP) and phosphorous (P) apparent digestibility and plasma urea and creatinine concentration in growing and finishing male Ripollesa lambs fed different CP concentrations in the diet. Twenty-four male Ripollesa lambs with 14.5 kg body weight (BW) were randomly assigned to two groups differing in CP content in the growing (14 to 19 kg of BW) and finishing (19 to 25 kg of BW) phases (20% vs. 18% CP and 19% vs. 17% CP, respectively). Faeces collected from the rectum and blood samples collected from the jugular vein were taken at 8:00 a.m., 12:00 p.m., and 4:00 p.m. During the growing period, the OM, CP and P apparent digestibility were higher in the lower CP diet ($p < 0.05$), but only P was affected by the sampling time, being highest at 8:00 a.m. ($p < 0.05$) compared to other sampling hours. During the finishing period, there were no differences in these digestibility coefficients between diets or sampling times ($p > 0.05$). Sampling time did not affect ($p > 0.05$) plasma urea concentrations either in the growing or finishing period. Plasma creatinine concentrations did not differ ($p > 0.05$) between lambs receiving 18% or 20% CP diets, but during the finishing period, it was lower at 4:00 p.m. in lambs fed 17% CP ($p < 0.05$) than those offered 19% CP. Overall, the results suggest that the collection schedule to evaluate the protein nutritional status can be shortened through one spot sample of faeces or blood in the morning.

1. Introduction

Spain is the top sheep producer within the European Union (Eurostat, 2020). In this country, the most common production system is a light lamb weaned at 30–60 days of age with 12–15 kg of body-weight (BW) and raised subsequently on a concentrate diet ranging from 15% to 21% crude protein (CP) on a dry matter (DM) basis (Bello et al., 2016) until 75–100 days of age with 24–28 kg of BW. This intensive feeding system could result in a high environmental load of nutrients, such as nitrogen and phosphorous (P). Therefore, these nutrients need to be adjusted not only to reduce their excretion and subsequent potential pollution on the environment, but also because high protein diets are very demanding of protein-rich ingredients, which have been demonstrated to boost the carbon footprint of livestock production (Kim et al., 2019), and also because P is a finite and non-renewable resource (Deng et al., 2020). Despite significant efforts for adjust these nutrients in diets for livestock, there is still the need to improve feeding animal practices to reduce manure nitrogen and P in intensive sheep production systems (Dourmad et al., 2020).

Animal nutritionists need to know how changes in the lambs' diet affect their metabolism and feed digestibility in commercial flocks. Therefore, to optimise the assessment of dietary CP in lamb's nutrition, it is necessary to develop a better knowledge of diurnal variation on nutrient apparent digestibility and blood metabolites related to nutrient status. Although urinary nitrogen could be helpful when dietary CP is studied, blood urea and creatinine have a more significant potential for utilisation because of their reliable reflection in the blood (Kohn et al., 2005). In this regard, blood urea concentration is used to evaluate the protein status of ruminants (Hammond, 1997) as it is linked to protein catabolism (Kaneko et al., 2008), and it does not differ during daylight in growing calves fed ad libitum (Devant et al., 2017). On the other hand, blood creatinine concentration reflects a loss of skeletal muscle mass (Megahed et al., 2019), but it may be affected by time of the day.

Regarding apparent digestibility, Fukumoto et al. (2007) concluded that only one daily faecal sample collection between 9 h and 13 h is needed to estimate the digestibility of nutrients in sheep fed ad libitum. The use of internal markers, such as the acid-insoluble ash (AIA) technique, to determine the digestibility of nutrients from faecal samples collected during the day has been uniformly accepted (Sales and Janssens, 2003), assuming that AIA is a natural component of feeds that is expected to flow with the digesta through the gastrointestinal tract of the animal. Additionally, it has been accepted that markers can be used throughout the faecal collection period for recording digestibility. However, the natural event of transit and degradation of ingested feed, although continuous in the rumen, may not be constant throughout the remainder of the digestive tract of ruminants (Sampaio et al., 2011). Even though Keulen and Young (1977) showed no evidence of a diurnal variation in AIA excretion in sheep, a potential interaction between sampling time and diet composition may not be discarded, since Morris et al. (2018) observed in ruminants a diurnal variability in AIA excretion when two forage diets were compared. This suggests that diet composition may influence the digestive process of marker excretion.

Hence, this study aimed to use a single faecal or blood sample to test the apparent digestibility of nutrients and blood metabolites related to protein status in growing and finishing lambs fed different CP concentration levels.

2. Materials and methods

2.1. Animals, diets and experimental design

The experiment was carried out in the experimental facilities of El Nial of the BonÀrea Agrupa (Guissona, Lleida, Catalonia, Spain, 41°46′32.2′′ N, 1°16′33.2′′ E; 484 m above sea level) between January and February 2018. A total of sixty weaned (45–60 days-old) male Ripollesa lambs weighing 14.5 ± 1.3 kg were housed in 12 shared pens (5 animals/pen; 1.04 m^2 per animal) and they were distributed in homogeneous groups according to their initial BW. Twenty-four lambs were randomly selected from the whole flock for this study (2 lambs/pen). In each pen, lambs had access to one longitudinal multiplace feeder with barley straw and a creep concentrate feeder. Lambs were fed in two periods according to their BW, the growing (14 to 19 kg BW) and finishing (19 to 25 kg BW) period, which lasted 21 days each. Four experimental diets with different CP levels were formulated and supplied to two treatment groups: half of the lambs (n = 12) were fed a diet containing 20.8% CP (CP20 group) and 19.1% CP (CP19 group) on dry matter basis (DM) during the growing and finishing periods, respectively, whereas the other half (n = 12) were fed diets containing 18.3% CP (CP18 group) and 17.4% CP (CP17 group) on a DM basis during the growing and finishing periods, respectively. Before the experiment started, lambs were fed standard commercial diets for five days adaptation concentrate containing coccidiostatic (decoquinate at 30 mg/kg). Ingredients and chemical composition of the experimental diets can be found in Pelegrin-Valls et al. (2020), and the organic matter (OM), CP, P and AIA contents are reproduced in Table 1. Briefly, pelleted diets were isoenergetic (1760 kcal of Net Energy for Ruminants/kg of concentrate) and they were formulated with the same ingredients and additives in the same manufacturing batch. Only the percentage of vegetable protein was modified. In both periods, lambs had free access to concentrate, water and barley straw.

Table 1. Dietary composition in the growing and finishing periods.

Item	Dietary Crude Protein		Barley Straw
	CP20/19	CP18/17	
Growing period			
OM (%)	94.3	93.9	94.8
CP (%, N × 6.25)	20.7	18.4	2.36
P (%)	0.42	0.45	0.07
AIA (%)	0.61	0.58	2.60
Finishing period			
OM (%)	94.5	94.5	95.6
CP (%, N × 6.25)	19.1	17.5	2.08
P (%)	0.42	0.42	0.07
AIA (%)	0.61	0.51	2.60

Note: %, on DM basis, unless otherwise stated. OM, Organic Matter; CP, Crude Protein; N, Nitrogen; P, Phosphorous; AIA, Acid-Insoluble Ash.

2.2. Sampling

The offered concentrate and straw were recorded daily, and the refused straw and concentrate were recorded once weekly on a pen basis. Thereby, cumulative feed disappearance was recorded, and feed intake was considered steady for the whole week. The concentrate was offered every morning in the creep feeder. Lambs were individually weighed once a week to calculate average daily gain (g/day) by regression of BW on time. In the last weeks of both the growing and finishing periods, faeces pools of approximately 50 g, coming from at least 3 lambs per pen (6 pen replicates/dietary treatment), were collected at 8:00 a.m., 12:00 p.m., and 4:00 p.m. by rectal stimulation to determine apparent digestibility coefficients of OM, CP and P. Samples of concentrate and straw were also collected at 8:00 a.m. All feed samples were kept at −20 °C until analysis. After thawing, samples were weighed and dried in a forced air stove at 60 °C for 72 h. The moisture of samples was determined by the weight difference between the fresh and DM. After drying, samples were ground in a knife mill to pass a 1 mm sieve and stored in watertight plastic bags until analysis. The total digestive tract apparent OM, CP and P digestibilities were calculated using the nutrient-to-marker ratio in the diet and faeces, as follows:

$$\text{Apparent digestibility coefficient (\%)} = 100 - [100 \times (\text{Marker}_{diet}/\text{Marker}_{faeces}) \times (Z_{faeces}/Z_{diet})]$$

where Z_{faeces} and Z_{diet} are the nutrient concentrations (%) in faeces and diet, respectively. The Marker_{faeces} and Marker_{diet} are the concentrations (%) of AIA in faeces and diet, respectively. The Z_{diet} and Marker_{diet} were calculated by considering the amount of concentrate and straw consumed per pen. The recovery rate of AIA in faeces was assumed to be complete (Sales and Janssens, 2003).

At the same time of faeces collection, blood samples were collected from two lambs per pen at 8:00 a.m., 12:00 p.m., and 4:00 p.m. The same lambs, that were randomly selected at the start of the trial, were sampled during the day and at both rearing periods. Vacuum tubes with EDTA (BD Vacutainer®, Becton,

Dickinson and Company, Plymouth, UK) were used to collect 5 mL of blood from the jugular vein (6 pen replicates /dietary treatment) and were centrifuged in situ at 3000× *g* for 10 min to obtain the plasma, which was stored in identified aliquots for each sampled lamb at −20 °C until metabolites analysis.

2.3. Chemical analyses

The ash content to calculate OM was determined with 2 g of feed and faeces samples in a muffle furnace at 550 °C for 3 h. CP content of diets (N × 6.25) was determined following the DUMAS procedure, using a nitrogen and protein analyser (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain). P was determined by ultraviolet-visible spectroscopy (ICP-OES, HORIBA Jobin Yvon, Activa family, with AS-500 Autosampler, HORIBA Scientific, Madrid, Spain). Apparent OM, CP and P digestibility coefficients were estimated by the AIA technique, which was determined following the procedure described by Álvarez-Rodríguez et al. (2017). Feed and faeces samples were analysed in duplicate.

Plasma urea and creatinine concentration (mg/dL) were analysed by an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). The kinetic method was used to quantify plasma urea which catalysed the hydrolysis of urea into ammonia and carbon dioxide. The test had a measurement range between 2 and 350 mg/dL and their mean intra- and inter-assay coefficients of variation were 2.8% and 2.7%, respectively. Plasma creatinine was quantified using the enzymatic method as final by-product of the muscular metabolism. The creatinine measurement range was 0.03 to 50 mg/dL with mean intra- and inter-assay coefficients with variations of 3.1% and 5.1%, respectively.

2.4. Statistical analysis

The cumulative concentrate and straw intake, in the growing and finishing periods, were analysed with standard least square means models with dietary treatment as fixed effect. Plasma metabolites and nutrient digestibility data, in each growing and finishing periods, were analysed with the statistical software JMP Pro13 (SAS Institute Inc., Cary, NC, USA), using mixed models with repeated measurements that included the dietary treatment, the sampling time and their interaction as fixed effects, and the pen as a random effect. Results are presented as least square means and their standard error. The comparison of means was carried out with the Tukey′s test. The level of significance was set at 5%.

3. Results

3.1. Animal performance, dietary and faecal AIA content and DM of faeces

The average concentrate and straw intake in the growing period did not differ ($p > 0.05$) between CP20 and CP18 groups (686 vs. 753 ± 27.3 g and 104 vs. 97 ± 7.3 g, respectively). In the finishing period, CP19 and CP17 groups also had a similar concentrate and straw intake (812 and 843 ± 16.7 g and 120 vs. 117 ± 8.5 g, respectively; $p > 0.05$). The overall forage to concentrate ratio was 13:87 and 12:88 for the growing and

finishing periods, respectively. The average daily gain did not differ between CP20 and CP18 diets either in the growing period (216 vs. 237 ± 17.9 g/day, respectively; $p > 0.05$), finishing period (296 vs. 272 ± 23.7 g/day, respectively; $p > 0.05$) or overall period (242 vs. 259 ± 12.0 g/day, respectively; $p > 0.05$).

Concentrate and straw AIA contents were analysed only from samples at 8:00 a.m. as it was considered that their contents did not change between hours of sampling. For the growing and finishing periods, the dietary AIA content was greater for the CP20 group than CP18 group and for the CP19 group than CP17 group (0.86 vs. 0.79 ± 0.01% and 0.87 vs. 0.77 ± 0.01%, respectively; $p < 0.05$).

Results of faecal composition are shown in Table 2. DM of faeces was similar for CP20 and CP18 group during the growing period, and in the CP19 and the CP17 group during the finishing period (Table 2; $p > 0.05$).

In the growing period, no significant differences were observed between the sampling times for DM of faeces. However, in the finishing period, DM of faeces was lower at 8:00 a.m. than at 12:00 p.m., and 4:00 p.m. (Table 2; $p < 0.05$).

OM and CP in the faeces content did not differ between treatments and sampling times (Table 2; $p > 0.05$). Nevertheless, faecal P content during the growing period was higher in the CP20 than in the CP18 group (Table 2; $p < 0.05$), although these differences were not distinguished in the finishing period. Similarly, in the sampling times, less P was observed in the faeces at 8:00 a.m. compared to 12:00 p.m. and 4:00 p.m. (Table 2; $p < 0.05$), but not in the finishing period.

The faecal AIA content in the growing period was lower in CP20 than CP18 groups ($p < 0.05$). However, in the finishing period, no differences were observed between the dietary treatments in faecal AIA content (Table 2; $p > 0.05$).

The faecal AIA content did not differ across sampling times in any period (Table 2; $p > 0.05$).

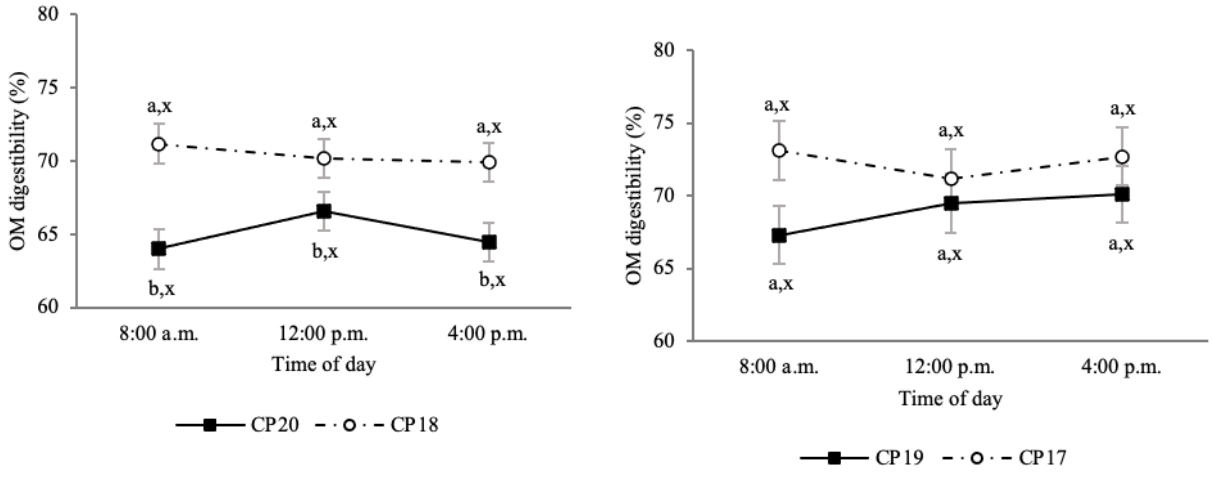
Table 2. Faecal composition in the growing and finishing periods.

Item	Sampling Time				Dietary Crude Protein			*p*-Value	
	8:00 a.m.	12:00 p.m.	4:00 p.m.	SD	CP20/19	CP18/17	SD	Hour	Diet
Growing period									
DM (%, on fresh-weight basis)	34.9	34.1	34.5	0.74	34.3	34.7	0.87	0.551	0.768
OM (%)	87.4	87.1	87.5	0.28	87.3	87.4	0.29	0.460	0.721
CP (%, N × 6.25)	21.3	20.5	20.4	0.51	21.3	20.3	0.43	0.444	0.111
P (%)	0.84 [a]	0.98 [b]	0.91 [b]	0.04	0.99 [a]	0.83 [b]	0.04	0.026	0.024
AIA (%)	2.39	2.43	2.36	0.05	2.28 [a]	2.51 [b]	0.05	0.491	0.019
Finishing period									
DM (%, on fresh-weight basis)	35.7 [a]	37.2 [a, b]	38.7 [b]	1.05	36.5	37.9	1.30	0.009	0.429
OM (%)	87.6	88.3	87.9	0.34	88.2	87.7	0.40	0.130	0.358
CP (%, N × 6.25)	19.1	18.6	19.4	0.51	19.3	18.8	0.54	0.463	0.441
P (%)	0.80	0.78	0.79	0.04	0.82	0.76	0.05	0.835	0.479
AIA (%)	2.61	2.62	2.72	0.12	2.69	2.61	0.17	0.259	0.755

Note: %, on DM basis, unless otherwise stated. The interaction between hour and diet did not affect any variable ($p > 0.05$). Within each row and effect, the statistical differences between means are described by different letters ([a, b]). SD, standard error; DM, Dry Matter; OM, Organic Matter; CP, Crude Protein; N, Nitrogen; P, Phosphorous; AIA, Acid-Insoluble Ash.

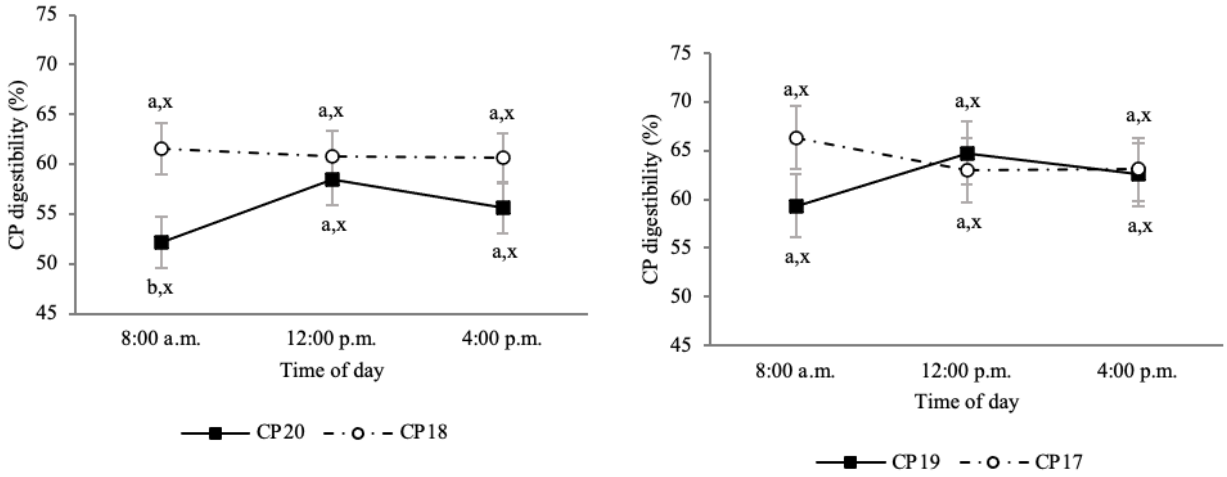
3.2. Nutrient digestibility and blood metabolites

During the growing period, diet OM digestibility in the CP20 group was lower than in CP18 group, regardless of sampling time (Figure 1A; $p < 0.05$). This effect was also observed in the CP20 group compared to CP18 group for diet CP apparent digestibility, but only at 8:00 a.m. (Figure 2A; $p < 0.05$). Moreover, the CP20 group had lower diet P apparent digestibility compared to CP18 group (Figure 3A; $p < 0.05$). On the contrary, plasma urea concentrations were greater in CP20 than CP18 group (Figure 4A; $p < 0.05$), but no differences were observed in plasma creatinine concentrations (Figure 5A; $p > 0.05$). The blood urea/creatinine ratio was also different between dietary treatments studied (51.9 vs. 43.9 ± 2.3 for CP20 and CP 18, respectively, $p < 0.05$).



(**A**) (**B**)

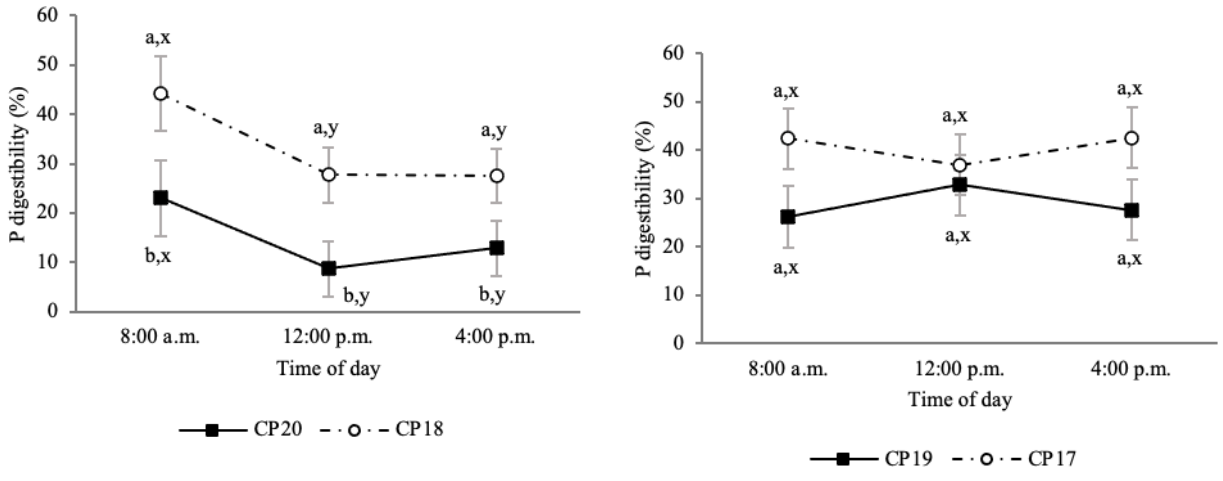
Figure 1. Digestibility of organic matter (OM) in growing (**A**) and finishing (**B**) periods in Ripollesa lambs fed with different concentrations of crude protein were compared between treatments (CP20 vs. CP18 and CP19 vs. CP17) and time of the day (least square mean ± standard error). Within each sampling hour, different letters (a, b) denote statistical differences ($p \leq 0.05$) between dietary treatments. Within each dietary treatment, different letters (x, y) denote statistical differences ($p \leq 0.05$) between sampling hours.



(**A**) (**B**)

Figure 2. Digestibility of crude protein (CP) in growing (**A**) and finishing (**B**) periods in Ripollesa lambs fed with different concentrations of crude protein were compared between treatments (CP20 vs. CP18 and CP19 vs. CP17) and time of the day (least square mean values ± standard error). Within each sampling hour, different letters (a, b) denote statistical differences ($p \leq 0.05$) between dietary treatments. Within each dietary treatment, different letters (x, y) denote statistical differences ($p \leq 0.05$) between sampling hours.

Sampling time did not affect ($p > 0.05$) apparent digestibility of OM and CP in any treatment during the growing period (Figures 1A and 2A). However, sampling time affected the apparent digestibility of P, which was greater at 8:00 a.m. ($p < 0.05$) compared to 12:00 p.m. and 4:00 p.m. (Figure 3A). On the other hand, urea and creatinine concentrations at different sampling times were similar ($p > 0.05$) between dietary treatments (Figures 4A and 5A). Likewise, the plasma urea/creatinine ratio was similar between sampling hours (50.7 at 8:00 a.m., 46.9 at 12:00 p.m. and 46.1 ± 2.07 mg/dL at 4:00 p.m.; $p > 0.05$).



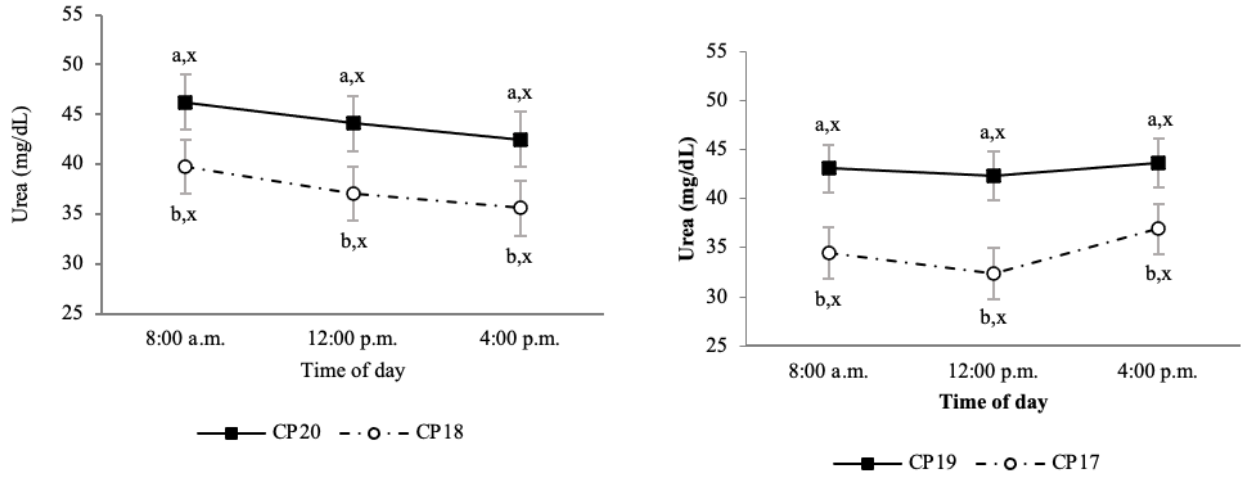
(**A**) (**B**)

Figure 3. Digestibility of phosphorous (P) in growing (**A**) and finishing (**B**) periods in Ripollesa lambs fed with different concentrations of crude protein were compared between treatments (CP20 vs. CP18 and CP19 vs. CP17) and time of the day (least square mean values ± standard error). Within each sampling hour, different letters (a, b) denote statistical differences ($p \leq 0.05$) between dietary treatments. Within each dietary treatment, different letters (x, y) denote statistical differences ($p \leq 0.05$) between sampling hours.

During the finishing period, the OM, CP and P apparent digestibility was similar between CP19 and CP17 groups (Figure 1B, 2B and 3B, respectively; $p > 0.05$). The CP19 group had a higher plasma urea concentration than CP17 group (Figure 4B; $p < 0.05$), nevertheless, plasma creatinine concentrations were similar between dietary treatments (Figure 5B; $p > 0.05$). On the contrary, plasma urea/creatinine ratio was higher in CP19 than in CP17 (51.4 vs. 39.6 ± 2.3, respectively, $p < 0.05$).

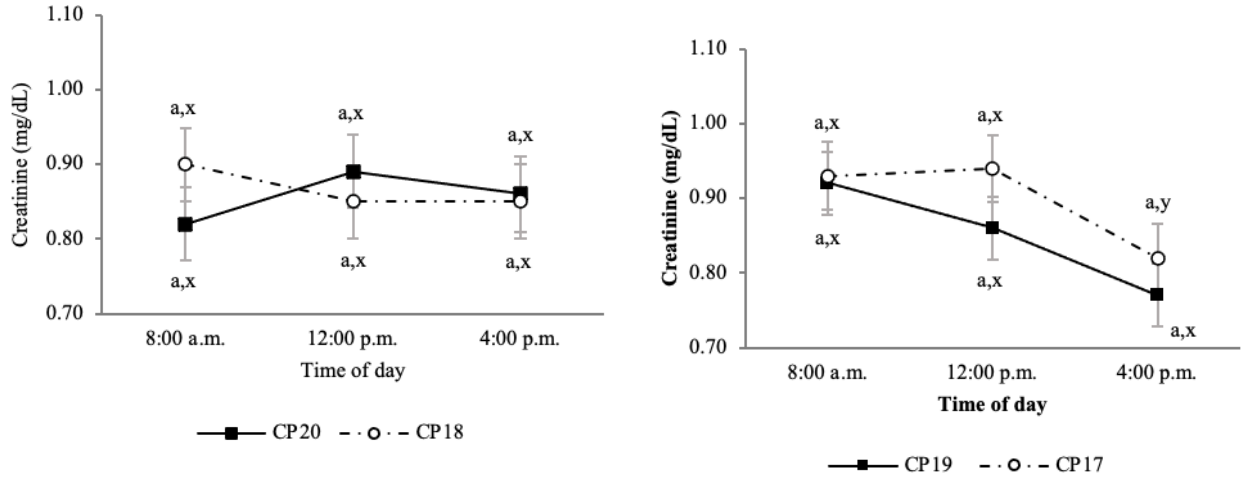
No differences existed between sampling times for OM, CP and P apparent digestibility in any of the groups studied during the finishing period (Figure 1B, 2B and 3B, respectively; $p > 0.05$). Likewise, sampling times did not affect plasma urea concentrations for any dietary treatment (Figure 4B; $p > 0.05$), but the CP17 group had higher plasma creatinine concentrations at 8:00 a.m. and 12:00 p.m. than at 4 p.m. (Figure 5B; $p < 0.05$), while no differences between sampling times were observed in the CP19 group ($p > 0.05$). In turn,

the urea/creatinine ratio was lower at 8:00 a.m. and 12:00 p.m. than at 16:00 p.m. (42.6 and 42.7 vs. 51.5 ± 2.42 mg/dL, respectively; $p < 0.05$).



(**A**) (**B**)

Figure 4. Plasma urea concentrations (mg/dL) in growing (**A**) and finishing (**B**) periods in Ripollesa lambs fed with different concentrations of crude protein were compared between treatments (CP20 vs. CP18 and CP19 vs. CP17) and time of day (least square mean values ± standard error). Within each sampling hour, different letters (a, b) denote statistical differences ($p \leq 0.05$) between dietary treatments. Within each dietary treatment, different letters (x, y) denote statistical differences ($p \leq 0.05$) between sampling hours.



(**A**) (**B**)

Figure 5. Plasma creatinine concentrations (mg/dL) in growing (**A**) and finishing (**B**) periods in Ripollesa lambs fed with different concentrations of crude protein were compared between treatments (CP20 vs. CP18 and CP19 vs. CP17) and time of day (least square mean values ± standard error). Within each sampling hour, different letters (a, b) denote statistical differences ($p \leq 0.05$) between dietary treatments. Within each dietary treatment, different letters (x, y) denote statistical differences ($p \leq 0.05$) between sampling hours.

4. Discussion

The objective of this study was to determine the effect of sampling time on the apparent nutrient digestibility and blood metabolites related to nutritional status in Ripollesa light lambs fed different levels of CP. Most of the evaluated variables did not show interaction between daily sampling time and dietary CP, and thus these effects are discussed separately.

4.1. Dietary CP effect on nutrient digestibility and plasma metabolites

In a previous study, we reported that CP content of lamb feed can be reduced (10% below the current commercial standards) without a negative effect on growth performance or carcass yield of local Spanish breeds (Pelegrin-Valls et al., 2020). However, the impact of a reduction in the amount of dietary protein on metabolism should be carefully evaluated and adequate methods are needed, as many metabolic processes in mammals have been described as being influenced by eating frequency (Asher and Schibler, 2011), which could impair the proper metabolic synthesis of proteins, and consequently, the growth of lambs.

In this study, a reduction in dietary CP during growing and finishing periods did not affect the faecal consistency, as DM of faeces remained steady. However, the dietary AIA content was greater in lambs fed high CP diets in the growing period. Probably, these group ingested a little more straw, but these mild differences were not supported by statistical differences in total feed intake. To estimate the apparent digestibility of nutrients, the AIA marker is more accurate when the dietary AIA is higher than 0.75% (DM basis) (Thonney et al., 1985). This was indeed attained by both dietary treatments in the growing and finishing periods with an intensive concentrate inclusion (nearly 90% of the ration) and barley straw supplement. The reliability of internal markers such as AIA has been questioned as it occasionally overestimates digestibility coefficients when compared with other markers (Álvarez-Rodríguez et al., 2017; Morris et al., 2018). However, Pepeta et al. (2020) found that AIA could be used as an accurate and precise marker for estimating nutrient digestibility in sheep. To use AIA as digestibility marker in concentrate-based feeding systems, it is essential to supplement lambs' with roughages, as barley straw, at dietary levels >10%. Therefore, to accurately estimate the nutrient digestibility through internal markers, it is necessary to take into account the feeding frequencies and dietary components (Vanzant et al., 1998).

The role of reducing the CP in the lambs' diet on the digestive efficiency may be evaluated through the apparent digestibility of nutrients. Accordingly, during the growing period, it was found that animals fed the CP18 diet had better OM digestibility. Similar results were obtained by Haddad et al. (2001), who observed that heavy lambs improved OM digestibility when fed diets containing CP level was less than 18%. In addition, bone (rich in P) and lean (rich in CP) tissues had early developing allometric growth coefficients ($b < 1$) in relation to carcass weight of lambs (Álvarez-Rodríguez et al., 2009). Thereby, these two tissues are mostly developed during the growing period of light lambs, which may support the differences in CP and P

apparent digestibility between dietary CP levels in this stage. However, this improvement in the faecal apparent OM digestibility with the reduction in dietary CP was not observed during the finishing period.

The P apparent digestibility in the growing period was lower in the CP20 group than in CP18 group. Borges et al. (2008) noted that P digestibility depends on its diet content, nutrient sources and physiological status. Furthermore, it has been described that faeces are the principal pathway of P excretion in ruminants, and it is directly correlated with the diet P content (Borges et al., 2008). However, according to Dias et al. (2013), the faecal loss of P may be overestimated because of salivary P secretion, suggesting that the true available P was probably greater in both dietary treatments. Assuming that the urinary loss of this mineral is minimal, the metabolic P requirements may be related to the mineral proportion of skeletal body weight gain (INRA, 2018). As these lambs had similar average daily gain, the greater P apparent digestibility in CP18 lambs compared to CP20 lambs may be the result of a slightly higher dietary concentration of this nutrient (0.45% vs. 0.42%, respectively) and a lower salivary P secretion to buffer rumen pH conditions, which can represent as much as 80% of the endogenous secretion in the rumen (Borges et al., 2008). On the contrary, in the finishing period, no differences were observed in OM, CP and P apparent digestibility between experimental diets, which suggests that the CP17 group met its nutritional requirements to a greater extent.

Plasma urea concentration was greater in lambs fed the highest CP diets. This is in line with Mahmoud et al. (2013) who concluded that heavy lambs fed diets with high CP levels had greater plasma urea concentrations than those fed lower CP levels. Plasma urea concentration reflects the amount of protein ingested and thus the absorption of ruminal ammonia (Hatfield et al., 1998). Hence, the reduction of dietary CP decreases the protein catabolism. However, plasma creatinine concentrations showed no differences between dietary treatments and sampling periods. In this regard, the current results suggest that lower CP diet did not promote muscle degradation because of insufficient dietary protein (Bilancio et al., 2014).

4.2. Sampling time effect on nutrient digestibility and plasma metabolites

It has been reported that feed intake of lambs is reduced at night as a result of an instinctive fear of predation (Moyo et al., 2019), so it would result in less excretion of faeces early in the morning. On the other hand, according to Sampaio et al. (2011), feed degradation and transit in the rumen are continuous but influenced by actual feed intake. These authors observed that feed degradation and faecal elimination increase during feed intake periods, which causes more inconsistency in the faecal content. Additionally, two peaks of eating and drinking occur in feedlot lambs (Shreffler and Hohenboken, 1980) and grazing lambs (Álvarez-Rodríguez et al., 2017), which occur near sunrise (about 8:00 a.m.) and sunset (about 8:00 p.m.). The faecal sampling schedule was planned to gather the potential digestive turnover differences after the morning eating time under ad libitum feeding conditions. During the finishing period, faeces DM was lower at 8:00 a.m. However, this variation did not have any relationship with the outcomes of digestibility coefficient calculations throughout the daylight, as discussed below.

A lack of representativity in the collection of the faecal samples may cause an estimation bias in the digestibility estimates (Sampaio et al., 2011). In this regard, only a slight variation in diurnal faecal excretion of the AIA marker has been seen over several days in rabbits (Furuichi and Takahashi, 1981). Similarly, Kanani et al. (2015) reported that cattle were not affected by sampling time and there was no diet by sampling time interactions, even though diet affected faecal AIA concentrations, which is in agreement with the present outcomes in sheep.

The sampling time in the light lambs of the present study showed no effect on OM and CP apparent digestibility in any of the periods studied. This would mean that the marker digestibility estimations were uniformly distributed throughout the entire faecal sample collection period. Similar results were obtained by Paternostre et al. (2019) in growing pigs, who concluded that only one spot-sampling was enough (either at 9:00 a.m. or 2:00 p.m.) to estimate the faecal OM digestibility by markers in animals fed ad libitum. On the contrary, in the growing period, P apparent digestibility was greater early in the morning (8:00 a.m.) while in the finishing period, this difference did not persist. As stated earlier, faecal P may include much of the P secreted by saliva that is not reabsorbed (Humer and Zebeli, 2015), and early morning faecal samples may reflect an increase in undigested P that would be linked to increased salivary P turnover, which is induced by rumination that is usually carried out at night (Moyo et al., 2019).

Plasma urea concentrations showed no differences between sampling times in any of the periods studied. Piccione et al. (2006) found, in sheep, that both salivary and blood urea profiles were high during the light phase and low during the dark phase of the natural light–dark cycle. Within 12 h after feeding, Valkeners et al. (2008) observed minimal variations in blood urea concentration in calves, which in turn showed a 3 h delay after ruminal ammonia synthesis. In this regard, Oliveira et al. (2020) described lower rumen ammonia nitrogen concentrations in lambs fed with low CP (13% on DM); however, they pointed out that this CP level ensured adequate nitrogen supply for ruminal microbial protein synthesis in hair sheep raised in a tropical environment.

In contrast, in our study, plasma creatinine concentration in the growing period did not differ between sampling hours, but in the finishing period it was higher at 8:00 a.m. and 12:00 p.m. than at 4:00 p.m., especially in the CP17 group. This could be explained by the postprandial variation in this catabolite in blood, maybe reflecting, in this study, a blood creatinine clearance and higher urinary turnover excretion before the last sampling (4:00 p.m.), which would follow the eating and drinking morning peak as they may affect the endogenous creatinine concentrations (Braun et al., 2010). Accordingly, Vivian et al. (2017) observed a decrease in blood creatinine concentration between 0 h and 12 h after feed intake in the finishing period of heavy lambs, which is in agreement with the present results. Consequently, the urea/creatinine ratio of the finishing period was higher at 4:00 p.m. than before, which would reflect a higher urea synthesis than creatinine in the last sampling, possibly linked to postprandial digestion. Other works have observed that

blood creatinine concentration usually had few diurnal fluctuations (6:00 a.m. to 6:00 p.m.), but it was higher during diurnal than nocturnal sampling periods (Dos Santos et al., 2018).

5. Conclusions

Overall, a single sample of faeces and blood could be used to assess OM, CP and P apparent digestibility, as well as urea and creatinine in light lambs fed ad libitum, and they were not affected by the interplay between sampling time and dietary CP level, although the CP digestibility estimate may be more affected by dietary CP in the morning (8:00 a.m.) during the growing period.

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V. CHAPTER 3

Is the inclusion of carob (*Ceratonia siliqua* L.) pulp in the concentrate of weaned light lambs worth it?

Abstract: This study aimed at evaluating the effects of dietary carob (*Ceratonia siliqua* L.) pulp, a by-product of the fruit processing industry, on light lamb performances, time budget behaviour, coefficients of total tract apparent digestibility (CTTAD) and blood metabolites. A total of 144 crossbred lambs with 41 ± 5.5 days of age were monitored in two consecutive batches. They were housed in groups of 6 lambs (3 females and 3 males) balanced according to weaning body-weight and assigned to one of three isoenergetic and isoproteic concentrate-based diets: C0 (without carob pulp), C15 (150 g/kg of carob pulp) and C30 (300 g/kg of carob pulp). At 50, 65 and 80 days of age, feed, faeces and blood samples were collected, and behaviour was recorded. The type of concentrate did not have effect on lamb growth and concentrate intake ($P > 0.05$). However, the concentrate feed conversion ratio (FCR) was higher for C15 and C30 compared to C0 ($P < 0.05$). The time budget behaviours were not affected by diets ($P > 0.05$). The time spent lying resting and standing static increased throughout the fattening period ($P < 0.05$), but self-grooming, feeding, exploration, movement and positive social interactions decreased with age ($P < 0.05$). Regarding the CTTAD of dry matter (DM), organic matter (OM) and crude protein (CP), it was noted that C30 had the lowest digestibility of these nutrients with respect to C0 and C15 ($P < 0.05$). Furthermore, the total condensed tannins (CT) content in faeces was higher in C30 compared to C0 and C15 ($P < 0.05$). The age of lambs affected the CTTAD of DM, OM, CP and phosphorus, which were lower at 50 days than in the rest of control days ($P < 0.05$). Blood metabolites were not affected by experimental diets ($P > 0.05$). However, urea concentrations increased significantly from 50 to 65 days of age, while glucose decreased concomitantly ($P < 0.05$). In conclusion, dietary carob pulp inclusion up to 300 g/kg does not negatively affect animal daily gains, daily activity budgets and metabolic nutritional status, but reduces diet digestibility and increases the concentrate FCR. Although a carob pulp inclusion of up to 150 g/kg in the energy-rich concentrate-based diet for weaned light lambs increases the concentrate FCR while keeping the daily gain, this dietary strategy would be a good trade-off between animal performances and circular economy principles.

1. Introduction

To address the environmental impact of sheep production, the formulation of diets combining circular economy principles and key nutrients to mitigate climate warming would be desired (Guyomard et al., 2020). Carob (*Ceratonia siliqua* L.) is evergreen legume tree that has been cropped traditionally in the Mediterranean area because of its drought resistance, adaptation to soil salinity and low fertilization requirements, which may represent a carbon sink in arid environments. Spain is the top producer of carobs around the globe, with about 60,000 Tm of carob pods produced per year. Carob pod fruits are long, indehiscent, straight or curved, thickened at the sutures, and composed by two major parts, pulp (90 %) and seeds (10 %) (Malagón, 2020).

Carob products are purposed for several foods, pharmaceutical and cosmetic industry (Zhu et al., 2019). Carob pulp by-products are yielded during this processing applications, which are rich in sugar (260–550 g/kg dry matter, DM) and lignin (100–430 g/kg DM) contents, whilst are low in hemicellulose (6–33 g/kg DM), crude protein (20–70 g/kg DM) and lipid (1–15 g/kg DM) contents, which make them a potential source of fodder, mainly for ruminants (Albanell et al., 1991). Carob pulp is also deficient in phosphorus (Heuzé et al., 2016). In contrast, carob pods are considered an exceptionally rich source of non-extractable polyphenols (Silanikove et al., 2006). The main phenolic compounds in carob pulp (in decreasing importance) are gallic acid, myricetin- and quercetin- derivatives, and procyanidins galloyl esters (Rico et al., 2019; Stavrou et al., 2018).

In this framework, the most proven dietary strategies to reduce enteric methane emission are the supply of tannins and/or lipids (INRA, 2018). The dietary inclusion of carob pulp has been previously evaluated in heavy lambs (from 20 to 25–30–40 kg of slaughter body-weight, BW) fed with total mixed rations with forage sources (Benbati et al., 2021; Gobindram et al., 2015; Obeidat et al., 2011) or concentrate pellets with moderate energy content (Lanza et al., 2001; Priolo et al., 1998). In other studies, it has been supplied with polyethylene glycol to bind their proanthocyanidins (or condensed tannins, CT) and thereby disentangle their role in animal performance and digestion (Priolo et al., 2000; Priolo et al., 2002). Despite extensive descriptions of the detrimental effects of tannins on feed conversion (Priolo et al., 2000; Priolo et al., 2002; Silanikove et al., 2006), the ratio whereby carob pulp inclusion affects feed digestibility and productive performance in high energy concentrate diets remains to be determined.

Some Mediterranean countries, as Spain, produce early weaned lambs fed on energy-rich and concentrate-based pellet diets and slaughtered at light BW. In this production system, graded inclusion of dietary carob pulp has not been evaluated. Therefore, the goal of this study was to assess the effects of carob pulp level in the concentrate pellet on animal performances and budget behaviours, total tract digestibility of nutrients and blood metabolic indicators in light lambs from 15 to 25 kg of BW.

2. Materials and methods

The animals were handled and slaughtered in accordance with the Spanish Animal Protection Regulations RD 53/2013, which complies with European Union Directive 2010/63 with regard to the protection of animals used for experimental and other scientific purposes. The lambs were raised in commercial conditions following the Council Directive 98/58/EC concerning the protection of animals kept for farming purposes.

2.1. Animals, diets and experimental design

The experiment was conducted in the experimental facilities Nial of BonÀrea Agrupa, located in Guissona (Lleida, Catalonia, Spain, 41º46′32.2″ N, 1º16′ 33.2″ E; 484 m above sea level). A total of 144 weaned male and female crossbred lambs (14.7 ± 1.5 kg of BW; 41 ± 5.5 days of age) from two consecutive batches were allotted into 12 pens each batch (3 male and 3 female lambs per pen) in loose- housed sheds (0.86 m_2/lamb) until slaughter at a target BW of 25 kg. The study was replicated in two equal batches of 72 lambs each: batch 1 in winter, from January to February (6 weeks) and batch 2 in summer, from June to July (7 weeks). The lambs of both batches were distributed in homogeneous light and heavy pen groups according to their initial BW to minimize the within-pen BW variation. The average monthly temperature for January and February was 4.8 and 9.1 ◦C, and for June and July it was 19.4 and 24.6 ◦C, respectively.

The lambs were submitted to one of three isocaloric (1760 kcal of Net Energy for Ruminants/kg of concentrate) and isoproteic concentrates (175 g/kg of crude protein, CP) (Table 1), which had been formulated with graded carob pulp inclusion: C0 (without carob pulp), C15 (150 g/kg of carob pulp) or C30 (300 g/kg of carob pulp) with a total of 8 pen replicates per treatment (4 replicates/ treatment x 2 batches).

Table 1. Ingredients of the concentrate pellets (g/kg).

Ingredients	**C0**	**C15**	**C30**
Maize	280	280	227
Barley	289	150	50
Wheat	80	80	80
Wheat bran	40	20	10
Gluten maize feed	120	71	30
Soybean meal (470g CP/kg)	144	188	225
Carob pulp	0	150	300
Palm oil	9	30	54
CaCO3	25	18	11
Salt	10	10	10
Vitamin-mineral premix [a]	3	3	3

[a] The vitamin-mineral premix contained in each kilogram of feed: Vitamin A, 10620 UI; Vitamin D3, 2550 UI; All-rac-tocopheryl-acetate (vitamin E), 300 UI; Vitamin B1, 6.7 mg; Zn, 61.1 mg; Mn, 46.6 mg; Fe, 21.4 mg; Cu, 7.5 mg; I, 0.77 mg; Co, 0.72 mg; Se (Sodium selenite), 0.45 mg; Butylated hydroxytoluene (E321), 112.5 mg and Propyl gallate (E310), 13.5 mg.

The nutritional value and technological properties of the pellet concentrates is shown in Table 2. As a first mechanical process, the pods are crushed to separate the seeds from the pulp, and then the carob pulp is ground into simple pieces, resulting in a final product of tiny kibbles (Rodríguez-Solana et al., 2021). The carob pulp had 902 g DM/kg, 33 g ash/kg DM, 51 g CP/kg DM, 333 g of neutral-detergent fibre (aNDFom)/kg DM, 399 g of acid-detergent fibre (ADFom)/kg DM, 191 g lignin/kg DM, and 543 g total sugars/kg DM. The closeness between the aNDFom and ADFom values in carob pulp has been reported elsewhere (Albanell et al., 1991), and it may be because the method of ADFom determination leads to some polymerisation of the sugars with the fibre fraction, resulting in an artificial increase of the apparent ADFom content (Milad et al., 2010). These differences and the large variability in ADFom and lignin content could be also explained by the presence of tannins, as tannin-protein complexes may be detected as artificial fibre (Silanikove et al., 2006). Total polyphenol content was 21.5 eq-g tannic acid/kg and its total CT content was 263.4 eq-g CT carob pulp/kg DM.

Table 2. Chemical composition, colour attributes and physical pellet quality of the concentrates.

Analysed composition	**C0**	**C15**	**C30**
Gross energy (MJ/kg)	18.1	18.6	19.3
Dry matter (g/kg, DM basis)	884	882	880
Ash (g/kg, DM basis)	62	67	60
Crude protein (CP) (g/kg, DM basis)	173	180	176
Ether extract (g/kg, DM basis)	38	50	72
Starch (g/kg, DM basis)	467	415	294
Total sugars (g/kg, DM basis)	60	98	150
Neutral-detergent fibre (aNDFom) (g/kg, DM basis)	211	229	235
Acid-detergent fibre (ADFom) (g/kg, DM basis)	64	101	150
Lignin (sa) (g/kg, DM basis)	6.4	28.6	63.6
Phosphorus (g/kg, DM basis)	3.9	3.5	3.0
Total polyphenols (g tannic acid eq./kg freeze-dried)	8.0	9.9	12.1
Total condensed tannins (g carob pulp total CT-eq./kg freeze-dried)	4.1	20.6	54.0
Extractable (% out of total condensed tannins)	23.4	4.5	3.6
Protein-bounded (% out of total condensed tannins)	54.2	67.9	79.3
Fibre-bounded (% out of total condensed tannins)	22.4	27.6	17.1
CIELAB colour attributes			
L* (Lightness)	72.9	67.9	63.1
a* (Redness)	7.2	7.3	8.4
b* (Yellowness)	21.9	26.0	29.7
Physical pellet quality			
Fine particles (%)	1.0	0.8	5.6
Durability (%)	97.1	96.7	96.2

Before the experiment started, the lambs were fed a standard commercial concentrate without coccidiostatic for a 5-day adaptation period. The experimental concentrates were formulated with the same

ingredients and additives and they were manufactured in the same batch. The feed presentation was granulated with a pellet diameter of 3.5 mm (Fig. 1) and the granulation temperature was 60 ºC.

The lambs had free access to concentrate pellets and, wheat straw through separate feeders, and they were supplied water ad libitum in circular drinking bowls. The nutritional value of the used wheat straw was: 51 g ash/kg DM, 21 g CP/kg DM, 5 g phosphorus/kg DM. Total polyphenol content of wheat straw was 13.5 eq-g tannic acid/kg DM, and its total CT content was 5.7 eq-g CT carob pulp/kg DM.

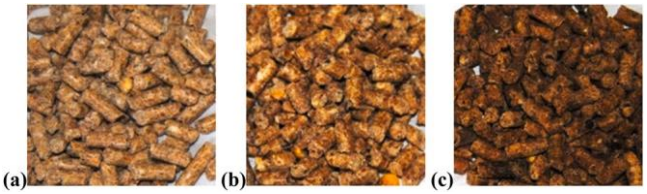

Figure 1. Concentrate pellet views: C0 (0 g of carob pulp/kg of concentrate) (a), C15 (150 g of carob pulp/kg of concentrate) (b) and C30 (300 g of carob pulp/kg of concentrate) (c).

2.2. Animal performance

Lambs were weighed individually once weekly to calculate their average daily gain (ADG, g/day). At the target slaughter BW, the lambs were transported to the commercial abattoir (3 km away from the farm), where they had water ad libitum. The fasting period lasted 3–4 h. Pre-slaughter BW and hot carcass weight were recorded to calculate the carcass dressing percentage.

The supplied dietary concentrate and straw was recorded daily, their orts were measured once weekly on a pen basis. The feed conversion rate (FCR, g/g) of concentrate was calculated as the ratio between average daily concentrate intake (as-fed basis) and ADG of lambs on a pen basis. In addition, total FCR was also calculated by including straw intake in the previously used formula.

2.3. Faecal sampling and calculation of apparent nutrient digestibility

Individual faeces samples were collected by rectal stimulation in all lambs of each pen at 50, 65 and 80 days of age between 8:00 and 9:00 a.m. in each batch, pooled (50–70 g approximately per pen) and stored in plastic food bags following the method described in Pelegrin-Valls et al. (2021a). Samples of concentrate and straw were also collected on a pen basis at the same time for technological assessment and chemical analyses. All feedstuffs and faecal samples that were submitted to subsequent chemical determinations were kept at -20 ºC until analysis. Acid-insoluble ash (AIA) was analysed in them to estimate the coefficients of total tract apparent digestibility (CTTAD) of DM and selected nutrients by the nutrient-marker ratio method, as follows:

$$CTTAD = 1 - [(Marker_{diet}/Marker_{faeces}) \times (Z_{faeces}/Z_{diet})]$$

where Z_{faeces} and Z_{diet} are the nutrient concentrations in faeces and diet (both concentrate and straw),

respectively. The $Marker_{faeces}$ and $Marker_{diet}$ are the concentrations of AIA in faeces and diet, respectively. The Zdiet and Markerdiet were calculated by considering the amount of concentrate and straw consumed per pen. The recovery rate of AIA in faeces was assumed to be complete (Sales and Janssens, 2003).

2.4. Behaviour recording

At the age of 50, 65 and 80 days, behaviour recordings were performed by a single observer by scan sampling of experimental pens between 9:00 a.m. and 2:00 p.m. at 10 min intervals, following the procedure of Averós et al. (2014). Briefly, for each observation day, the experimental pens were observed in a random order, starting at 9:00 a.m. Each experimental pen was observed twice (10 min x 2 replicates) through a series of continuous scan samplings. During each 10 min scan sampling, the behaviour of all lambs within the pen were sequentially collected. An average of 10 scan samplings was collected per 10 min observation. The ethogram used for scan sampling comprised: drinking, exploring the pen fixtures, eating concentrate, eating straw on the feeder or bedding, moving around the pen, negative social interactions, positive social interactions, lying resting, self-grooming, and standing static.

2.5. Blood sampling

Blood samples were collected from two lambs per pen (one female and one male) the day after animal behaviour recordings (days 50, 65 and 80 of age). The same lambs, that were randomly selected at the start of the experiment, were sampled the same three times. Vacuum tubes with EDTA were used to collect 4 mL of blood from the jugular vein (8 pen replicates/dietary treatment) and were centrifuged in situ at 3000g for 10 min to obtain the plasma, which was stored in identified aliquots for each sampled lamb at - 20 ºC until metabolites analysis.

2.6. Analyses

Selected technological characteristics, as the colour and the pellet quality of the concentrates, were assessed in triplicate. The colour attributes were determined after chopping the pellets by the CiELab colour coordinates (L*, lightness; a*, redness; and b*, yellowness) with spectrophotometer (CM-2600d Konica Minolta Sensing Inc., Osaka, Japan). A screen sieve of 2 mm was used to determine the percentage of feed non-pelletized dust particles after a 30-second shaking of a 300 g-sample. The pellet durability was evaluated subsequently with a 150 g-sample in a Pfost durability tester (weight of pellets after 10 min of tumbling 5 min x 50 rpm/ weight of pellets before tumbling x 100).

The feedstuffs and faecal samples that had been frozen were thawed, weighed again and dried in a forced air stove at 60 ºC for 72 h. Their moisture was determined by the weight difference between the fresh and DM. After drying, samples were ground in a knife mill to pass a 1 mm sieve to analyse AIA, organic matter (OM), CP, ether extract (EE), and phosphorus to calculate the CTTAD.

The gross energy of concentrates was analysed with a calorimeter bomb (Gallenkamp auto bomb CBA-305–010 M; Sanyo Gallenkamp PLC, Sussex, UK). Furthermore, the ash content to calculate OM was determined with 2 g of sample in a muffle furnace at 550 ºC for 3 h. The AIA of concentrates and straw was analysed following a standard procedure (BOE, 1995). CP content (N × 6.25) was determined following the Dumas procedure, using a nitrogen and protein analyser (Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain). EE was determined with an XT10 Ankom extractor (Ankom Technology Corporation, Fairport, NY, USA). Phosphorus was determined by ultraviolet-visible spectroscopy (ICP-OES, HORIBA Jobin Yvon, Activa family, with AS-500 Autosampler, HORIBA Scientific, Madrid, Spain). In addition, concentrates and straw were sequentially analysed for ADFom and lignin (sa), whereas the concentrate was analysed for aNDFom and the straw was alternatively analysed for NDFom, using the Ankom 200/220 fibre analyser (Ankom Technology Corporation, Fairport, NY, USA). The aNDFom was assayed with a heat-stable amylase and expressed exclusive of residual ash, whereas ADFom was expressed exclusive of residual ash and lignin was determined by solubilization of cellulose with sulphuric acid. Total starch and total sugars in the concentrates were determined by the approved polarimetry (EWERS) and Luff-Schoorl methods, respectively (BOE, 2000).

In addition, total polyphenols and CT composition were analysed in the feedstuffs and faeces. The total polyphenols and CT fractions were extracted in dried feedstuffs and faeces samples according to Rufino-Moya et al. (2019). Samples and standard calibration were measured with a Heλios β spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) at 725 nm, and polyphenol contents were expressed as tannic acid equivalents. The extractable CT, protein-bound CT and fibre-bound CT were fractioned following the method of Terrill et al. (1992), and quantified by the colorimetric HCl-butanol method described by Grabber et al. (2013). The standard used for the quantification of the samples was extracted and purified from frieze-dried carob pulp using the method described by Wolfe et al. (2008). Finally, samples and standard calibration were measured with the Heλios β spectropho- tometer at 550 nm and CT concentrations were expressed as carob pulp CT-equivalents. Feedstuffs and faeces chemical determinations were analysed in duplicate.

Plasma urea, creatinine, glucose, triglycerides and cholesterol were analysed by an automatic analyser (GernonStar, RAL S.A., Barcelona, Spain). Protocols and reagents were provided by the analyser manufacturer (RAL, Barcelona, Spain). The mean intra- and inter-assay coefficients of variation for these metabolites were < 4.4 % and < 7.7 %, respectively.

2.7. Statistical analyses

The productive performance from 11 lambs (2 lambs from C0, 5 lambs from C15, and 4 lambs from C30) were removed from the statistical dataset due to death (n = 3) or wasting syndrome (n = 8, reduction of BW from the start to the end of the experiment). These lambs belonged to the winter (n = 2) and summer

batch (n = 9), and they had not been involved in blood sampling. The FCR variable considered the pen average concentrate or total feed intake and the mean ADG of the remaining lambs in the pen.

The data were analysed with the statistical software JMP Pro15 (SAS Institute Inc., Cary, NC, USA). Concentrate and total feed intake and FCR were analysed with least square models including the fixed effects of dietary treatment (C0, C15, or C30) and the batch (1 vs. 2). Growth performance, behavioural parameters, plasma metabolites and nutrient digestibility variables were analysed through mixed models with repeated measurements that included the dietary treatment (C0, C15, or C30), the lamb age (50, 65 or 80 days), and the batch (1 vs. 2) as fixed effects, as well as the animal (for plasma metabolites) or pen (for behaviour and nutrient digestibility) nested within dietary treatments and batch as a random effect. The single interactions between fixed effects were also included in the models but they are only reported in the results section if they resulted significant ($P < 0.05$). The lamb sex effect and its interactions with diet, age and batch were also considered in growth performance and plasma metabolic variables, that were recorded in individual replicates. Least square means and their standard errors are presented. The separation of means was carried out with the Tukey′s test. The level of significance was set at 5 %. The association between dietary treatments and wasting syndrome or mortality of lambs was evaluated through Pearson's contingency coefficients.

3. Results

3.1. Animal performances and behaviour

The inclusion of dietary carob pulp did not affect most of the lamb performances (Table 3), except the concentrate FCR, that was impaired in C15 and C30 compared to control (C0) ($P < 0.05$). The straw to concentrate ratio decreased linearly throughout the fattening period (226, 192 and 133 ±5.5 g of straw/kg of diet in C0, C15 and C30, respectively; $P < 0.05$). On the other hand, female lambs had lower ADG than male lambs (211 vs. 244 ±9.5 g/day, $P < 0.01$), but the carcass weight did not differ across sexes (11.7 vs. 12.3 ±0.29 kg, respectively, > 0.05).

Table 3. Effect of dietary carob pulp inclusion on lamb performances during the fattening period.

	Carob pulp treatment [a]				
Items	**C0**	**C15**	**C30**	**SEM**	***P*-value**
Age at start (days of age)	41.6	41.0	41.2	0.82	0.89
Initial BW (kg)	14.7	14.8	14.7	0.23	0.97
Within-pen coefficient of variation of initial BW (%)	5.54	5.95	6.35	0.675	0.70
ADG (g)	230	232	220	11.6	0.76
Slaughter BW (kg)	25.4	25.6	25.0	0.61	0.76
Within-pen coefficient of variation of slaughter BW (%)	15.6	11.6	12.6	1.80	0.28
Carcass weight (kg)	12.2	12.2	11.6	0.35	0.39
Carcass dressing (%)	46.4	47.6	46.3	0.72	0.37
Concentrate intake (g/day, as-fed basis)	730	783	793	42.6	0.54
Straw supply (g/day)	184	186	184	4.1	0.94
Concentrate FCR (g/g)	3.44[b]	3.99[a]	3.95[a]	0.150	0.03
Total FCR (g/g)	4.09	4.33	4.55	0.205	0.31
Wasting syndrome (%)	4.17	6.25	6.25	-	0.87
Mortality (%)	0.00	4.17	2.08	-	0.36
Female/male ratio of lambs reaching target slaughter date	24/24	24/22	23/24	-	0.95

[a] C0: concentrate without carob pulp, C15: concentrate with 150 g carob pulp/kg and C30: concentrate with 300 g carob pulp/kg. SEM = Standard error of the mean. The concentrate feed conversion ratio (FCR) was calculated as the average daily concentrate intake divided by the average daily gain (ADG). The total FCR was calculated as the average daily concentrate and straw intake divided by the ADG. Different letter denotes statistical differences between dietary treatments in feed conversion ratio ($p < 0.05$).

The dietary treatment did not affect the activity budget or behaviour of lambs (Table 4), that was mainly affected by the lamb age. The time spent lying resting and the time standing static increased throughout the fattening period ($P < 0.05$). In contrast, the time spent eating straw and concentrate, as well as the time exploring the pen fixtures and moving around the pen, decreased concomitantly ($P < 0.05$). Finally, the time spent in self-grooming and the positive social behaviours decreased throughout the fattening period ($P < 0.05$).

Table 4. Effects of dietary carob pulp level and lamb age on the activity budget and behaviours (%, proportion of time, 9:00-14:00 h).

	Carob pulp treatment [a]					Days of age				
Items [b]	**C0**	**C15**	**C30**	**SEM**	***P*-value**	**50**	**65**	**80**	**SEM**	***P*-value**
Lying resting	49.2	52.8	44.4	6.72	0.67	30.5^{b}	51.2^{a}	64.7^{a}	6.04	<0.001
Eating straw on the feeder or the bedding	21.4	17.5	22.2	3.56	0.60	27.8^{a}	22.7^{a}	10.5^{b}	3.56	0.003
Eating concentrate	10.2	12.1	15.6	2.23	0.24	15.9^{a}	12.5^{ab}	9.5^{b}	1.94	0.05
Standing static	8.70	5.60	5.81	1.780	0.41	6.80^{b}	4.10^{b}	9.14^{a}	1.510	0.04
Exploring the pen fixtures	6.73	6.30	7.30	1.371	0.87	11.1^{a}	5.80^{b}	3.31^{b}	1.371	<0.001
Drinking water	1.10	0.62	1.13	0.267	0.34	1.41^{a}	0.93^{ab}	0.53^{b}	0.267	0.06
Moving around the pen	1.10	2.74	1.53	0.588	0.12	3.33^{a}	0.94^{b}	1.10^{b}	0.518	<0.001
Self-grooming	0.90	1.00	1.00	0.216	0.94	1.30^{a}	1.00^{ab}	0.60^{b}	0.216	0.08
Negative social interactions	0.65	0.90	0.40	0.196	0.24	1.05^{a}	0.24^{b}	0.62^{ab}	0.196	0.02
Positive social interactions	0.24	0.45	0.70	0.170	0.21	0.70^{a}	0.60^{ab}	0.13^{b}	0.170	0.07

[a] C0: concentrate without carob pulp, C15: concentrate with 150 g carob pulp/kg and C30: concentrate with 300 g carob pulp/kg. SEM = Standard error of the mean. Within each row and effect, different letter denotes statistical differences between dietary treatments or lamb ages ($p < 0.05$).

[b] Negative social interactions were considered as sudden, strong head contact with other lambs or kick another lamb to leave the resting place, while positive social interactions were considered as sniffing, nosing, grooming, nudging or licking other lambs.

3.2. Nutrient digestibility and faecal condensed tannins composition

The interaction between dietary carob inclusion and lamb age on the CTTAD of OM, CP, EE and phosphorus is shown in Fig. 2. The CTTAD of OM and CP did not differ among treatments at 50 days of age, but they were higher in C0 than in C30, while C15 was similar between diets at day 65 of age (Fig. 2 A and B, respectively, $P < 0.05$). However, at day 80 of age both C0 and C15 were higher than C30 ($P < 0.05$). Moreover, the CTTAD of EE did not change between dietary treatments at 50 and 65 days of age ($P > 0.05$), but it was higher in C0 than in C30 at 80 days of age (Fig. 2 C, $P < 0.05$). Finally, the CTTAD of phosphorus was lower in C15 than C0 and C30 at day 50 of age ($P < 0.05$) whereas no differences were observed at the subsequent days of age (Fig. 2 D, $P > 0.05$).

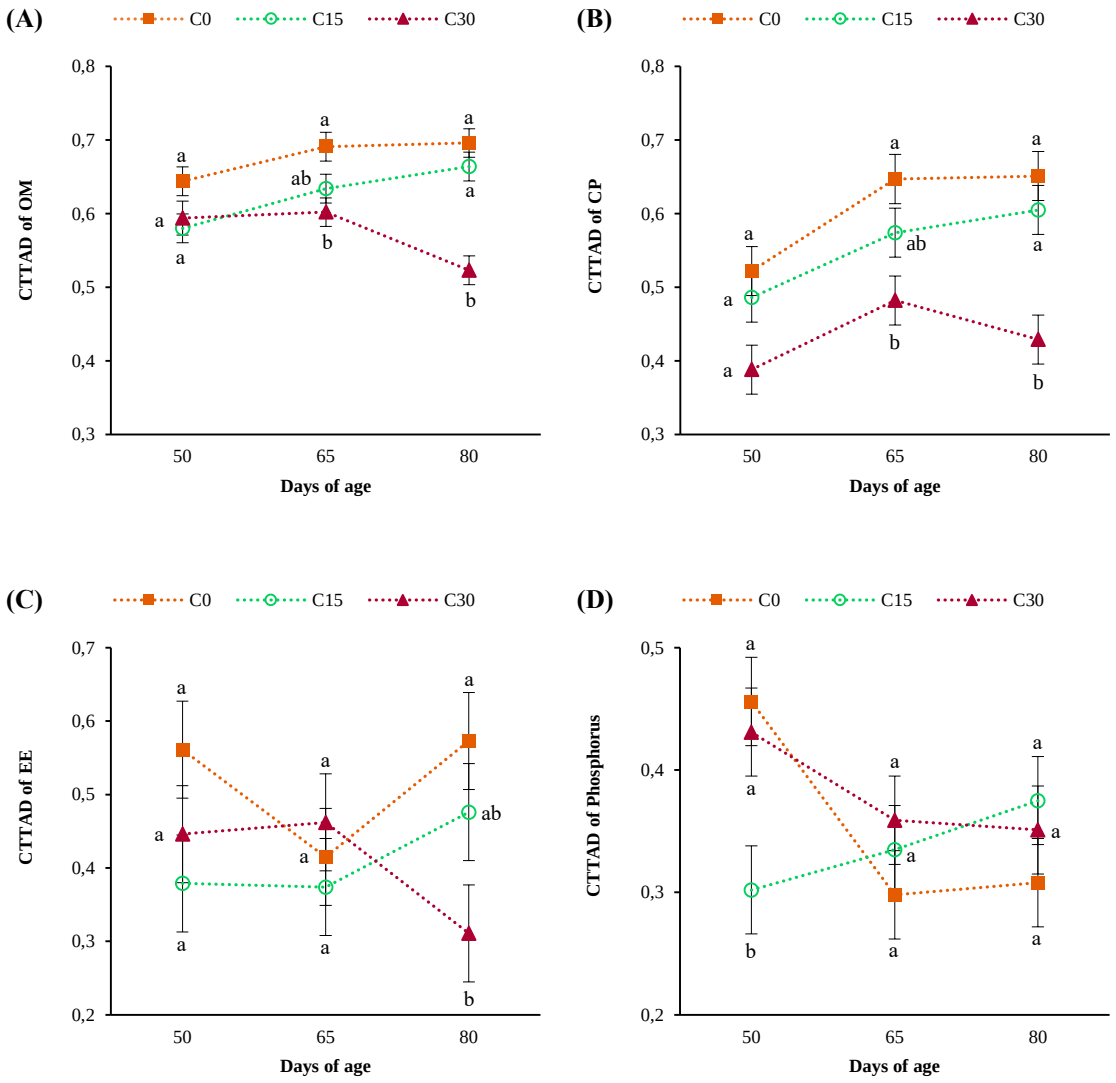

Figure 2. Interactions between dietary carob pulp inclusion (C0 = 0 g of carob pulp/kg of concentrate, C15 = 150 g of carob pulp/kg of concentrate and C30 = 300 g carob pulp/kg of concentrate) and lamb age on the coefficients of total tract apparent digestibility (CTTAD) of organic matter (OM) (A), crude protein (CP) (B), ether extract (C) and phosphorus (D) (least square mean values ± standard error). Within each sampling day, different letters (a, b) denote statistical differences ($p < 0.05$) between dietary treatments.

The independent effects of dietary carob pulp level and lamb age on the CTTAD of DM and CT composition are shown in Table 5. The following variables were not affected by the interaction between treatment and age period, thereby their main significant effects are exposed separately. Concerning the main effect of dietary carob, the CTTAD of DM were lower in C30 compared to C0 and C15 ($P < 0.05$). The total CT in faeces was higher in C30 compared to C0 and C15 ($P < 0.05$). The extractable and protein-bound CT fraction decreased linearly while the fibre-bound CT fraction increased linearly in the faeces with increasing the dietary carob pulp in the concentrate pellet ($P < 0.05$).

With regard to the effect of lamb age, the CTTAD of DM increased from 50 days of age onwards ($P < 0.05$). However, the total CT in faeces was not steady throughout the fattening period. Whilst the extractable CT fraction did not differ with lamb ages ($P > 0.05$), protein-bound CT fraction decreased at 80 days of age ($P < 0.05$) while fibre-bound CT fraction was lowest at 65 days of age ($P < 0.05$).

Table 5. Effects of dietary carob pulp level and lamb age on the faecal coefficient of total tract apparent digestibility (CTTAD) of dry matter and condensed tannins (CT) composition.

	Carob pulp treatment [a]					Days of age				
Items	**C0**	**C15**	**C30**	**SEM**	***P*-value**	**50**	**65**	**80**	**SEM**	***P*-value**
CTTAD of faecal DM	0.86[a]	0.83[a]	0.77[b]	0.013	<0.001	0.79[b]	0.83[a]	0.83[a]	0.008	<0.001
Faecal CT composition										
Total CT (g CT-eq/kg DM)	13.6[c]	31.9[b]	83.2[a]	4.16	<0.001	43.2[b]	36.8[c]	48.7[a]	2.70	<0.001
Extractable CT (% out of total CT)	11.8[a]	5.30[b]	1.80[c]	0.511	<0.001	6.05	6.40	6.41	0.380	0.64
Protein-bound CT (% out of total CT)	48.8[a]	28.4[b]	21.5[c]	1.60	<0.001	33.5[a]	34.6[a]	30.6[b]	1.24	0.01
Fibre-bound CT (% out of total CT)	39.4[c]	66.3[b]	76.7[a]	1.92	<0.001	60.4[ab]	59.0[b]	63.0[a]	1.42	0.03

[a] C0: concentrate without carob pulp, C15: concentrate with 150 g carob pulp/kg and C30: concentrate with 300 g carob pulp/kg. SEM = Standard error of the mean. Within each row and effect, different letter denotes statistical differences between dietary treatments or lamb ages ($p < 0.05$).

3.3. Blood metabolites

The dietary treatments did not affect plasma urea, creatinine, glucose, triglycerides and cholesterol concentrations (Table 6, $P > 0.05$), whereas the lamb age influenced plasma urea, which increased from 50 to 65 days of age in the fattening period ($P < 0.05$) and glucose, that decreased concomitantly ($P < 0.05$). The lamb sex only affected plasma urea levels, that were higher in female than male lambs (34.0 vs. 30.9 ±1.22 mg/dL, respectively, $P < 0.05$).

Table 6. Effects of dietary carob pulp level and lamb age on plasma metabolites.

	Carob pulp treatment [a]					Days of age				
Items	**C0**	**C15**	**C30**	**SEM**	***P*-value**	**50**	**65**	**80**	**SEM**	***P*-value**
Urea (mg/dL)	32.9	33.9	30.5	1.53	0.27	29.3[b]	33.1[a]	34.9[a]	1.26	0.002
Creatinine (mg/dL)	0.81	0.76	0.78	0.028	0.37	0.81	0.78	0.76	0.025	0.45
Glucose (mg/dL)	78.6	82.4	78.5	2.00	0.29	82.3[a]	78.6[b]	78.6[b]	1.42	0.01
Triglycerides (mg/dL)	4.25	3.68	4.38	1.055	0.88	5.03	4.47	2.81	0.916	0.15
Cholesterol (mg/dL)	29.6	32.4	25.8	2.97	0.30	29.1	27.8	30.8	2.14	0.39

[a] C0: concentrate without carob pulp, C15: concentrate with 150 g carob pulp/kg and C30: concentrate with 300 g carob pulp/kg. SEM = Standard error of the mean. Within each row and effect, different letter denotes statistical differences between dietary treatments or lamb ages ($p < 0.05$).

4. Discussion

Currently, there is no specific recommendation about the optimum dietary carob pulp that may be added to lamb diets in Mediterranean environments. The presence of CT in carob pulp may have adverse effects on digestion when it is included in high proportion in the diets of ruminants (Richane et al., 2022). The present study aimed at establishing the effects of dietary carob inclusion in concentrate-based diets on the growth performance, behaviour and metabolic markers of nutritional status in weaned light lambs.

The main constraint when including carob pulp is the low CP and high fibre content, that urges to balance the feed mixture with protein concentrates and oil sources to assure similar nitrogen and net energy supply to the animals. To overcome this drawback, the use of concentrate compound pellets may be an optimum feed presentation to improve the ADG and FCR compared to forage-based rations (Blanco et al., 2015). The study proved that daily growth rates of lambs were similar among dietary treatments and this led to similar final BW and carcass performance. This is in agreement with earlier studies using concentrate pellets (Priolo et al., 1998; Lanza et al., 2001) or total mixed rations for heavy lambs (Benbati et al., 2021; Gobindram et al., 2015; Obeidat et al., 2011). In this study, the concentrate pellets were formulated following the commercial nutrient recommendations for finishing light lambs in Spain (Ferret et al., 2008), which imply high energy and protein contents. However, the experimental diets of most of the afore-mentioned references included between 10 % and 35 % of carob pulp and were compared with control diets which had lower energy content than in the current work. When calculating the total CT contents of concentrates on the basis of carob pulp analysis and level of inclusion, their values were lower than expected (ranging from 31.6 % to 47.8 %). The reduction of CT content after pelleting may be explained by CT oxidation due to high temperature. In this process, plant cells would be damaged, allowing the release of previously sequestered soluble CT from the vacuole into the cytosol and forming complexes with proteins and fibres that may increase the protein-bound and fibre-bound CT fractions (Girard et al., 2018).

In the present study, the concentrate pellet intake was numerically greater when carob pulp was included in the diet, leading to a negative effect on concentrate FCR that was significantly increased in C15 and C30 compared to control (C0) treatment. In this regard, the dietary inclusion of carob pulp may have implications on feeding costs depending on certain feed price scenarios, as more protein and oil sources have to be added when a high proportion of carob pulp (C30) is included in the concentrate. An economic analysis was conducted by taking into account the highest, average and lowest price scenarios of feed ingredients and carcasses in 2020. The inclusion of carob pulp up to 300 g/kg did not affect the incomes and gross margin per lamb. In the lowest feed ingredients price scenario, the feeding costs per lamb did not differ across diets. However, in the average and highest feed ingredient price scenario, the feeding costs per lamb in C30 (11.1€) were around 1€ to 2.5€ higher than in C15 and C0, respectively. Therefore, the inclusion of carob pulp above 150 g/kg would affect the feed cost, but depending on the income per carcass it may have no impact on the gross margin, even with an inclusion of 300 g/kg (Pelegrin-Valls et al., 2021b). Likewise, the proportion of

lambs showing wasting syndrome or mortality were kept similar across diets.

Apparently, dietary treatment did not have effect on the activity budget and behaviours. In this regard, Gobindram et al. (2015) observed that including up to 35 % of carob in heavy lamb diets did not affect the overall feed intake throughout the day but modified the feeding behaviour by modulating the rate of ingestion (delaying the number of visits to the feeder, not attending soon after daily supply). As the overall concentrate and straw intake did not differ among dietary treatments, it would be feasible that carob-fed lambs reduced their eating rate compared to control diet in ad libitum feeding conditions, but no differences were detected in the time spent consuming concentrate or straw. The current results would support the hypothesis of temporal distribution of feeding activity according the avoidance-tolerance continuum mechanism (Iason and Villalba, 2006). Hence, behavioural strategies appear to be self-organized in ad libitum feeding in order to overcome the toxic effects of plant secondary metabolites, as could be CT.

Although, digestibility procedures fit the analytical standards, the effect of sampling time was evaluated within one day (Pelegrin-Valls et al., 2021a), but the variability between days could affect digestibility estimation (Morris et al., 2018; Thonney et al., 1985). For this reason, the results must be interpreted with caution as in some cases it would be interesting to obtain grab samples over several days when using low AIA diets. This study revealed a linear decrease in total tract digestibility of OM, CP and faecal CT content in lambs with increasing carob supply in the concentrate. However, the EE and phosphorus total tract digestibility were less affected by diet, which would imply the contribution of both nutrients to keep similar growth rates in lambs, regardless of carob inclusion. Priolo et al. (2002) found lower total tract digestibility of nutrients when including 56 % of carob pulp in ground mixed diets including 26 % of alfalfa hay for light lambs, which was attributed to the carob CT contents. This outcome was less marked in Awassi heavy lambs during fattening from 18 to 32 kg with an inclusion rate of 25 % of dietary carob pods replacing barley grain, as they could stand with total tract digestibility of nutrients (Obeidat et al., 2011). Reduced straw to concentrate ratio may also have contributed to the dif- ferences in the CTTAD of nutrients as the lambs grew. However, the inclusion of 300 g/kg of carob pulp in the diet of lambs would worsen nutrient digestibility (especially CP) probably as a result of the presence of high CT level, which may reduce proteolysis rate and inhibit the growth of proteolytic rumen microbes (Obeidat et al., 2012).

If the FCR were expressed as Digestible DM intake/kg ADG, the values would have been numerically similar across dietary treatments (3.0, 3.1 and 3.0 for C0, C15 and C30, respectively), suggesting that differences in animal performance would be related with the differences in nutrient digestibility.

The higher faecal CT content in carob-fed lambs (C15 and C30) may be beneficial to the environment when using this manure as fertilizer, as there is a delayed OM breakdown in soils and long-term build-up carbon sequestration and slow organic nitrogen release (Fagundes et al., 2021) that may mitigate nitrogen loss in crop or pasture systems by slowing nitrogen mineralization (Clemensen et al., 2020). Moreover, Gobindram et al. (2015) observed that although lambs were able to cope with a diet including up to 35 % of

carob pulp without hindrance to animal performance, they were probably under some form of metabolic stress, as they increased linearly the peripheral urea and reduced their triglycerides and cholesterol concentrations. However, the present study did not reveal any sign of undernutrition in the dietary treatments, which supports the coping abilities of feedlot light lambs to boost resilience and proves the diversification of feed ingredients in the concentrates as a viable strategy.

Finally, concerning the lamb age effect on animal behaviours, CTTAD of nutrients and metabolic status, this experiment observed an overall increase in passive activities that was concomitant to less time eating and drinking, and less time for social interactions, which proved that animal welfare was not compromised with advancing lamb fattening, since reduced lying and increased standing have been considered as indicators of discomfort in feedlot environments (Commun et al., 2012). In addition, CTTAD of nutrients (except phosphorus) increased throughout the fattening period, which may be linked to improved digestive function and metabolic adaptation to energy-rich concentrate feeding, as glucose oxidation by rumen epithelial cells isolated from lambs is normally low at birth, peaks at 14 days of age, and then decreases to adult levels between 42 and 56 days of age (Baldwin and Jesse, 1992). The reduction of blood glucose throughout the fattening period may reflect a gradual rumen adaptation to digest more carbohydrates which produce more volatile fatty acids as a source of energy to the detriment of their intestinal hydrolysis. Even though blood glucose decreases, the apparent DM digestibility of growing lambs can be kept steady (Giráldez et al., 2021), which is in line with the present study. This may suggest that other gastrointestinal nutrients uptake or their liver synthesis sustains the energy balance in fattening lambs.

5. Conclusions

The results of this study indicate that dietary carob pulp inclusion up to 300 g/kg does not negatively affect animal daily gains, behaviour daily activity budgets and metabolic nutritional status, but reduces diet digestibility. However, the concentrate feed conversion ratio increases above 150 g/kg of carob pulp inclusion. Thereby, the dietary inclusion of up to 150 g/kg of carob pulp in the energy-rich concentrate-based diet for weaned light lambs would be a good trade-off to keep the digestibility of nutrients, without any detrimental effect on behaviour time budgets or peripheral markers of energy and protein metabolism. This feeding strategy would have positive implications to boost circular economy principles within the Mediterranean sheep production systems.

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VI. CHAPTER 4

Impact of carob (*Ceratonia siliqua* L.) pulp inclusion and warm season on gastrointestinal morphological parameters, immune-redox defences and coccidiosis in concentrate-fed light lambs.

Abstract: This study aimed to evaluate the effects of dietary carob (*Ceratonia siliqua* L.) pulp and warm season on gastrointestinal morphological parameters, immune-redox defences and coccidiosis in concentrate-fed light lambs. Weaned lambs were assigned to one of three concentrate-based diets: C0 (without carob pulp), C15 (150 g/kg of carob pulp) and C30 (300 g/kg of carob pulp) from 40 to 80 days of age during two consecutive cold and warm batches. Blood samples were collected at the end of fattening (80 days) to determine the metabolic status. Rectal faeces were sampled at 50, 65 and 80 days of age to determine consistency and oocyst count per gram (OPG). Rumen, jejunum, and ileum samples were collected at slaughter for histological evaluation and gene expression analyses. The inclusion of carob pulp in lamb diets did not affect animal growth but reduced coccidia oocyst excretion and improved faecal consistency and gastrointestinal morphological parameters, particularly enhancing the ruminal thickness of the papilla living strata and reducing the darkness of the epithelium colour. Moreover, carob condensed tannins in the lambs' diet enhanced the expression of antioxidant SOD2 in rumen, while down-regulating NRF2, SOD1, CAT and PPARG in ileal tissues. However, the inclusion of up to 300 g of carob pulp/kg of concentrate increased the blood oxidative status marker, namely malonaldehyde. There was no interaction between the treatments and season in the evaluated variables. Lambs from the warm season exhibited reduced growth performance, altered ruminal epithelium, lower circulating iron levels, increased protein concentrations and higher susceptibility to coccidiosis. In addition, regulatory immune and antioxidant mechanisms to counterbalance reactive oxygen species production in gastrointestinal tissues were evident. Our results suggest that dietary inclusion of carob pulp (150 and 300 g/kg) in lamb diets improved gastrointestinal health and homeostasis but did not ameliorate the deleterious effects of warm season.

1. Introduction

Animal production will face significant challenges in terms of feeding systems due to global warming (Godde et al., 2021). Therefore, the feeding strategies utilized in the Spanish meat sheep sector will be crucial in mitigating these effects. While high-concentrate diets can improve ruminant production efficiency, they can also promote metabolic disorders and an altered ruminal environment (Askar et al., 2014; Metzler-Zebeli et al., 2013; Steele et al., 2012). The use of polyphenols, and specifically condensed tannins (CT), in ruminant production has been studied as a result of their influence on nutrition, productivity and health, with both beneficial and detrimental effects (Huang et al., 2018; Mueller-Harvey, 2006; Pelegrin-Valls et al., 2022a). CT have been shown to improve ruminal morphological traits, gut health and regulate certain stressors such as gastrointestinal parasites (Arroyo-López et al., 2014; Burke et al., 2013; Ouyang et al., 2019). However, their effect depends on local environmental conditions or the CT composition since they can be transformed or digested while travelling through the digestive tract (Quijada et al., 2018; Tretola et al., 2023). Furthermore, the presence of the two CT groups (prodelphinidins and procyanidins) in varying proportions in roughages can determine different biological efficacies (Patra and Saxena, 2011; Ropiak et al., 2016).

Carob (*Ceratonia siliqua* L.) is a tree native to the Mediterranean region that promotes the circular economy, as Spain is one of the main world producers (Pelegrin-Valls et al., 2022a). The pulp of the carob fruit, which is considered an agrifood waste, has been used specially in ruminant diets as a source of CT (Silanikove et al., 2006). Carob CT are predominantly prodelphinidins (96.7 % prodelphinidins/3.3 % procyanidins) with a high number of galloyl groups (41.1%) (Saratsi et al., 2020). Galloylation increases their ability to interact with other compounds as proteins (Mueller-Harvey, 2006), and contributes to their antiparasitic activity (Ramsay et al., 2016; Ropiak et al., 2016). Consequently, the increased formation of tannin-protein complexes causes reduced rumen protein degradation and higher dietary protein digestion in the small intestine, that might enhance host immune defences against gastrointestinal parasites (Ramírez-Restrepo et al., 2010).

Intensive rearing of lambs may lead to metabolic and physiological stress that could benefit parasites such as coccidia. These protozoan parasites of the genus *Eimeria* colonise the small and large intestine of young lambs. Following infection, the *Eimeria* life cycle takes 1-3 weeks (Bangoura and Bardsley, 2020) and begins with an initial invasion that targets the intestinal villi, destroying the crypts and promoting cell infiltration (Khodakaram and Hashemnia, 2017). Diarrhea is an important clinical sign of the disease that lead to poor weight gain or mortality. In this regard, the inclusion of carob pulp in lamb concentrates could be useful to control coccidiosis infection. Previous studies have shown how CT enhance mainly γδ T-cell populations, that regulate host innate immune responses and maintain tissue homeostasis and epithelial wound repair at mucosal surfaces (Tibe et al., 2012; Williams et al., 2016).

The gastrointestinal mucosa faces high metabolic demands during stressful conditions such as lamb fattening or heat stress. Under normal conditions, cellular antioxidant defences control the production of free

radicals with the help of antioxidant-rich food (Chauhan et al., 2016). Inflammation and oxidative stress are closely related, and thus, maintaining redox balance is crucial for optimal animal health. The most commonly reported antioxidant mechanisms of carob CT are related to their radical scavenging ability and absorption of metal ions (Ioannou et al., 2023), preventing hydroperoxide formation (Goulas and Georgiou, 2019), and promoting the enzyme antioxidant defences (Abu Hafsa et al., 2017; Martić et al., 2022). Accordingly, anti-inflammatory effects of carob-CT are associated with the inhibition of the expression of inflammatory mediators, such as TNF-α, IL-1β, as well as their antioxidant mechanisms, since they supress neutrophil myeloperoxidase activity (thereby preventing the conversion of H_2O_2 into HOCl) and in vitro reactive oxygen species (ROS) production (Rtibi et al., 2015). However, under heat stress conditions, ruminants need to carry out a series of physiological responses to maintain homeostasis that involve stress, energy metabolism and immune responses (Lu et al., 2019; Slimen et al., 2019). Besides the nutritional challenges, heat stress may also modify the normal function of gastrointestinal physiological mechanisms impairing the oxidative balance (Sejian et al., 2021). When ROS are formed in biological systems, one of the main lines of antioxidant defence strategy under heat stress might be led by the enzymes SOD and CAT, among others (Ighodoro and Akinloye, 2018).

Therefore, this study aimed to examine the effects of carob pulp CT on gastrointestinal morphological parameters, coccidiosis and the relationship with immune-antioxidant homeostasis. Furthermore, the inclusion of carob pulp was evaluated both in the cold and warm season to analyse potential interactions between heat stress and dietary carob antioxidants.

2. Materials and methods

Experimental lambs were managed and slaughtered according to the Spanish Animal Protection Regulations RD 53/2013, which complies with European Union Directive 2010/63 concerning the protection of animals used for experimental and other scientific purposes. All animal procedures used in this study were carried out in accordance of ARRIVE guidelines and approved by the Ethics Committee of the University of Lleida (CEEA 01-03/21).

2.1. Lambs, diets and experimental design

In this study, samples were collected for multiple determinations with several lambs involved, therefore a further description of the lambs or replicates per treatment is provided in the following sections. The experiment was carried out in the experimental facilities of El Nial of the BonÀrea Agrupa (Guissona, Lleida, Catalonia, Spain). The experiment was conducted in two equal consecutive batches, during the cold and warm seasons with a total of one hundred forty-four weaned crossbred lambs (41 days-old and 14.6 ± 0.32 kg initial body weight -BW). Lambs were not vaccinated against any disease before or during the experiment. No anticoccidial or antihelmintic synthetic drugs were used before or during the trial. The lambs were housed in

previously cleaned pens, which were disinfected and cleaned using calcium hydroxide between batches. The lambs were distributed in homogeneous groups according to their initial BW and sex-balanced (3 males and 3 females per pen) in 12 shared pens for each batch (6 lambs/pen; 0.86 m2 per lamb).

The lambs were fattened on three randomly assigned experimental diets consisting of C0 (concentrate with 0 g/kg of carob pulp inclusion), C15 (concentrate with 150 g/kg of carob pulp inclusion) and C30 (concentrate with 300 g/kg of carob pulp inclusion). Before the experiment started, lambs were fed standard commercial diets for five days with coccidiostat-free adapting concentrate. Briefly, pelleted experimental diets were formulated with the same ingredients and additives in the same manufacturing batch. The percentage inclusion of some ingredients was modified to keep all the diets isoenergetic (1760 kcal of Net Energy for Ruminants/kg of concentrate) and isoproteic (Pelegrin-Valls et al., 2022a). The lambs had ad libitum access to concentrate, water and barley straw throughout fattening, and their BW and feed intake were measured weekly to calculate average daily gain (ADG). The total proanthocyanidins content was 4.1, 20.6 and 54.0 g carob pulp total CT-eq./kg freeze-dried concentrate) for the group C0, C15 and C30, respectively.

2.2. Determination of blood metabolites

Blood samples were withdrawn from the jugular vein in vacuum tubes Z (BD Vacutainer®, Becton, Dickinson and Company, Plymouth, UK) (5 mL) and vacuum tubes with heparin (5 mL) (BD Vacutainer, Berkshire, UK) from two lambs per pen and batch, randomly selected at day 80 (1 male and 1 female; n = 16 replicates per dietary treatment). Samples were then centrifuged in situ at 3000 x-g and 4 ºC for 10 min to obtain serum and plasma, respectively, and stored at -20 °C until metabolite analysis.

Serum concentrations of total proteins (mg/dL), albumin (mg/mL), iron (mg/mL) and fructosamine (µmol/L) were determined as potential indicators of oxidative and parasitism status. All metabolites were analysed by an automatic analyzer (GernonStar, RAL/TRANSASIA, Dabhel, India).

In addition, malonaldehyde (MDA) and ABTS (2,2'-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid) were analyzed as pro- and antioxidative markers in plasma. The procedure for determination of total MDA, free MDA and protein-bound MDA has been described in Yonny et al. (2016). More details of the chromatographic conditions used are described in Bertolin et al. (2019). The ABTS radical cation (µmol Trolox-eq./mL) was obtained from a dilution of the plasma to 1/25 and its addition with the ABTS blend and subsequent calibration with Trolox as reference standard (Jiménez-Escrig et al., 2003).

2.3. Faecal sample analysis

In both batches, individual faecal samples (approximately 50 g) of all lambs were taken by rectal stimulation and pooled per pen at 50, 65 and 80 days of age (n = 8 replicates/treatment). After sampling, faecal samples were stored at 4 ºC until coprological analyses were performed at the following day.

2.3.1 Faecal coccidian oocysts

The number of coccidia in faeces (oocysts/g) was evaluated using a modification of the McMaster method (Bowman, 2020). Briefly, one gram of faeces was homogenized in 14 mL of zinc sulphate flotation solution (specific gravity = 1.18 g/cm^3) and filtered through cotton gauze. Once the solution was homogenized, each chamber of McMaster was filled with 3 mL. Then, the concentration of coccidia oocysts was estimated by screening two complete McMaster flotation chambers with microscope at 10x and the oocysts were multiplied by 100 to obtain the oocysts per gram of faeces (OPG) values. Lambs were classified according to OPG values, using arbitrary thresholds of 3,000, 10,000 and 50,000 oocysts/g of faeces. The coprology only considered coccidian count because the intensively-fed lambs have a short fattening period with low gastrointestinal helminths infection risk and the farm facilities were previously submitted to a sanitary break.

2.3.2. Dry matter content

The rest of the faecal sample was used for dry matter (DM) content determination, that was dried in a forced air stove at 60 ºC for 72 h. The moisture of samples was determined by the weight difference between the fresh and DM.

2.4. Sampling and processing of gastrointestinal tissues

The entire gastrointestinal tracts of 30 lambs were randomly sampled at slaughter (C0 = 10 lambs, C15 = 10 lambs and C30 = 10 lambs; 5 males and 5 females per dietary treatment), and quickly placed in an ice tray. Ruminal, jejunal and ileal tissues were collected for histology and gene expression analysis.

For histological and morphological examination, 5-cm^2 samples from the rumen cranial dorsal sac, jejunum and ileum were taken, rinsed with phosphate-buffered saline solution (PBS) and fixed in a 10% formalin solution. Tissue samples were trimmed and embedded in paraffin blocks to prepare 5-µm cross-sections using a rotary Microtome (Thermo Scientific Microm HM 325, Massachusetts, USA) and stained with hematoxylin-eosin (H/E).

For gene expression, a portion of ruminal tissue from the rumen cranial dorsal sac and a 2-cm section from both the mid jejunum and the ileum proximal to ileocecal valve were divided, rinsed with PBS, incubated in RNAlater (Invitrogen, Madrid, Spain) for 24 h at 4 ºC and finally stored at −80 °C until analysis.

2.5. Morphological and histological analysis of the gastrointestinal tract

Ruminal epithelium colour was visually analysed to assess the subjective degree of parakeratosis using a colour scale as follows: Light (1), brown (2) and dark (3) colours according to Álvarez-Rodríguez et al., (2012). The presence of rumen parakeratosis in the lambs was determined by the dark colour and the rest of the lambs were classified as without presence of parakeratosis by the brown and light colours.

From each rumen sample, two sections with 5 or more papillae were examined with a Motic BA310E microscope and digital pictures were taken at 10x and 40x magnification with a digital camera (Moticam

1080). Thickness of total rumen epithelium, thickness of rumen living strata layers (which contains the stratum basal, spinosum and granulosum, without corneum strata) and papilla epithelium keratinization degree was measured at 20 different sites in each picture using the image processing and analysis software (Motic Images Plus 3.0 ML, Kowloon, Hong Kong) (Scocco et al., 2013; Steele et al., 2011). Epithelial keratinization degree was calculated as the proportion that represented the keratin layer with respect to the thickness of rumen epithelium. Furthermore, lambs were classified according to the percentage of parakeratinisation in the rumen, based on the average percentage observed in concentrate-fed light lambs (Pelegrin-Valls et al., 2020).

Digital pictures were taken in jejunum and ileum at 10x magnification, and thickness of muscle layer was measured at 4 different sites using the image processing and analysis software (Motic Images Plus 3.0 ML, Kowloon, Hong Kong).

2.6. Quantitative real time polymerase chain reaction analysis (qPCR)

Total RNA was extracted with E.Z.N.A. kit (Omega BIO-TEK®, Norcross, GA) from 100 mg of ruminal, jejunal and ileal samples according to the manufacturer's instructions. Concentrations and purity of the total RNA were determined by NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Complementary DNA was synthesized from 1 μg of total RNA in the presence of random primers using the RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

In order to avoid genomic contamination and whenever it was possible to meet other primer stability requirements, the primers were designed to span an intron. For each gene, a standard curve was generated by amplifying serial dilutions of a control cDNA (copy DNA) to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplification was conducted using the SYBR green method of the ABI PRISM 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) under the conditions specified by the manufacturer. The qPCR conditions were carried out by an initial activation and denaturation step of 10 min at 95 °C followed by 40 cycles consisting of 10 s at 95 °C and 1 min at 60 °C. PCR reactions were run using 3 μL of 30-fold diluted cDNA as template in a total volume of 8 μL containing 1 x Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) as reported elsewhere (Pelegrin-Valls et al., 2020). Each measurement was carried out in triplicate and the average used to calculate the relative gene amount. Data were normalized and analysed by the $2^{-\Delta\Delta Ct}$ method using the real efficiency value obtained for each of the genes studied. Gene expression values were analysed in log form but expressed as relative quantification (González-Calvo et al., 2014).

Messenger RNA expression was determined by qPCR for the following target genes: *toll-like receptor 2* (*TLR2*), *toll-like receptor 4* (*TLR4*), *interleukin 10* (*IL10*), *transforming growth factor-β1* (*TGFB*), *tumor*

necrosis factor-α (*TNFA*), *interferon-γ* (*IFNG*), *nuclear factor E2-related factor 2* (*NRF2*), *peroxisome proliferator activator receptor-γ* (*PPARG*), *nuclear factor kappa β* (*NFKB*), *superoxide dismutase* 1 (*SOD1*), *superoxide dismutase 2* (*SOD2*), *catalase* (*CAT*), *glutathione peroxides 1* (*GPX1*), *glutathione peroxides 2* (*GPX2*), Na^+/H^+ *exchange isoform 1* (*NHE1*) and *down regulated in adenoma* (*DRA*), *β-actin* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ*) and *dystrobrevin binding protein 1* (*DTNBP1*). Primer sequences, studied tissues and the origins of the primers is shown in Table 1. Sequences of primers for DTNBP1, TLR2, TLR4, PPARG, NHE1 and DRA were designed with the Primer3Plus and Primer-Blast tools (PrimerBlast; Primer3Plus) and synthesized by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany). Gene normalization was achieved utilizing the three most stably expressed reference genes in each tissue according to NormFinder (Molecular Diagnostic Laboratory, Aarhus, Denmark) software: *GAPDH*, *YWHAZ* and *DTNBP1* in rumen, and *GAPDH*, *YWHAZ* and *ACTB* in jejunum and ileum.

Table 1. Primer sequences used for quantitative real-time PCR.

Gene	General gene role	Target tissue	Primer sequence (5′–3′)	bp	GenBank Accession	E (%)	nM	Source
GAPDH	Housekeeping	Jejunum – Ileum – Rumen	F: ATCTCGCTCCTGGAAGATG R: TCGGAGTGAACGGATTCG	200	NM_001190390.1	1.90	600 300	Puech et al., 2015
ACTB	Housekeeping	Jejunum – Ileum	F: CTGGACTTCGAGCAGGAGAT R: GATGTCGACGTCACACTTC	194	NM_001009784	1.94	600	Puech et al., 2015
YWHAZ	Housekeeping	Jejunum – Ileum – Rumen	F: TGTAGGAGCCCGTAGGTCATCT R: TTCTCTCTGTATTCTCGAGCCATCT	102	AY970970.1	2.07	200	Garcia-Crespo et al., 2005
DTNBP1	Housekeeping	Rumen	F: TGTCCCATGTTTGCCGAAGT R: GTGGAGTGCAGCCCATGTAT	76	NM_001126349.1	2.09	200	Primer3plus
TLR2	Inflammation regulation	Rumen	F: CGACGCCTTTGTGTCCTACA R: GGAGGGTTGAAGTGCTCCAG	84	NM_001048231.1	2.03	200	Primer3plus
TLR4	Inflammation regulation	Rumen	F: ACCCTTGCGTACAGGTTGT R: CAGGTCCAGCATCTCGGTTG	104	NM_001135930.1	2.10	200	Primer3plus
IL10	Anti-inflammatory	Jejunum – Ileum	F: TTAAGGGTTACCTGGGTTGC R: TTCACGTGCTCCTTGATGTC	109	NM_001009327.1	1.96	200	Pelegrin-Valls et al., 2020
TGFB	Anti-inflammatory	Jejunum – Ileum – Rumen	F: TTGACGTCACTGGAGTTGTG R: CGTTGATGTCCACTTGAAGC	120	NM_001009400.2	2.04	200	Pelegrin-Valls et al., 2020
TNFA	Pro-inflammatory	Jejunum – Ileum – Rumen	F: CAAATAACAAGCCGGTAGCC R: TGGTTGTCTTTCAGCTCCAC	118	NM_001024860.1	1.96	200	Pelegrin-Valls et al., 2020
IFNG	Pro-inflammatory	Jejunum – Ileum	F: AAGTTCTTGAACGGCAGCTC R: TTGGCGACAGGTCATTCATC	130	NM_001009803.1	1.91	500	Pelegrin-Valls et al., 2022b
NRF2	Redox system	Jejunum – Ileum – Rumen	F: GAGCCCAGTCTTCAATGCTC R: TCAGCCAGCTTGTCATTTTG	171	XM_015093345.2	1.97	200	Pelegrin-Valls et al., 2022b
PPARG	Transcription factor	Jejunum – Ileum	F: GATAAAGCGTCAGGGTTCCA R: TATGAGACATCCCCACAGCA	187	NM_001100921.1	1.98	200	Primer3plus
NFKB	Transcription factor	Jejunum – Ileum – Rumen	F: CTACACCTTGCCTGTGAGCA R: AAGGACACCAACAGCTCCAC	173	NM_001166184.1	1.93	300	Pelegrin-Valls et al., 2022b
SOD1	Antioxidant enzyme	Jejunum – Ileum – Rumen	F: CACCATCCACTTCGAGGCAA R: GCACTGGTACAGCCTTGTGT	126	NM_174615.2	2.06	200	Lesage-Padilla et al., 2017
SOD2	Antioxidant enzyme	Jejunum – Ileum – Rumen	F: GGATCCCCTGCAAGGAACAA R: TGGCCTTCAGATAATCGGGC	110	NM_201527.2	2.03	200	Lesage-Padilla et al., 2017
CAT	Antioxidant enzyme	Jejunum – Ileum	F: TTCCGTCCTTTATCCACAGC R: CCATTGGCATTAACCAGCTT	199	XM_004016396.5	2.04	300	Pelegrin-Valls et al., 2022b
GPX1	Antioxidant enzyme	Jejunum – Ileum	F: GGCATCAGGAAAACGCCAAG R: GGGGACCACGTGATGAACTT	217	XM_004018462.5	1.98	300	Pelegrin-Valls et al., 2022b
GPX2	Antioxidant enzyme	Jejunum – Ileum	F: ATTGAGAATGTGGCCTCGCT R: CCAGGGCGGACATACTTGAG	179	XM_004010720.5	2.07	300	Pelegrin-Valls et al., 2022b
NHE1	pH regulation and/or SCFA	Rumen	F: CGAAGAGGACCAGAACGAACA R: GAATCTGAACCATCCCCTTGT	117	XM_027963353.2	2.03	300	Primer3plus
DRA	pH regulation and/or SCFA	Rumen	F: CCCAGTCAGGAAGTAGAAGCA R: TTTTGTTTCAATTGGTACCTGACAT	96	NM_001184899.1	1.93	200	Primer3plus

Note: F = Forward; R = Reverse; bp = amplified product length in base pairs; E (%) = Efficiency; nM = Optimal primer concentration; SCFA = Short-chain fatty acids.

2.7. Climate variables

The average experimental period ambient temperature and relative humidity (RH) for January and February (cold season) was 6.9 ºC (89% RH) and 9.0 ºC (83% RH), and for June and July (warm season) it was 19.4 ºC (72,5% RH) and 23.3 ºC (58%RH), respectively.

Climatic data such as daily mean, maximum and minimum temperatures and relative humidity, were obtained throughout the experimental period from a meteorological station located ≤10 km from the barn. Temperature–humidity indices (THI) were calculated from ambient temperature (T; ºC) and relative humidity (RH%/100) according to the formula reported by Marai et al. (2001):

$$THI = T - \{(0.31 - 0.31\ RH) \times (T - 14.4)\}$$

The values obtained indicate the following: <22.2 = absence of heat stress; 22.2 to <23.3 = moderate heat stress: 23.3 to <25.6 = severe heat stress and 25.6 and more = extreme severe heat stress (Marai et al., 2001).

2.8. Statistical analysis

The data were analysed with the statistical software JMP Pro15 (SAS Institute Inc., Cary, NC, USA). Blood oxidative and parasitic markers, rumen and gut histomorphometric measurements and gastrointestinal gene expression markers at 80 days of age were analysed with least square models including the fixed effects of dietary treatment (C0, C15 or C30) and the season (cold vs. warm). Faecal DM and OPG were analysed through mixed models with repeated measurements that included the dietary treatment (C0, C15 or C30), the lamb age (50, 65 or 80 days), and the batch (cold vs. warm) as fixed effects, as well as the pen (nested within batch) as a random effect. The single interactions between fixed effects were also included in the models but they are only reported in the results section if they resulted significant ($P < 0.05$). Pearson tests on contingency tables were used to evaluate the association between OPG threshold values in lambs and the carob inclusion level within ages. Gastrointestinal gene expression data were log-transformed to meet the assumption of normality and homoscedasticity. Tissues from one lamb (a male from C0) were discarded from the gene expression analyses due to a traceability failure in slaughterhouse. Least square means and their standard errors are presented. The separation of means was carried out with the Tukey′s test. The level of significance was set at 0.05, but tendencies are reported if the level of significance was below 0.10.

3. Results

3.1. Weather conditions, lamb growth and blood metabolites

The average THI values for January and February (cold season) were 7.1 and 9.3, while for June and July (warm season) were 18.9 and 22.0, respectively. Similarly, the maximum THI values for January and February were 13.7 and 16.4, while for June and July were 31.0 and 31.9, respectively. Consequently, lambs in maximum THI warm season suffered extreme severe heat stress at certain times of the day.

The statistical analysis did not reveal any interactions between dietary treatment and season for any of the variables studied, and therefore, the results are presented separately. The overall ADG of lambs did not differ among dietary treatments (247, 233 and 249 ± 20 g/day in C0, C15 and C30, respectively, $P > 0.05$), but it was higher in cold than in warm season (278 and 208 ± 16.5 g/day, respectively, $P = 0.006$).

Serum total proteins, albumin, fructosamine and iron were not significantly affected by dietary carob pulp inclusion (Table 2, $P > 0.05$). Treatment did not have a significant effect on antioxidant capacity (ABTS) ($P > 0.05$). However, lower protein-bounded MDA and total MDA were observed in lambs from the C0 and C15 groups with respect to the C30 group ($P < 0.05$).

No significant effects of the season were observed on albumin, fructosamine, protein-bounded MDA and total MDA (Table 2, $P > 0.05$). However, lambs from the cold season showed higher serum iron concentration but lower serum concentrations of total protein to those from the warm season ($P < 0.05$). Additionally, the ABTS radical cation concentration also differed between seasons, with lower levels in the cold season compared to the warm season ($P < 0.05$).

Table 2. Serum metabolites and antioxidant status markers in crossbred lambs at slaughter (80 days of age) according to carob pulp inclusion level and season.

	Treatment					Season			
Item[1]	**C0**	**C15**	**C30**	**SE**	***P*-value**	**Cold**	**Warm**	**SEM**	***P*-value**
n	16	16	16			24	24		
Total proteins (mg/dL)	5.28	5.41	5.37	0.08	0.57	5.13[a]	5.58[b]	0.07	< 0.0001
Albumin (mg/dL)	3.06	3.11	3.19	0.04	0.13	3.15	3.09	0.03	0.33
Fructosamine (µmol/L)	295	307	306	7.82	0.46	299	307	6.39	0.36
Iron (mg/dL)	192	165	185	13.9	0.37	203[a]	158[b]	11.3	0.008
Protein-bounded MDA (µM/L)	5.82[a]	6.00[a]	6.69[b]	0.23	0.03	6.18	6.16	0.19	0.92
Total MDA (µM/L)	6.17[a]	6.33[a]	7.07[b]	0.23	0.02	6.57	6.48	0.19	0.73
ABTS (µmol TROLOX-eq/ml)	9.43	8.57	9.52	0.36	0.27	7.92[a]	10.5[b]	0.29	< 0.0001

Note: C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. Within each row, different letter denotes statistical differences ($p < 0.05$) between dietary treatments. SEM = Standard error of mean. [1] MDA= malonaldehyde; ABTS=2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid.

3.2. Faecal analyses

Faecal DM differed between dietary treatments and seasons. The faecal consistency of lambs in the C0 group was similar to the C15 group, but it was lower than the C30 group (319, 334 and 348 ± 7.9 g of DM/kg of fresh faeces, respectively $P < 0.05$). On the other hand, the average DM content of faeces was higher in the cold season compared to the warm season (348 vs. 318 ± 6.4 g of DM/kg of fresh faeces, respectively, $P = 0.004$).

The lambs were weaned at 40 days of age and they were assigned to dietary treatments with a mean starting OPG of 11,302 ± 8,755 oocysts/g ($P > 0.05$). No differences were observed between dietary treatments at 50 and 80 days of age, but at the age of 65 the OPG was higher in the C0 than the C15 and C30 groups (Fig. 1A, $P < 0.05$).

When the OPG was analysed as the percentage of lambs excreting ≥3,000, 10,000 and 50,000 oocysts/g faeces on days 50, 65 and 80 of age, it was found that a higher rate of lambs from the C0 shed more >50,000 oocysts/g at day 65 compared to the C15 and C30 groups ($P < 0.05$). Similarly, at day 80 of age, there was a higher proportion of lambs from the C0 and C15 groups shedding more >3,000 oocysts/g compared to the C30 group ($P < 0.05$) (Table 3).

The mean evolution of OPG throughout the fattening of lambs between the cold and warm season batches was similar at 40, 50 and 80 days of age (Fig. 1B, $P > 0.05$). However, at 65 days of age, lambs in the cold season batch exhibited lower OPG excretion compared to the warm season batch (Fig. 1B, $P < 0.05$). When the OPG was analysed as the percentage of lambs excreting ≥3,000, 10,000 and 50,000 oocysts/g faeces on days 50, 65 and 80 of age, a higher rate of lambs from the warm season shed >50,000 oocysts/g at day 65, and >10,000 oocysts/g at day 80 compared to lambs from the cold season ($P < 0.05$) (Table 3).

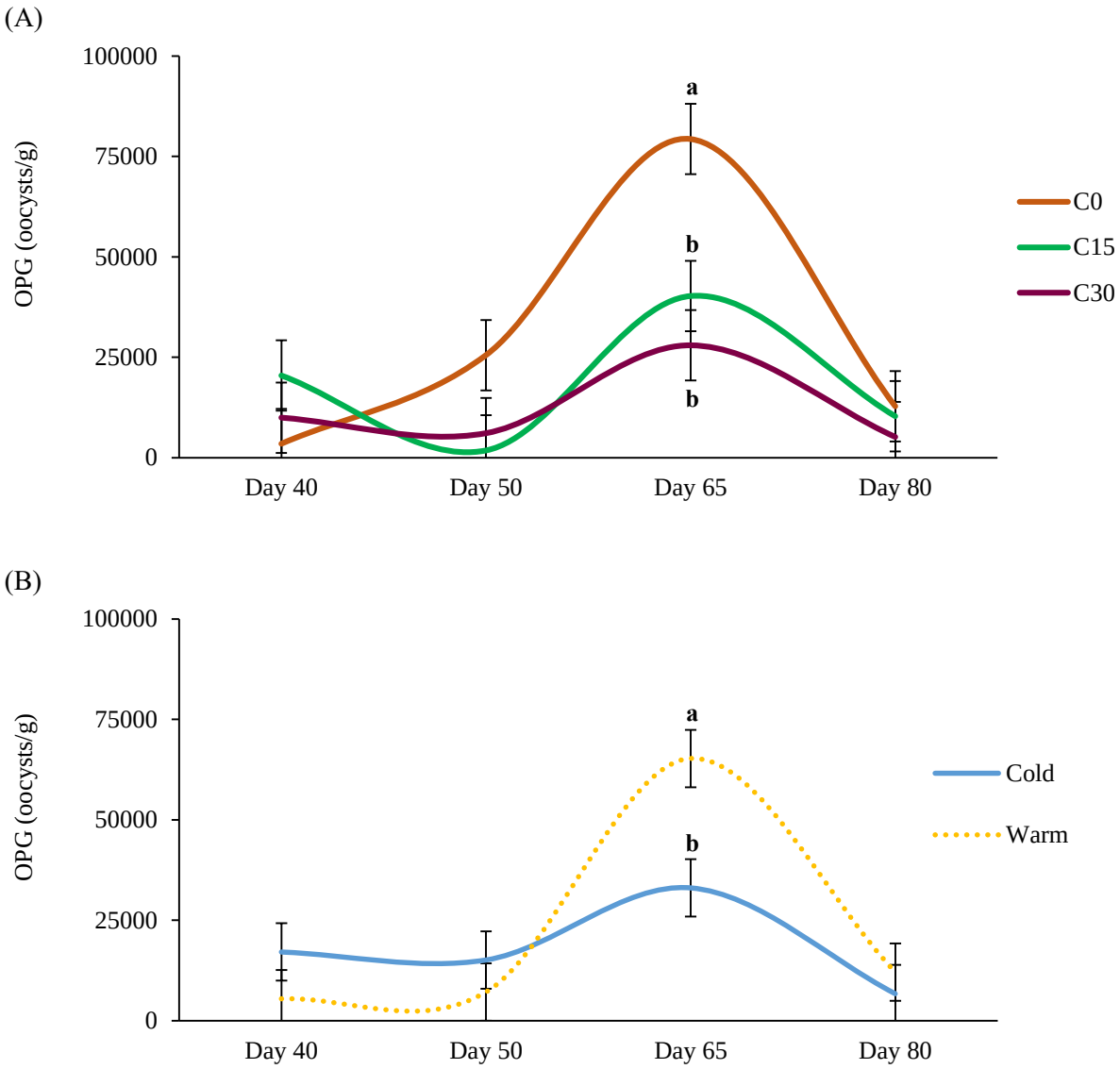


Figure 1. Oocysts count per gram (OPG) evolution in crossbred lambs according to the age and inclusion of carob pulp (A) and season (B). C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. [a,b] Denote statistical differences ($p < 0.05$) between inclusion of carob pulp or season within each age period.

Table 3. Percentage of crossbred lambs excreting ≥3,000, 10,000 and 50,000 OPG at 50, 65 and 80 days of age according to carob pulp inclusion level and season.

	Treatment				Season		
Item	**C0**	**C15**	**C30**	**P-value**	**Cold**	**Warm**	**P-value**
n	8	8	8		12	12	
Lambs excreting coccidian faecal oocysts (%)							
Day 50 of age							
>3,000 OPG in faeces	37.5	12.5	50.0	0.26	25.0	41.7	0.39
>10,000 OPG in faeces	37.5	0.00	12.5	0.12	25.0	8.3	0.27
>50,000 OPG in faeces	25.0	0.00	0.00	0.11	8.3	8.3	1.00
Day 65 of age							
>3,000 OPG in faeces	100	100	100	1.00	100.0	100.0	1.00
>10,000 OPG in faeces	100	87.5	100	0.35	91.7	100.0	0.31
>50,000 OPG in faeces	75.0[a]	50.0[b]	12.5[b]	0.04	25.0[b]	66.7[a]	0.04
Day 80 of age							
>3,000 OPG in faeces	100[a]	100[a]	62.5[b]	0.03	91.7	83.3	0.54
>10,000 OPG in faeces	50.0	50.0	12.5	0.20	16.7[b]	58.3[a]	0.04
>50,000 OPG in faeces	100	100	100	1.00	0.0	0.0	1.00

Note: C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. [a,b] Denote statistical differences ($p < 0.05$) between inclusion of carob pulp or season.

3.3. Gastrointestinal histology

Morphological and histological analysis of the rumen and small intestine are presented in Table 4. Regarding rumen tissues, the dietary treatments had no significant effect on the thickness of the epithelium and muscular layer ($P > 0.05$). However, the thickness of the papilla living strata (comprising basal, spinous, and granular stratum) was lower in C0 group compared to C30 ($P < 0.05$), although C15 showed no differences with the other two treatments ($P > 0.05$).

The analysis of rumen papilla keratinisation degree showed no differences between treatments ($P > 0.05$). However, when the data were analysed as the percentage of lambs with ≥30% parakeratosis, it was observed that lambs from the C0 group tended to have more parakeratosis than C30, with C15 showing similar results to both treatments ($P < 0.10$). Additionally, the application of the colour scale to determine parakeratosis (Fig. 2) demonstrated that most lambs in the C0 group had more parakeratosis compared to those in C15 and C30 (Table 4, $P < 0.05$). The muscular layer of the jejunum did not any changes with carob pulp inclusion ($P > 0.05$). However, lambs from the C0 group showed increased thickness of the ileum muscular layer compared to those from C15 group ($P < 0.05$).

Regarding the effect of the season, lambs from the cold season exhibited lower development of the rumen papilla compared to lambs from the warm season. Additionally, a lower percentage of papillae keratinisation and a lower percentage of lambs with ≥30% parakeratosis were observed in cold season compared to the warm season ($P < 0.05$). However, both cold and warm season

lambs had a similar level of parakeratosis based on the colour grading *(P* > 0.05). The thickness of smooth muscle layer of the small intestine did not show any variation between seasons (*P* > 0.05).

Table 4. Morphological and histological characteristics of the gastrointestinal tract in crossbred lambs at slaughter (80 days of age) according to carob pulp inclusion level and season.

	Treatment					Season			
Item[1]	**C0**	**C15**	**C30**	**SEM**	***P*-value**	**Cold**	**Warm**	**SEM**	***P*-value**
n	9	10	10			11	18		
Dorsal rumen characteristics									
Thickness of epithelium (µm)	60.6	64.7	63.9	2.22	0.38	51.9^{a}	74.1^{b}	1.85	<0.0001
Thickness of living strata (µm)	42.9^{a}	46.8^{ab}	48.8^{b}	1.96	0.05	38.9^{a}	53.5^{b}	1.63	<0.0001
Thickness of muscle layer (µm)	820	927	903	99.4	0.72	696^{a}	1071^{b}	82.8	0.004
Papilla average keratinization (%)	17.7	17.9	15.0	1.70	0.42	13.0^{a}	20.7^{b}	1.40	0.0009
Lambs with parakeratosis >30%	55.6^{x}	20.0^{xy}	11.1^{y}	-	0.08	10.0^{x}	38.9^{y}	-	0.09
Lambs with parakeratosis according to the colour scale (%)	77.7^{a}	10.0^{b}	0.00^{b}	-	0.05	27.3	27.8	-	0.97
Small intestine characteristics									
Muscular layer of jejunum (µm)	245	193	219	22.9	0.30	202	236	18.9	0.22
Muscular layer of ileum (µm)	294^{a}	200^{b}	225^{ab}	26.9	0.05	218	262	22.3	0.18

Note: C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. Within each row, letters ^{a,b} denotes statistical differences ($p < 0.05$) and letters ^{x,y} denotes statistical tendencies between dietary treatments ($p < 0.10$). SEM = Standard error of mean. [1] Thickness of epithelium values are the sum of all the strata of the epithelium, including the stratum corneum. Thickness of living strata values are the sum of SB = Stratum basal, SS = Stratum spinosum and SG = Stratum granulosum, without stratum corneum layer.

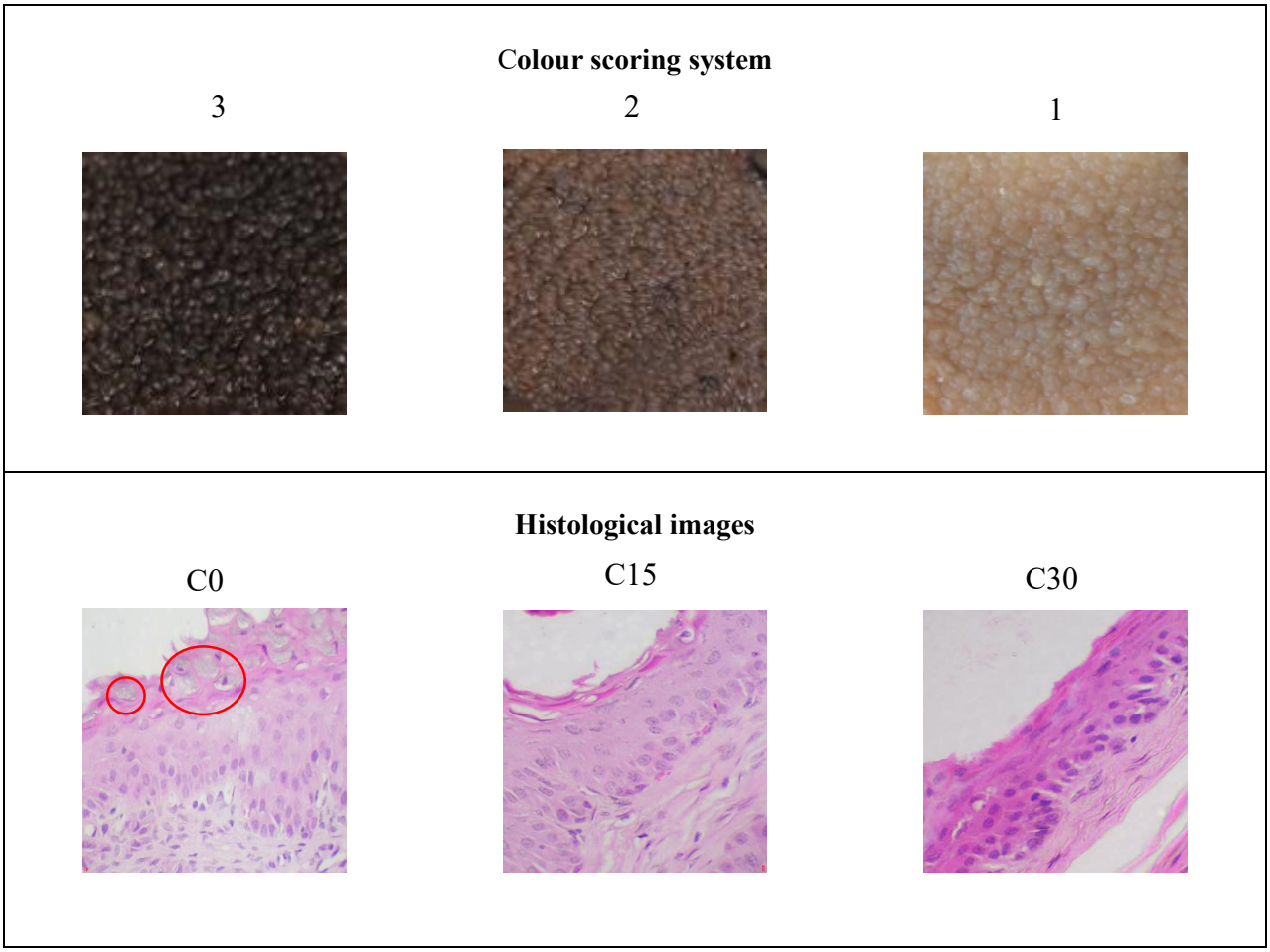


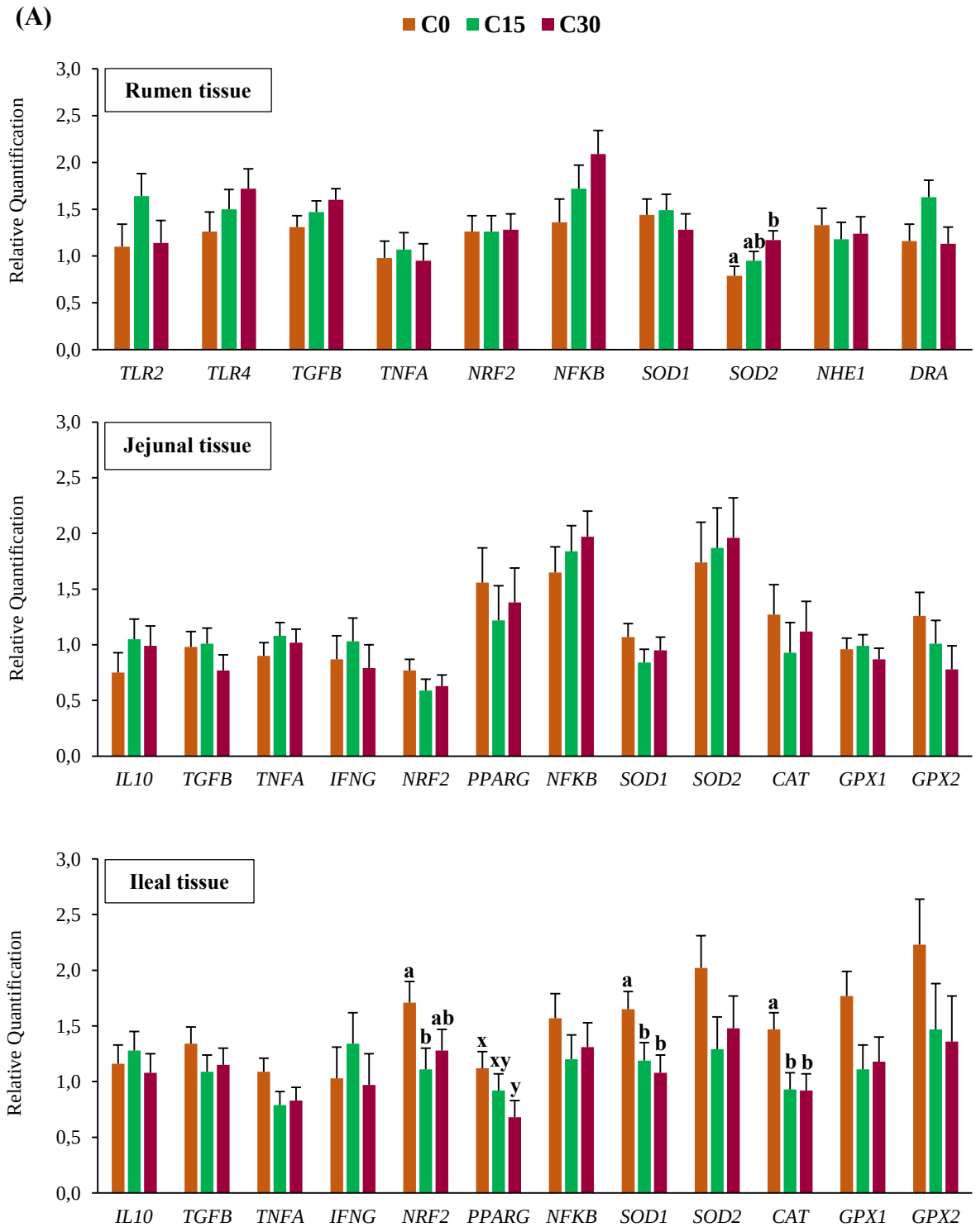
Figure 2. Colour scoring system used for subjective classification of rumen parakeratosis and histological images (40x) of the rumen according to the inclusion of carob pulp in the diet of lambs. The colour scoring system was categorised as follows: Dark colour (3; presence of parakeratosis), brown colour and Light-coloured (2 and 1, respectively; without presence of parakeratosis). C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. The red circles in the histological sample of the C0 rumen show some examples of parakeratosis vacuoles found during microscopic analysis.

3.4. Gene expression analysis

Figure 3A shows the differences in gene expression observed in the cranial dorsal rumen sac, jejunum and ileum between the dietary treatments. In the rumen, the expression of SOD2 was higher in lambs from C30 compared to C0 ($P < 0.05$). There was no significant effect of the treatments on gene expression in the jejunum ($P > 0.05$). However, treatment did influence the expression of NRF2, PPARG, SOD1 and CAT expression in the ileum. The expression of NRF2 was higher in the C0 group compared to the C15 group ($P < 0.05$), while PPARG expression tended to be higher in C0 compared to C30 ($P < 0.10$). Furthermore, higher expression of SOD1 and CAT were observed in C0 compared to C15 and C30 ($P < 0.05$). The remaining

genes analysed in the three tissues did not exhibit significant differences between the treatments ($P > 0.05$, Fig. 3A).

Figure 3B presents the differences in gene expression found in the cranial dorsal rumen sac, jejunum and ileum between the analysed seasons. In the rumen, expression levels of TLR2 and TGFB were higher in lambs from the warm season than those from the cold season ($P < 0.05$). In jejunal tissue, lower GPX2 and PPARG expression ($P < 0.05$ and $P < 0.10$, respectively), and higher TGFB and TNFA expression ($P < 0.05$ and $P < 0.10$, respectively) were observed in lambs from the warm season. In ileum, expression levels of NRF2, PPARG, NFKB, SOD1, SOD2 and CAT ($P < 0.05$) and IFNG ($P < 0.10$) were higher in lambs from the warm season compared to lambs from the cold season.


(A)
C0
C15
C30
Rumen tissue
Relative Quantification
3,0
2,5
2,0
1,5
1,0
0,5
0,0
TLR2
TLR4
TGFB
TNFA
NRF2
NFKB
SOD1
SOD2
NHE1
DRA
a
ab
b
Jejunal tissue
IL10
TGFB
TNFA
IFNG
NRF2
PPARG
NFKB
SOD1
SOD2
CAT
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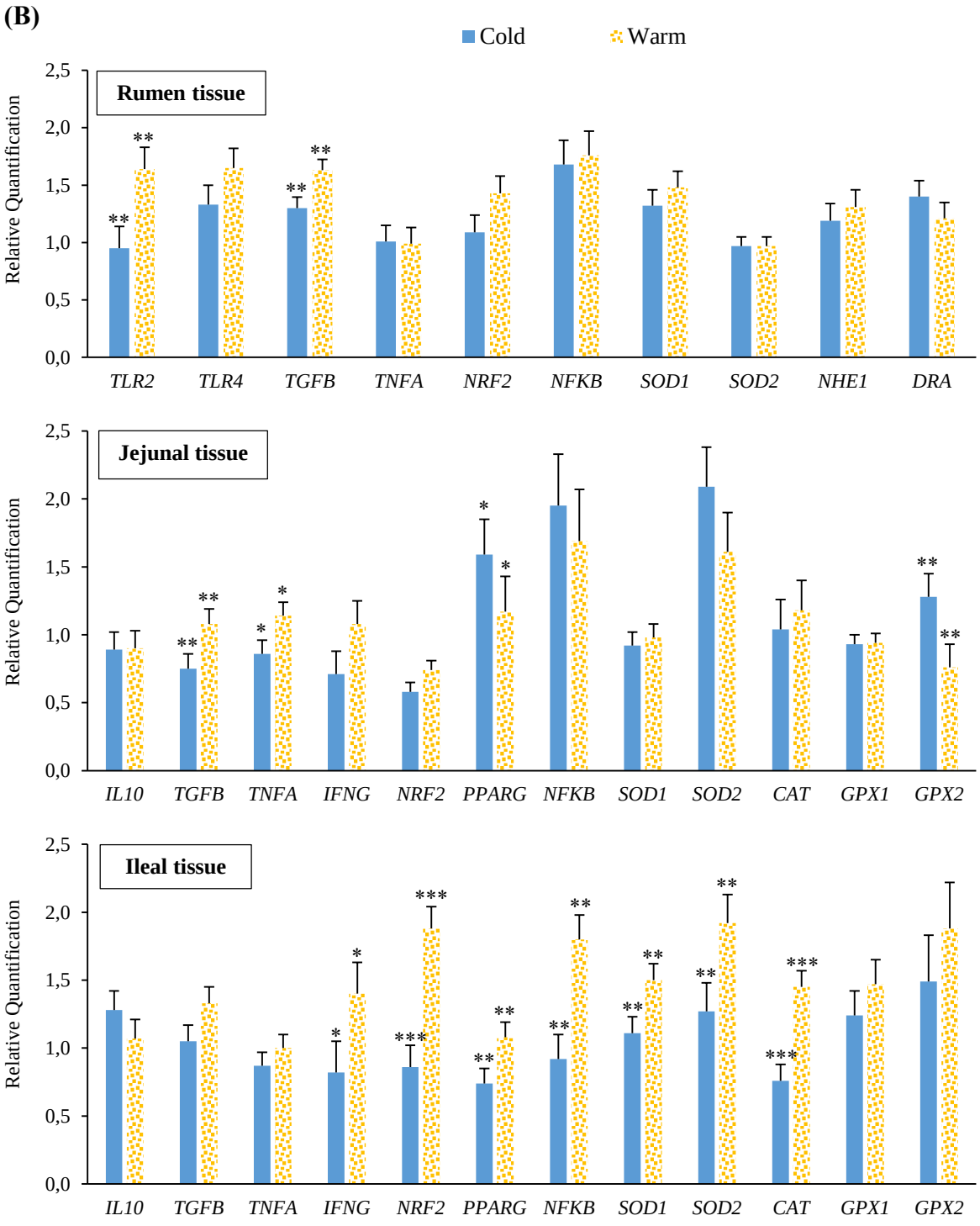

Figure 3. Relative quantification gene expression for rumen cranial dorsal sac, jejunum and ileum tissues in crossbred lambs according to carob pulp inclusion level (A) and season (B). C0 = concentrate without carob pulp, C15 = concentrate with 150 g carob pulp/kg and C30 = concentrate with 300 g carob pulp/kg. Bars represent least square mean values ± SE. ^{a,b} denote statistical differences ($p \leq 0.05$) and ^{x,y} denote tendencies ($P \leq 0.10$) between inclusion of carob pulp. The level of significance in the figure of the seasons is expressed as follows: *$P \leq 0.10$; **$P \leq 0.05$; ***$P \leq 0.0001$.

4. Discussion

In this study, we characterized the effect of dietary CT on the incidence of coccidiosis, as well as the health and antioxidant-immune crosstalk within the digestive tract of light crossbred lambs. Notably, the inclusion of carob pulp CT was associated to improved ruminal and intestinal health and decreased faecal oocyst excretion during patent infection. Moreover, CT in the lambs' diet modulated the expression of antioxidant and immune-mediating genes in ruminal and ileal tissues. The inclusion of carob pulp CT in the diets was expected to ameliorate the deleterious effects of heat stress. However, we did not observe any interaction between the treatments and season in the evaluated variables.

In the intensive ruminant production industry, high-concentrate diets lead to morphological and molecular adaptations that imply an increased surface area of ruminal papillae, reduced thickness of the living stratum and organization, a premature transition of rumen epithelial cells to the stratum corneum, and consequently increased keratinization (Jin et al., 2018; Steele et al., 2011). This increase in keratinisation can be macroscopically identified as a darker rumen mucosa (Álvarez-Rodriguez et al., 2012; Blanco et al., 2015). Condensed tannins have been demonstrated to modulate rumen microbial population and the fermentation process (Patra and Saxena, 2011; Petrič et al., 2022), and promote rumen epithelium development, especially the stratum basale (Ouyang et al., 2019). Accordingly, the presence of carob pulp in the lambs' diet was associated with macroscopically lighter rumen colour. At the same time, dietary carob pulp inclusion up to 300 g/kg increased the thickness of the living stratum and reduced the stratum corneum. Interestingly, no difference was observed in the gene expression of the intracellular pH regulators DRA and NHE, which are involved in SCFA absorption in the rumen epithelium (Grosse-Brinkhaus et al., 2016; Yan et al., 2014). It is possible that the astringency of the diets with high carob pulp content might have induced a slower feeding pattern rate by mixing concentrate and straw more frequently. However, dietary carob pulp inclusion did not negatively affect lamb daily gains nor feeding time (Pelegrin-Valls et al., 2022a). In addition, gene expression analysis revealed increased SOD2 gene expression with dietary carob pulp inclusion. In redox balance, the superoxide radical ($^{*}O_2$) and the singlet oxygen radical ($^{1}O_2^{-}$) are catalysed by SOD to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Ighodaro and Akinloye, 2018). Therefore, these results suggest that dietary inclusion of carob pulp may contribute to improving not only epithelium integrity but also the oxidative status in the rumen by enhancing antioxidant defences, as SOD is a key player in cellular defences against oxidative stress (Sgorlon et al., 2006).

The inclusion of carob pulp in lambs' diet has been demonstrated to have potential coccidiostatic effects in weaned lambs (Saratsis et al., 2016). Weaning period is a major cause of physiological stress that predisposes to clinical coccidiosis (Chartier and Paraud, 2012). Our results suggest that the level of environmental exposure or reactivation of some latent infection associated with the weaning period promoted a patent infection with a peak on Day 65 and a subsequent decrease in faecal egg counts (Keeton and Navarre, 2018). In this regard, the inclusion of dietary carob pulp (up to 150 and 300 g/kg) was associated with reduced

oocyst excretion rates on Days 65 and 80. It is known that the infection severity and the anatomical area involved determines the speed and degree of recovery (Bangoura and Bardsley, 2020). Overall, the infection was not sufficiently advanced to cause serious physiological disorders such as anemia or diarrhea, as no important alteration in iron levels or episodes of diarrhea were observed in the lambs (Green et al., 1993; Johns and Heller, 2021). Coccidial infection reduces faecal consistency because nutritional exchange mechanisms in the intestinal villi are altered (Khodakaram and Hashemnia, 2017). The presence of CT helped to control eimeriosis in both C15 and C30 groups. However, only the inclusion of dietary carob pulp up to 300 g/kg improved the faecal DM consistency, which would also be related to fewer signs of coccidiosis due to the CT content as there was a significant lower excretion on Day 80 in C30. Therefore, including CT sources in weaned lambs would help to reduce the use of coccidiostats at the farm level (Burke et al., 2013; Kommuru et al., 2014). In Spain, the most frequently identified *Eimeria* species tend to exhibit a propensity for the small and large intestine (Bangoura and Bardsley, 2020; Carrau et al., 2018; Díaz et al., 2010). For this reason, an increase in the thickness of ileal muscular layer, responsible for the peristaltic movements of intestinal contents towards the colon, could be associated to enhanced intestinal motility in group C0, that showed higher susceptibility to coccidiosis. The thickness of the muscular layer may also be associated with cytokines secreted by immune cells present between muscle cells (Santos et al., 2018).

In ruminants, the immune response to eimeriosis involve Th1 immune responses in primary infection and the formation of specific antibodies in the later phase (Reeg et al., 2005; Taubert et al., 2008). However, proinflammatory responses may also cause pathological and potentially harmful changes in the tissue. Our results showed that dietary carob CT reaching the gut helped to modulate immune responses and endogenous antioxidant defences in the ileum. Dietary inclusion of carob pulp (up to 150 and 300 g/kg) down-regulated the anti-inflammatory mediators NRF2 and PPARG, as well as the antioxidant enzymes SOD1, that converts the reactive superoxide radical to H_2O_2, and CAT, which has H_2O_2 degrading capacity. NRF2 is an anti-inflammatory mediator involved in the regulation of the antioxidant response (Ahmed et al., 2017), while PPARG plays a role in the regulation of inflammation by inhibiting inflammatory NFKB expression and preserving the integrity of the intestinal mucosa (Dubuquoy et al., 2002). As oxidative stress and inflammation are interdependent, it is possible that lambs in the C0 group needed to activate regulatory immune and antioxidant responses associated with parasitosis. Thus, that is feasible that dietary inclusion of carob pulp diminished the regulatory immune responses and endogenous antioxidant defences by hindering the local production of ROS thanks to their ability to scavenge free radicals and block MPO-activity (Rtibi et al., 2015). Condensed tannins are efficient inhibitors of lipid peroxidation, as lower levels of protein-bound MDA and total MDA were observed in goats fed CT-rich diets (with 32 g of ether extract/kg of feed) (Yonny et al., 2016). However, the inclusion up to 300 g/kg of carob pulp increased both protein-bound MDA and total MDA plasmatic concentrations, probably, due to the increase of dietary lipids (until 72 g of EE/kg of feed) to adjust isoenergetic diets. In this regard, dietary treatments did not affect triglycerides and cholesterol

concentrations (Pelegrin-Valls et al., 2022a).

Oxidative stress increases when animals are exposed to environmental factors such as heat stress, increased exposure to pathogens or intensive husbandry conditions (Celi and Gabai, 2015). In this context, the inclusion of carob CT was expected to alleviate negative effects associated with high temperatures, but not interactions were observed. According to our results, lambs from warm season suffered extreme severe heat stress at high daily temperatures and were able to activate adaptive mechanisms, since they developed higher antioxidant capacity, and no differences were detected in MDA concentrations. However, lambs in this season exhibited altered ruminal epithelium, reduced growth performance, lower circulating iron levels, and increased protein concentrations. Upregulated genes regulating stress reactions and higher susceptibility to coccidiosis were also evidenced.

Heat stress induces a reduction in visceral blood flow, that can lead to varying degrees of hypoxia and impaired barrier function of the gastrointestinal tract (Eslamizad et al., 2020; Hall et al., 1999; Koch et al., 2021). This situation, along with the depression of voluntary feed intake was associated with the decrease in ruminal pH and reduced motility (Bernabucci et al., 2009; Odongo et al., 2006). Therefore, functional adaptations of ruminal papillae in lambs from the warm season may be required to compensate the nutrient uptake capacity, as ruminal papillae were more developed and displayed rumen epithelium morphological alterations consistent with ruminal parakeratosis. Furthermore, the expression of TLR2 and TGFB was increased in ruminal tissues of extreme severe heat-stressed lambs. The activation of TLR signalling pathways has been proposed as an essential non-specific mechanism to protect against invading microorganism in heat-stressed farm ruminants (Paul et al., 2015; Sophia et al., 2016). Thus, the upregulation of TGFB could be involved in regulatory growing cell and differentiation pathway, concomitantly with TLR2 gene expression (Foley et al., 2012).

In a previous study we characterized the complex and region-dependent homeorhetic mechanisms of the intestinal tract in suckling lambs (Peregrin-Valls et al., 2022b). These regional differences probably are influenced by the selective recruitment of immune cells to the intestinal tissue in the development of immune responses against invading pathogens (Ibeagha-Awemu et al., 2021). In jejunum, extreme severe heat stress diminished the gene expression of gastrointestinal GPX2, which is essential for peroxide removal in the intestine (Florian et al., 2001), and increased the expression of the anti-inflammatory TGFB, probably to counterbalance the pro-inflammatory TNFA in jejunum, where most of the nutrient absorption occurs (Koch et al., 2019). It is possible that mucosal hypoxia associated with thermal stress increased cell dysfunction, subsequently impairing the equilibrium between oxidative stress and antioxidant defence mechanisms in the jejunum (Koch et al., 2019; Song et al., 2018). Meanwhile, ileal tissues from lambs in warm season exhibited a higher gene expression of transcription factors NRF2, PPARG and NFKB and antioxidant enzymes SOD1, SOD2 and CAT. The ileum is characterized by a strong immune signature associated with the presence of Peyer's patches, compared to other parts of gastrointestinal tract (Bush et al., 2019). So, it is likely that under

heat stress conditions, the recruitment of phagocytes and the accumulation of ROS triggered NFKB signalling pathways (Koch et al., 2021; Lingappan, 2018). Consequently, the impaired intestinal barrier of the ileum required a NRF2-mediated oxidative stress response, that involved increased expression of antioxidant enzymes and PPARG, to moderate heat stress (Alemu et al., 2018; Dubuquoy et al., 2002).

However, the increased oxidative stress during warm season causes the deregulation of immune cell responses, resulting in a deterioration of the disease resistance. Our results indicate that heat stress during fattening phase predisposes lambs to coccidiosis, as positive correlation was observed between the temperature and OPG counts, likely due to the influence of temperature on the course of sporulation (Graat et al., 1994; Ruiz et al., 2006). Accordingly, lower faecal consistency was also recorded. The damage in the intestinal mucosa might also lead to decreased intestinal iron absorption in duodenum, causing systemic iron dysregulation (Moustafa et al., 2021). Nevertheless, both inflammation and hypoxia at gut level might have affected the modulation of macrophage iron homeostasis, favoring iron transport in macrophages due to the ability of iron to catalyze the production of ROS and promote antimicrobial activity (Koch et al., 2021; Nairz et al., 2017). We also detected higher concentration of total blood protein during the warm season, while albumin concentrations were unaffected. This suggests a metabolic response involving increased amino acid catabolism to sustain energy requirements at the expense of protein mobilisation (Cozzi et al., 2011; Slimen et al., 2019).

Further studies are necessary to improve nutritional interventions based on carob pulp that could enhance the adaptation of lambs to thermal stress.

5. Conclusions

In rumen, the dietary inclusion of carob pulp (150 and 300 g/kg) improved the thickness of the papilla living strata and reduced the darkness of the epithelium colour. In addition, increased expression of radical scavenger enzymes, such as SOD2, along with the differences observed in epithelium integrity biomarkers, suggest that dietary inclusion of carob pulp in lamb diets improved rumen health. The inclusion of dietary carob pulp also reduced the severity of coccidiosis, improved faecal consistency (up to 300 g/kg) and helped to modulate immune responses and endogenous antioxidant defences in the ileum. Nevertheless, carob pulp was not sufficient to generate a positive adaptive status to the extreme severe heat stress scenario.

The warm season had negative effects on growth of lambs, rumen histological parameters, and coccidiosis resistance. The cytokines that regulate inflammation and antioxidant defence system were increased in lambs from the warm season, modulating the pro-inflammatory and oxidative effects associated to warm season.

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VII. CHAPTER 5

Effect of maternal dietary condensed tannins from Sainfoin (*Onobrychis viciifolia*) on gut health and antioxidant-immune crosstalk in suckling lambs.

Abstract: Ewes fed sainfoin (a source of condensed tannins "CT") may influence the homeostasis of the gastrointestinal tract of suckling lambs. This study investigated the effects of CT from sainfoin in the maternal diet on plasma fructosamine, faecal coccidian excretion, and gene expression of immune and antioxidant markers in jejunum and ileum of suckling lambs. Twelve Rasa Aragonesa lambs with their dams were selected. The maternal diet was based on fresh sainfoin (SAINFOIN, $n = 6$) and sainfoin + polyethylene-glycol (SAINFOIN + PEG, as a CT-binder, $n = 6$) plus a daily supplement of 200 g barley in both groups. A lower percentage of lambs that shed more than 10 oocysts/g faeces was observed in SAINFOIN compared to the SAINFOIN + PEG group ($p = 0.07$). Jejunal gene expression of transforming growth factor-β1, tumour necrosis factor-α, and glutathione peroxidase (*GPX*) 1 and 4 were lower in the SAINFOIN group ($p < 0.05$). In contrast, ileal catalase and *GPX2* expression were increased in the SAINFOIN group ($p < 0.05$). Overall, the results suggest that the presence of CT in the dams' diets has a positive effect on reducing excreted coccidian oocysts and favours antioxidant-immune crosstalk at gut level in suckling lambs.

1. Introduction

The production of suckling lambs (around 12 kg of body weight; BW) is based on the mother's milk until slaughter and is an economically very important system in some regions, such as in the Mediterranean area (Sañudo et al., 1998). However, as in other species, there is a trade-off between ewe and lamb characteristics and immune function that impact on lamb performance (Lima et al., 2020). In this context, sustainable animal production requires the use of natural plant resources that diminish predisposition to disease (Gioxari et al., 2022; Hoste et al., 2015).

Ovine coccidiosis is a common enteric disease caused by a protozoan parasite from the genus *Eimeria*. When coccidiosis occurs in young animals, the most common clinical signs are diarrhoea and inefficient weight gain (Keeton and Navarre, 2017), causing an important economic impact. These clinical signs are caused by the destruction of parasitised cells and their subsequent mucosa damage that impairs nutrient absorption (Bangoura and Bardsley, 2020; Chartier and Paraud, 2012). In recent years, there has been an increasing interest in alternative nutrients with antiparasitic properties, as a result of the presence of drug residues in food and widespread drug resistance, mainly to chemical coccidiostatics (Hoste et al., 2015; Mueller-Harvey et al., 2019; Roila et al., 2019; Saratsis et al., 2016). For this reason, the use of antioxidant-rich natural sources in animal feeding is nowadays considered to improve not only lamb health but also meat's oxidative stability (Álvarez-Rodríguez et al., 2022). Several studies encourage the use of anticoccidials of vegetable origin (Blomstrand et al., 2022; Pérez-Fonseca et al., 2016; Saratsis et al., 2016), such as sainfoin, which integrates condensed tannins (CT; syn. proanthocyanidins) in its structure (Hoste et al., 2015; Mueller-Harvey et al., 2019; Saratsis et al., 2016).

Sainfoin (*Onobrychis viciifolia*) is a perennial legume extensively used in the Mediterranean area that has been successfully used as a source of CT in ruminants (Quijada et al., 2018; Rivaroli et al., 2019). The CT structures present in sainfoin, mostly prodelphinidins (ratio prodelphinidins/procyanidin 75/25) (Quijada et al., 2018), have been associated with changes in the ruminal microbiome, and with a reduction of CH_4 emission and protein degradability (Petrič et al., 2022; Niderkorn et al., 2020). In this regard, the inclusion of sainfoin in ewes' diet has been demonstrated to have potential coccidiostatic effects in lactating lambs (Saratsis et al., 2016). Inflammation and oxidative stress are closely related, being the maintenance of redox equilibrium that is essential to avoid impaired animal health (Celi and Gabai, 2015). Immunomodulatory effects of CT involve inhibition of inflammatory cytokines and promotion of antioxidant defences through down-regulation of nuclear factor kappa β (NF-κB) activation and increase of nuclear factor erythroid-2-related factor 2 (Nrf2) activity/expression (Koudoufio et al., 2021; Rajput et al., 2019; Stefanson et al., 2014).

Gut homeostasis is dependent on equilibrium between pro- and anti-inflammatory cytokines which help in maintaining tolerance to commensal microbiota and dietary antigens but at the same time coordinate effective immune responses against pathogens (Chassaing et al., 2014; Maynard et al., 2012). The period of transition from pre-ruminant to ruminant occurs from around 3 to 8 weeks of age when the lamb rumen reaches full functionality; however, the pathways preserving gut homeostasis during the oesophageal groove to

forestomach function are complex and poorly understood in ruminants (Bush et al., 2019; Steele et al., 2016). At present, little is known about the physiological mechanism through which maternal dietary CT can improve the intestinal environment in suckling lambs with a yet incipient rumen function. For this reason, to test the efficacy of this CT it is necessary to understand the consequences of the effect of CT on ewes' diet on milk. The present study is a companion paper to a series of investigations performed in lactating ewes fed with fresh sainfoin during the raising period. According to these studies, sainfoin CT did not negatively affect dam dry matter (DM) intake, milk production, and parasitism (Baila et al., 2022a), nor the growth and carcass and meat characteristics of their suckling lambs Baila et al., 2022b).

The objective of the present study was to examine the effects of CT from sainfoin fed to dams (using polyethylene-glycol-PEG as a blocking agent in a control treatment) on selected markers of gut health and homeostasis of their suckling male lambs. To determine the effects of maternal dietary CT we examined blood fructosamine, coccidian faecal oocyst excretion, and the expression of immune and antioxidant markers in jejunal and ileal tissues of suckling lambs at slaughter. Further anatomopathological analyses were performed.

2. Materials and methods

2.1. Study site

The experiment was conducted between April and May 2019 in the experimental facilities of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (Zaragoza, Spain, 41°39′23" N, 0°5′36" O; 199 m above sea level). The average monthly temperature for April and May was 14.1 and 17.7 °C, respectively.

2.2. Animals, diets and experimental design

After lambing, twelve Rasa Aragonesa ewes and their male lambs were selected from a broader study (Baila et al., 2022b). The ewe–lamb pairs were distributed in two homogeneous groups according to ewe initial BW, body condition score, lambing date, and lamb BW at birth, and were assigned to a different feeding programme: fresh sainfoin (SAINFOIN group, $n = 6$) and fresh SAINFOIN + PEG group ($n = 6$). Sainfoin was offered ad libitum with a supplement of barley (200 g/day) distributed in two meals. Before each meal, ewes from the SAINFOIN + PEG group were orally administrated with a solution of PEG to inactivate the effects of the CT from fresh sainfoin (50 g of PEG 4000/100 mL), while ewes from the SAINFOIN group were orally dosed with water.

Lambs were kept permanently with their dams in individual indoor cages (2.2 m^2) and nourished mainly by suckling during the whole experimental period. The lambs were slaughtered at a target BW of 10–12 kg. A full description of the animals, feedstuffs, and milk composition depending on the presence of CT, the methodology used to determine the CT content and the week of lactation can be found in Baila et al.

(2022a). Briefly, the total CT, protein-bound CT, and fibre-bound CT were analysed in freeze-dried sainfoin and barley samples, fractioned, and quantified by the colorimetric HCL-butanol method. Similarly, milk-content polyphenols were determined by the Folin-Ciocalteu method. The CT content of sainfoin was 34.0 ± 6.1 g CT-equivalents sainfoin/kg DM, while the CT of barley grain was 2.1 ± 0.7 g CT-equivalents sainfoin/kg DM. On the other hand, the polyphenols content in milk was 42.3 and 51.8 ± 6.07 µg eq. gallic acid/g of milk for the SAINFOIN and SAINFOIN + PEG group.

2.3. Blood sample collection and fructosamine analysis

Blood samples were collected individually from lambs in vacuum tubes with heparin (5 mL) (BD Vacutainer, Berkshire, UK) from the jugular vein before the slaughtering. Immediately, the samples were centrifuged in situ to obtain the plasma and stored at −20 °C until analysis.

Plasma concentrations of fructosamine (µmol/L), an indicator of non-enzymatic glycation of circulating proteins, were determined with an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). The measurement range of the fructosamine was 1 to 1000 µmol/L and intra-assay and inter-assay coefficients of variation were 2.2% and 2.1%, respectively.

2.4. Coccidian faecal egg count

Fresh faecal samples were collected directly from the final rectal portion at slaughter, stored in plastic bags, and kept at 4 °C until coprological analyses were performed at the following day. Faecal samples were examined individually by using the modified McMaster method (Bowman, 2020). Briefly, one gram of faeces was mixed and diluted in 14 mL of zinc sulphate flotation solution (specific gravity, 1.18 g/cm^3) and filtered through double cotton gauze. The concentration of oocyst was estimated by screening two complete McMaster flotation chambers under a microscope (10×). The average faecal oocyst counts were multiplied by 100 to obtain the oocysts per gram of faeces (OPG) values. Animals were classified according to OPG values, using arbitrary thresholds of 0, 10, and 500 oocysts/g faeces.

2.5. Histology and RT-qPCR

Jejunal and ileal tissues were aseptically collected immediately after slaughter for histology and Quantitative Real-Time PCR (qPCR) analysis. For histological examination, a 2-cm segment was excised approximately from the middle part of the jejunum, and a 2-cm segment of the ileum was aseptically excised proximal to the ileocecal valve. The samples were then cut open, rinsed with phosphate-buffered saline (PBS) solution and fixed in a 10% formalin solution. Additional sections (<0.5 cm) of mid jejunum and terminal ileum were aseptically excised, rinsed with PBS solution, incubated in RNAlater for 24 h (RNAlater® Solution, Ambion Inc., Austin, TX, USA) and stored at −80 °C for gene expression analysis.

2.6. Histopathological examination and microscopic lesion scoring

Formalin-fixed tissue samples were trimmed and processed according to standard histological procedures, and sections were stained with haematoxylin–eosin.

Slides were reviewed and scored by a veterinary pathologist in a blinded analysis. Intestinal injury scores were measured using the following aspects of inflammation: inflammatory changes (type of inflammatory infiltrate, intensity, location, fibrin exudation, lymphangiectasis, crypt abscesses and villous blunting), tissue destruction (enterocyte loss, ballooning degeneration, oedema, mucosal atrophy, loss of crypts) and tissue repair (hyperplasia, angiogenesis, granulomas, and fibrosis) adapted from Dommels et al. (2007). The histopathological lesions were scored on a scale from 0 to 3 (0, no change from normal tissue; 1, focal, occasional; 2, numerous (<50%); 3, intense/severe, change involved most areas and all the layers of the intestinal section (≥50%)). The sum of inflammatory lesions, tissue destruction, and tissue reparation score were used to calculate the total histological injury score (HIS) for each tissue according to the adapted method of Dommels et al. (2007).

2.7. RT-qPCR analysis

Total RNA was extracted from 100 mg of jejunal and ileal tissues with E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. RNA purity and quantity were determined spectrophotometrically using NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). Samples were treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, USA) to eliminate contaminating genomic DNA. First- strand DNA synthesis was carried out with 1 µg of total RNA and random hexamer primers using the Maxima RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

The messenger RNA expression was determined by qPCR for target genes: interleukin 10 (*IL10*), transforming growth factor-β1 (*TGFB*), tumor necrosis factor-α (*TNFA*), interferon- γ (*IFNG*), nuclear factor erythroid-2-related factor 2 (*NRF2*), nuclear factor kappa β (*NFKB*), superoxide dismutase 1 and 2 (*SOD1* and *SOD2*), catalase (*CAT*) and glutathione peroxidase 1, 2, and 4 (*GPX1*, *GPX2,* and *GPX4*). The genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) were used as reference genes. Primer sequences and the origins of the primers are shown in Table 1. The *IFNG*, *NRF2*, *NFKB*, *CAT*, *GPX1*, *GPX2*, and *GPX4* primers were designed with the Primer 3 Plus and Primer-Blast tools (Primer-Blast; Primer3Plus) and synthesised by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany). For each gene, a standard curve was generated by amplifying serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplification was conducted in an ABI PRISM 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) and performed in triplicate using 3 µL of 30-fold diluted cDNA as a template in a total volume of 8 µL containing 1 × Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) as described elsewhere (Pelegrin-

Valls et al., 2020). Data were normalised and analysed by the $2^{-\Delta\Delta Ct}$ method using the mean Ct value obtained for the two reference genes and the Ct values for each target gene (Schmittgen and Livak, 2008).

Table 1. Primer sequences used for quantitative Real-Time PCR.

Gene	Tissue-function ratio	Forward and Reverse primer (5′–3′) [1]	bp [2]	Access. No.	E (%) [3]	nM [4]	Source
GAPDH	Reference genes	F: ATCTCGCTCCTGGAAGATG R: TCGGAGTGAACGGATTCG	200	NM_001190390.1	1.90	600 300	Puech et al., 2015
ACTB	Reference genes	F: CTGGACTTCGAGCAGGAGAT R: GATGTCGACGTCACACTTC	194	NM_001009784	1.94	600	Puech et al., 2015
IL10	Anti-inflammatory	F: TTAAGGGTTACCTGGGTTGC R: TTCACGTGCTCCTTGATGTC	109	NM_001009327.1	1.96	200	Pelegrin-Valls et al., 2020
TGFB	Anti-inflammatory	F: TTGACGTCACTGGAGTTGTG R: CGTTGATGTCCACTTGAAGC	120	NM_001009400.2	2.04	200	Pelegrin-Valls et al., 2020
TNFA	Pro-inflammatory	F: CAAATAACAAGCCGGTAGCC R: TGGTTGTCTTTCAGCTCCAC	118	NM_001024860.1	1.96	200	Pelegrin-Valls et al., 2020
IFNG	Pro-inflammatory	F: AAGTTCTTGAACGGCAGCTC R: TTGGCGACAGGTCATTCATC	130	NM_001009803.1	1.91	500	Primer-Blast; Primer3Plus
NRF2	Redox system	F: GAGCCCAGTCTTCAATGCTC R: TCAGCCAGCTTGTCATTTTG	171	XM_015093345.2	1.97	200	Primer-Blast; Primer3Plus
NFKB	Transcription factor	F: CTACACCTTGCCTGTGAGCA R: AAGGACACCAACAGCTCCAC	173	NM_001166184.1	1.93	300	Primer-Blast; Primer3Plus
SOD1	Antioxidant enzyme	F: CACCATCCACTTCGAGGCAA R: GCACTGGTACAGCCTTGTGT	126	NM_174615.2	2.06	200	Lesage-Padilla et al., 2017
SOD2	Antioxidant enzyme	F: GGATCCCCTGCAAGGAACAA R: TGGCCTTCAGATAATCGGGC	110	NM_201527.2	2.03	200	Lesage-Padilla et al., 2017
CAT	Antioxidant enzyme	F: TTCCGTCCTTTATCCACAGC R: CCATTGGCATTAACCAGCTT	199	XM_004016396.5	2.04	300	Primer-Blast; Primer3Plus
GPX1	Antioxidant enzyme	F: GGCATCAGGAAAACGCCAAG R: GGGGACCACGTGATGAACTT	217	XM_004018462.5	1.98	300	Primer-Blast; Primer3Plus
GPX2	Antioxidant enzyme	F: ATTGAGAATGTGGCCTCGCT R: CCAGGGCGGACATACTTGAG	179	XM_004010720.5	2.07	300	Primer-Blast; Primer3Plus
GPX4	Antioxidant enzyme	F: GGGAGTAATGCGGAGATCAA R: CCACACAGCCGTTCTTATCA	199	XM_027970172.1	2.04	300	Primer-Blast; Primer3Plus

[1] F = forward; R = reverse; [2] bp = amplified product length in base pairs; [3] E (%) = efficiency; [4] nM = optimal primer concentration.

2.8. Statistical analysis

Statistical analysis was conducted using the statistical package JMP Pro13 (SAS Institute Inc. Cary, NC, USA). The data were analysed with a one-way least square model considering the maternal diet as a fixed effect. When necessary, data were log-transformed to meet the assumption of normality and homoscedasticity. The student's t-test was used to compare fructosamine, OPG values, and relative mRNA gene expression according to maternal diet. Histopathological injury scoring was analysed with a mixed model considering the fixed effects of maternal diet, intestinal tissue and their interaction. Data are reported as least square means and their standard error. The level of significance was set at 0.05, but tendencies were commented

on if the level of significance was below 0.10. Pearson tests on contingency tables were used to evaluate the association between OPG threshold values in lambs and the maternal diet. Spearman correlations between microscopic lesion scores and OPG values were also evaluated.

An additional principal component analysis (PCA) was used as a dimension-reduction technique, as well as an exploratory data analysis tool to study the relationship among the twelve genes' expression markers in jejunal and ileal tissues. Principal components of log-transformed gene expression relative quantifications were identified by computing the eigenvectors of the covariance matrix of the log-transformed values for relative gene expression quantification. A Bartlett test was applied to perform variance homogeneity tests for each eigenvalue, which represents a partition of the total variation in the multivariate sample. Using the PCA and Varimax rotation method, a factorial analysis was applied to study the relationship among the twelve gene expression markers in jejunal and ileal tissues. The maternal dietary treatment was included as a supplementary variable in PCA.

3. Results

3.1. Lamb performance and plasma fructosamine

The age and slaughter BW did not differ across maternal dietary treatments (31 ± 3.2 days old and 11.2 ± 0.3 kg in the SAINFOIN + PEG group vs. 29.5 ± 3.9 days old and 11.3 ± 0.3 in the SAINFOIN group, respectively, $p > 0.05$). Regarding to fructosamine values at slaughter, there was no difference between SAIFOIN + PEG and SAINFOIN groups (185.1 vs. 194.7 ± 8.15 μmol/L, respectively, $p > 0.05$).

3.2. Faecal oocyst count

The effects of CT from fresh sainfoin fed to ewes on the level of coccidia in lambs are shown in Table 2. The average excretion of OPG was similar for both groups ($p > 0.05$). However, a trend toward significance was observed for an increased percentage of lambs that shed more than 10 oocysts/g faeces in the SAINFOIN + PEG group compared to the SAINFOIN group ($p = 0.07$).

Table 2. Effect of maternal experimental diets (SAINFOIN + PEG vs. SAINFOIN) on faecal oocyst count in suckling lambs at slaughter and percentage of lambs excreting 0, 10 and 500 oocyst/g faeces.

Faecal oocyst count	SAINFOIN	SAINFOIN+PEG	P-value
Log-transformed OPG [1]	19	219	0.28
(Log-values ± SE [2] between parenthesis)	(1.28 ± 0.65)	(2.34 ± 0.65)	
Lambs excreting oocyst (%)			
>0 oocysts/g faeces	50	83.3	0.21
>10 oocysts/g faeces	33.3	83.3	0.07
>500 oocysts/g faeces	16.7	33.3	0.50

[1] OPG = oocyst per gram of faeces; [2] SE = standard error.

3.3. Histopathological analysis

Upon histopathological examination, there were no remarkable differences with regards to the average of the total HIS due to the presence of CT in the dams' diets (Table 3; $p > 0.05$). No significant association was observed between faecal oocyst counts and average total HIS in jejunum ($p = 0.23$; $p = 0.46$) and ileum ($p = 0.24$, $p = 0.44$).

Table 3. Histopathological injury score of suckling lambs according to maternal experimental diets (SAINFOIN + PEG vs. SAINFOIN) and tissue (jejunal and ileal).

	Treatment [2]		**Tissue** [2]		**P-value** [3]		
Item (µm) [1]	**SAINFOIN**	**SAINFOIN+PEG**	**Jejunal**	**Ileal**	**SE**	**Treatment**	**Tissue**
Total inflammatory	4.3	4.3	5.1	3.5	0.23	1.0	< 0.0001
Total tissue destruction	2.1	2.0	3.3	0.8	0.23	0.61	< 0.0001
Total Tissue repair	0.01	0.04	0.04	0.01	0.03	0.32	0.32
∑ HIS	6.4	6.3	8.4	4.3	0.42	0.83	< 0.0001

[1] Total inflammatory section = intensity, fibrin exudation, lymphangiectasis, location (layer), crypt abscesses, and villous blunting; total tissue destruction section = enterocyte loss, oedema, mucosal atrophy, loss of crypts; total tissue reparation: hyperplasia, angiogenesis, granulomas and fibrosis; HIS = total histological injury score. [2] Values are expressed as mean ± SE. [3] No interactions between treatment and tissue were observed.

Independently of maternal diet, the jejunum presented higher total HIS scores compared to the ileum ($p < 0.001$). Jejunal lesions were characterised by mixed cell infiltration (eosinophils, lymphocytes, macrophages, plasma cells), lymphangiectasis, villous blunting, mucosal atrophy, and enterocyte loss. In the case of the ileum, eosinophils were the predominating cell population. In both organs, the histological changes were restricted to the mucosa layer.

3.4. Gene expression

In jejunal tissues, significantly lower expression of *TNFA* ($p = 0.07$, trend), *TGFB, GPX1*, and *GPX4* were observed in the SAINFOIN lambs when compared to SAINFOIN + PEG lambs (Figure 1a; $p < 0.05$). In ileal tissues, significantly higher expression of *GPX2* and *CAT* were observed in the SAINFOIN lambs compared to the SAINFOIN + PEG lambs (Figure 1b; $p \leq 0.01$). The presence of CT in the dams' diet did not affect jejunal *IL10*, *IFNG*, *NRF2, NFKB*, *SOD1, SOD2*, *CAT*, and *GPX2* expression ($p > 0.10$), nor ileal *IL10*, *TGFB*, *TNFA*, *IFNG*, *NRF2, NFKB, SOD1*, *SOD2*, *GPX1*, and *GPX4,* expression ($p > 0.10$).

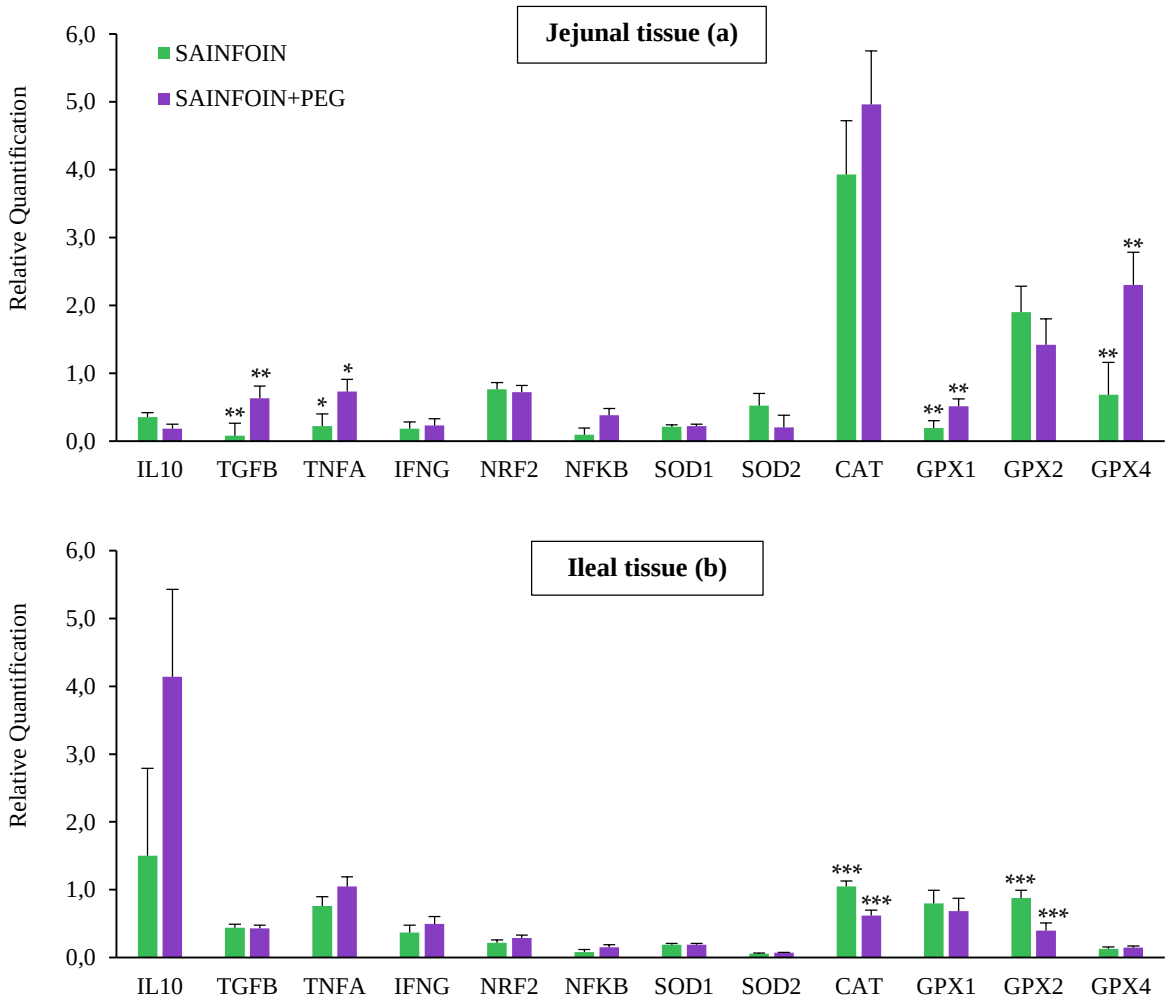

Figure 1. Relative quantification of gene transcripts in jejunum (**a**) and ileum (**b**) in suckling lambs according to maternal dietary (SAINFOIN vs SAINFOIN+PEG.). Bars represent least square mean values ± SE. The level of significance is expressed as follows: * $P \leq 0.10$; ** $P \leq 0.05$; *** $P \leq 0.01$.

3.5. Principal components analysis

Principal components are new variables that are constructed as linear combinations of the initial variables. In jejunal tissues, seven principal components accounted for 95.9% of the variation in the gene expression values, while the total number of components extracted (eigenvalues), based on the amount of variance contributed by each component, was reduced from 4.49 to 0.46. Component 1 accounted for 37.4% of the total variation, while component 2 accounted for 19.7% variation (Figure 2a). Similarly, in ileal tissues, seven principal components accounted for 99.0% of the variation in the gene expression values, while the total number of components extracted (eigenvalues), based on the amount of variance contributed by each component, was reduced from 4.46 to 0.59. Component 1 accounted for 37.2% of the total variation,

while component 2 accounted for 18.8% variation (Figure 2b). In both tissues, the left quadrant of component 1 was mainly represented by SAINFOIN individuals, while the right quadrant was mainly represented by lambs whose dams had been fed SAINFOIN + PEG. Likewise, the upper quadrant of component 2 was mainly represented by SAINFOIN individuals, while the bottom quadrant was mainly represented by lambs whose dams had been fed SAINFOIN + PEG.

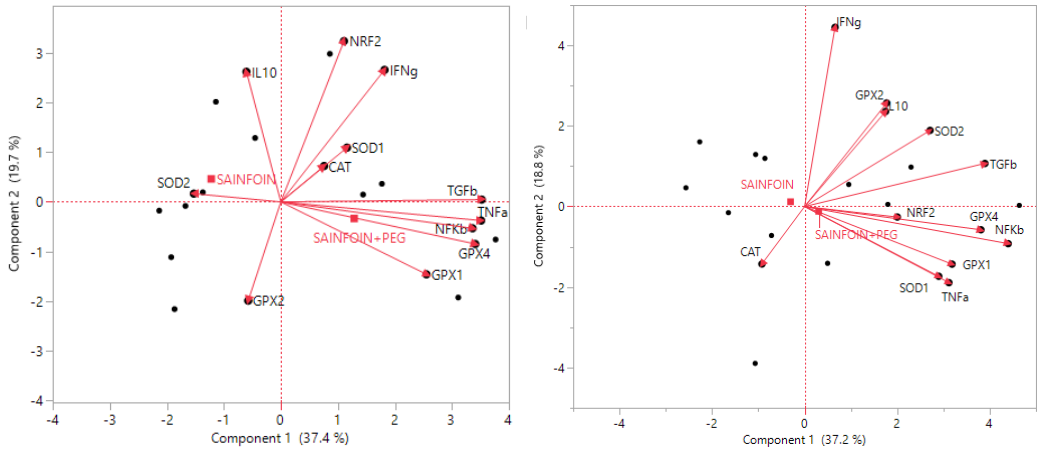

Figure 2. Biplot of principal components of gene expression markers in jejunum (**a**) and ileum (**b**).

The PCA identified three clusters based on gene markers in jejunum. The first cluster was related with the pro-inflammatory marker *TNFA* (69% out of total cluster variation, 6 gene members), while the second and third cluster were mainly related with *NRF2* (54.5% out of total cluster variation, 4 genes) and *CAT* (74.5% out of total cluster variation, 2 genes) (Table 4).

Table 4. Summary of PCA clusters of gene expression markers in jejunum.

Cluster	Gene members	Most representative variable	Proportion of explained cluster variation	Proportion of total variation
1	*TNFA, TGFB, GPX4, NFKB, GPX1* and *SOD2*	*TNFA*	0.69	0.35
2	*NRF2, IFNG, IL10* and *SOD1*	*NRF2*	0.55	0.18
3	*CAT* and *GPX2*	*CAT*	0.75	0.12

In ileal tissues, the PCA also identified four clusters. Cluster 1 was mostly represented by *NFKB* (64.9% out of total cluster variation, 6 gene members), while cluster 2 was mostly represented by *IFNG* (64.4% out of total cluster variation, 3 genes). The third cluster was mainly represented by antioxidant enzyme *CAT* (70.9% out of total cluster variation, 2 genes) and the fourth cluster was represented by single *NRF2* (100% out of total cluster variation, 1 gene) (Table 5).

Table 5. Summary of PCA clusters of gene expression markers in ileum.

Cluster	Gene members	Most representative variable	Proportion of explained cluster variation	Proportion of total variation
1	*NFKB, GPX4, GPX1, TGFB, TNFA* and *SOD1*	*NFKB*	0.65	0.32
2	*IFNG, GPX2* and *SOD2*	*IFNG*	0.64	0.16
3	*CAT* and *IL10*	*CAT*	0.71	0.12
4	*NRF2*	*NRF2*	1	0.08

4. Discussion

In this study, we characterised the effect of maternal dietary CT on coccidiosis and health and antioxidant-immune crosstalk at a gut level in suckling lambs. Our main findings were that: (1) maternal dietary CT tended to reduce faecal oocyst excretion in lambs; (2) the presence of CT in the dams' diet affected differently the expression of the antioxidant defence system and regulatory immune genes in intestinal tissues; and (3) based on PCA analysis, gut homeostasis in suckling lambs involves a tissue-dependent interaction of pro-inflammatory and antioxidant mediators in both jejunal and ileal tissues.

A large body of published work has demonstrated the antiparasitic properties of CT in gastrointestinal coccidian infection (Blomstrand et al., 2022; Pérez-Fonseca et al., 2016). For example, the use of sainfoin delayed the onset of coccidian infections in both pre-weaned (Saratsis et al., 2016) and weaned lambs (Rivaroli et al., 2019; Saratsis et al., 2012). In our study, the percentage of lambs that excreted more than 10 oocysts/g at slaughter age tended to be higher in the SAINFOIN + PEG group than in the SAINFOIN group. This suggests that the chelate used in ewes' diet of the SAINFOIN + PEG group reduced the protection of CT against coccidia along the intestinal tract of naturally infected lambs. Lambs are infected in early life (with the onset of excretion at 13–15 days) as a result of an oocyst contaminated environment, however, the presence of large numbers of oocysts in young lambs is not necessarily indicative of disease (Pout et al., 1966; Saratsis et al., 2011). Clinical coccidiosis seems to be directly linked to the species of coccidian involved, the number of oocysts ingested, significant asexual multiplication in the host, and possible physiological stress (Chartier and Paraud, 2012). In our study, oocyst excretion was not correlated with the observed microscopic lesion scores in the jejunum and ileum of naturally infected lambs, and both treatments reached the target BW at slaughter with similar age and, thereby, they had similar growth rates (Baila et al., 2022a). It has been suggested an interplay between passive maternal immunity and active immunisation during the lactation period in infected lambs (Reeg et al., 2005). On the other hand, solid feed is naturally starting at around 3 weeks of age (Álvarez-Rodríguez et al., 2007), leading to important anatomical and physiological changes in the gastrointestinal tract. Therefore, the lambs of this study could be familiarising with sainfoin supplied to the dams, and thereby, some other feed components apart of those from ewe milk could be reaching the small intestine. However, the amount of solid feed consumed at the time of slaughter (4–5 weeks of age) may

be very low (about 50 g/day) (Faichney, 1992). Furthermore, the jejunum is the part of the intestine where most of the nutrient absorption occurs, so these events could be disrupting the histopathology of the jejunum (Pond et al., 1995; Vi et al., 2004). Nevertheless, as the present study it was conducted under natural coccidian infection, further research on histopathological findings in suckling lambs are needed.

Coccidia cause damage to the intestinal mucosa by activating cellular immune responses (Bangoura and Bardsley, 2020; Ovington et al., 1995). However, the activation of an effective immune response can induce a strong competition for the maintenance of growth in parasitised lambs. The protective role of CT has been associated with a direct action on the modulation of specific immune cells and antioxidant activity (Yahfoufi et al., 2018). In this study, the inclusion of CT from sainfoin in the dams' diet diminished gene expression of cytosolic *GPX1* and phospholipid hydroperoxide *GPX4*, and anti-inflammatory *TGFB* and pro-inflammatory *TNFA* in the jejunum, but increased the gene expression of *CAT* and gastrointestinal *GPX2* in the ileum. Accordingly, PCA revealed that both immune and antioxidant responses are different in lambs from the SAINFOIN group when compared to SAINFOIN + PEG group. These results would indicate that there was a positive transfer of some polyphenols to the milk in the SAINFOIN ewes' diet, as observed in goats nursing kids (Jordán et al., 2010), due to the CT that may have been absorbed during digestion (Quijada et al., 2018). Hence, dams' diet CT may have immunomodulatory effects on the intestinal environment, causing an adverse scenario for the parasites without a negative impact on nutritional well-being.

Plant-derived compounds, as polyphenols, have the ability to prime Nrf2 expression and activity while inhibiting NF-κB activation (Stefanson et al., 2014). However, in this study, we observed a decreased production of both pro- and anti-inflammatory cytokines and antioxidant enzymes in the jejunum. The antioxidant properties of CT are linked to their ability to scavenge free radicals (Bagchi et al., 1997; Pérez-Fonseca et al., 2016). Thus, it is possible that the presence of CT in dams' diet, and accordingly some of the CT derivatives reaching the gut, helped to reduce local oxidative stress and to diminish immune responses and endogenous antioxidant defences by reducing the production of reactive oxygen species (ROS). This result is in agreement with studies performed in other species, where decreased activities of the oxidative stress-responsive transcription factors *NFKB* and *NRF2* were observed in the duodenal mucosa of pigs supplemented with polyphenol-rich grape by-products (Gessner et al., 2013). On the other hand, polyphenols also enhance antioxidant defences by increasing the activity of endogenous antioxidant enzymes (Mu et al., 2020; Puiggros et al., 2005). In mammalian models, marked regional variations of antioxidant capacity in the gastrointestinal tract have been described (Chedea et al., 2018; Moghadasian et al., 1996). Thus, the presence of polyphenols in the dams' diet could help in the detoxifying metabolism of hydroperoxides in suckling lambs by favouring the expression of *CAT* and *GPX2*, considered the most important of the four types of *GPX* for peroxide removal in the intestine (Florian et al., 2001). In swine, the 5% grape pomace diet leads to an increase of *GPX* activity in the colon with respect to the duodenum (Chedea et al., 2018), which was attributed to the presence of the unmetabolised procyanidin trimers. In this trial, the carcass and meat quality of suckling lambs were not affected by the inclusion of CT from sainfoin in the dams' diet (Baila et al., 2022b). In agreement with

previous studies, plasma concentrations of fructosamine in lambs, as indicator of nutritional status (Filipović et al., 2011), were not affected by the presence of CT in the dams' diet.

In ruminants, the small intestine is characterised by regional differences in the development of immune responses, probably influenced by selective recruitment of immune cells to the intestinal tissue (Ibeagha-Awemu et al., 2021) and gut colonisation (Hansen et al., 2012; Rajput et al., 2019) as a result of different undigested products reaching the intestine. Independently of the maternal experimental diet, PCA analysis defined dynamic/regulatory antioxidant-immune interactions involved in gut growth and development of suckling lambs. In the jejunum, the first cluster was related to the pro-inflammatory marker *TNFA* that may exert apoptotic and anti-apoptotic pathways (Lüpertz et al., 2008), while the second and third clusters were mainly related to *NRF2*, which is involved in redox homeostasis, and *CAT*, which has H_2O_2 degrading capacity. In ileal tissues, the first cluster was mostly represented by *NFKB*, involved in homeostatic and cell survival pathways, while the second cluster was mostly represented by *IFNG*, involved in cellular immunity. The third cluster was mainly represented by the antioxidant enzyme *CAT* and the fourth cluster was represented by a single *NRF2*. Oxidative stress and inflammation are interdependent since an excess of ROS can initiate a cascade of intracellular signalling, that involve NF-κB pathway and pro-inflammatory stimuli. In response to these stresses, it is necessary the activation of the Nrf2 pathway that leads to the activation of various antioxidant enzymes, such as *CAT*, as observed in the ileum of the current study. Given ROS can induce oxidative injury and also act in redox signalling (Lei et al., 2016), it has been described as a downregulation of *CAT* expression by *TNFA* in order to enhance NF-κB activation, that is stimulated in the presence of H_2O_2 (Lüpertz et al., 2008). This analysis suggests the presence of complex and compartment/region-dependent homeorhetic mechanisms of the intestinal tract in suckling lambs, in agreement with previous studies (Bush et al., 2019).

5. Conclusions

In conclusion, CT from sainfoin in the diet of ewes tended to reduce coccidian oocyst excretion in their suckling lambs. Maternal diets (SAINFOIN vs. SAINFOIN + PEG) did not affect the histopathological analysis of the jejunum and ileum of suckling lambs. However, the superoxide radical scavenging activity of CT in dams' diet caused a down-regulation of *TNFA* and *TGFB* and *GPX1* and *GPX4* in jejunal tissue while enhancing the H_2O_2 degrading capacity of *CAT* and *GPX2* in ileal tissues of lambs from SAINFOIN group. PCA analysis confirmed the presence of complex, and compartment/region-dependent homeorhetic mechanisms mediating the small intestine health in suckling lambs.

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VIII. GENERAL DISCUSSION

1. Current challenges of lamb production in Spain.

Europe is the third largest sheep producer in the world, following Asia and Africa (FAOSTAT, 2023), with the United Kingdom as the continent's leading producer. Spain is the top producer of lamb meat in the European Union (EU), with approximately 121,000 tonnes per year (MAPA, 2022). However, over the last decade, the Spanish population has decreased from eating 1.85 kg to 1.09 kg of lamb meat per capita (MAPA, 2022). This response contrasts with global expectations, where consumption of red meat such as lamb is expected to increase by approximately 200% by 2050 (Ponnampalam et al., 2019). These contradictions arise, among other factors, from the new eating habits of part of the population that avoids meat consumption for health reasons or due to ethical considerations regarding animal welfare (Bodas et al., 2021).

On the other hand, Spain has a serious problem of generational replacement in the agricultural sector, which directly affects activities such as pastoralism, which require a high level of temporary dedication. Furthermore, sheep production is closely linked to the use of autochthonous breeds, which are highly adaptable to extensive grazing systems, but have difficulties in intensive production. In this regard, the European Green Pact aims to transform the current EU food system towards a more sustainable model by 2030 (Farm to fork strategy). Among the various objectives proposed, it emphasises the achievement of carbon neutral or positive production in order to minimise climate change, guarantee access to safe and sustainable food, while generating economic returns and promoting the competitiveness of the sector (Europa.eu, 2023).

The last decade has seen fluctuations in feed costs for fattening lambs in intensive systems based on concentrate and straw. Nevertheless, even today, the feed formulation industries overestimate the real protein requirement of lambs according to their productive phases. In fact, it is estimated that crude protein (CP) inclusion requirements can be decreased without affecting the productive performance of livestock (de Evan et al., 2020; INRA, 2018; Saro et al., 2020). On the other hand, more recent research is focusing on the inclusion of ingredients containing bioactive components in lamb diets with the capacity to impact on lamb health and stimulate the circular economy, although at high levels, these ingredients could reduce productivity (Álvarez-Rodríguez et al., 2022; Baila et al., 2022).

In conclusion, all the challenges described previously affect the viability of sheep sector in Spain and in particular, fattening lamb. Hence, the present thesis has been combined several experiments in which the possibility of reducing the CP and the inclusion of carob pulp (*Ceratonia siliqua* L.) in the diet of light lambs was analysed, as well as the inclusion of sainfoin (*Onobrychis viciifolia*) in the diet of dams to test the effects on suckling lambs. These experiments focus on the animals' diets to try to improve their performance and positively influence production costs (Ponnampalam et al., 2019). In this respect, the reduction of CP can reduce feed costs, while the inclusion of alternative ingredients such as carob pulp or sainfoin can have a positive effect on the health of lambs and stimulate the circular economy. By integrating the different production phases of light and suckling lambs, these experiments made it possible to analyse the productive

effects of each of the challenges, assess the circadian rhythm of metabolites in blood and nutrients in faeces, study nutritional and oxidative metabolism, test the benefit of nutritional changes on intestinal health and animal behaviour and, finally, evaluate the gastrointestinal immune and antioxidant response of the lambs.

2. Animal performance.

Improved feeding strategies lead to increase productive performances and economic outcomes on farms (Ponnampalam et al., 2019; Theodoridis et al., 2021). Chapters 1, 3 and 5 report three studies aiming to investigate the reduction of CP, the inclusion of carob pulp and sainfoin in the diet of lambs and dams, respectively.

The results of the first study show that a 2% reduction of CP in the diet of light lambs does not influence the main productivity parameters such as average daily gain (ADG), body weight (BW), slaughter weight or concentrate and straw intake. However, the feed conversion ratio (FCR) tends to change during the finishing phase (Chapter 1, Table 4). This suggests that increasing the CP in the diet of lambs does not necessary have a direct impact on growth. In fact, in economic terms, resources would be wasted through the use of high dietary protein concentrates. It has been shown that FCR can be kept stable with the inclusion of 13-18% CP on dry matter (DM) in lambs slaughtered at 48.5 kg BW (Chapter 1; Bernard et al., 2019; Prima et al., 2019). However, local breed format and the preferences of consumers in Spain lead to the production of lambs that are much lighter, with a slaughter BW of up to 25-30 kg. According to Tullo et al. (2019), feeding strategies play a critical role to minimise the environmental impact of nitrogen and phosphorus accumulated in manure. For nitrogen, it is important to properly balance CP according to the production phase of the animal through a better understanding of nitrogen assimilation during the production phases.

According to Cannas et al. (2019), current nutritional models are more complex than in the past and require constant update, especially because of the multitude of innovative inputs that are being generated around the feeding of small ruminants. In the case of the inclusion of carob pulp in the lamb diet, it is important to note that, although this ingredient is rich in sugars and can promote intake due to its palatability, it contains low levels of fat and protein (Karabulut et al., 2006; Stavoru et al., 2018). However, it has been shown that the total CP content of the lamb diet can be significantly reduced compared to concentrate formulations. Currently, the concentrates may contain as much as 21% CP on DM, but this could be reduced to 17% CP on DM at the end of fattening of light lambs slaughtered at 25 kg BW (Chapter 1; Bello et al., 2016; Bernard et al., 2019; Joy et al., 2008). Therefore, the introduction of carob pulp in the lamb diet should also be analysed together with other ingredients to determine if it is a good alternative nutritional option for more economically and environmentally efficient production. When carob pulp was included in the diet of lambs with similar energy and protein contents, it was observed that performance parameters and final target BW were achieved equally as well as those fed without carob pulp inclusion (Chapter 3, Table 3).

As discussed in Chapter 3, lambs fed 150 g/kg (C15) and 300 g/kg (C30) of carob pulp showed an

increased FCR in the concentrate compared to the control group (C0). However, this negative effect on feed conversion was not observed when straw was introduced in the analysis variable, with all groups showing a similar FCR (Chapter 3, Table 3) and, when lambs' cumulative intake was analysed according to their BW, a higher slope of concentrate intake could be observed with increasing carob pulp in the diet (Figure 1). These variables demonstrate that including a high percentage of carob pulp in the lamb diet (C30) can increase the feed costs of a farm depending on various scenarios. Firstly, carob pulp contains condensed tannins (CT) in its structure, and a high level of inclusion may impair feed intake due to the astringent effect (Richane et al., 2022), which could affect the animals' feed conversion. Secondly, to determine the economic impact, the gross margin per animal has to be analysed according to the ingredients introduced in the diet at that time. Chapter 3 briefly describes an economic analysis of the diets used in the 2021 experiment. It shows how the feed costs of lambs fed the C30 diet were up to 2.5€ higher in the medium and high ingredient price range. Therefore, depending on carcass income, it might not even have any impact on the economic performance of the farm. The cost of lamb concentrates from 2018 to 2022 ranged between 248 and 415 €/t on average (MAPA, 2023). Thus, considering that protein concentrates represent up to 20% of total concentrate (Álvarez-Rodríguez et al., 2018), the protein cost of feed would be between 50 and 83 €/t for the year with lowest and highest prices, respectively. In this context, the decrease of CP in Chapter 1 would adjust the protein inclusion to 18% and 17% in the growing and finishing phase, respectively. Therefore, the costs derived from the CP would be reduced by between 5 and 8 €/t for the growing phase and between 8 and 13 €/t for the finishing phase, depending on whether the costs are analysed in the year 2018 or 2022.

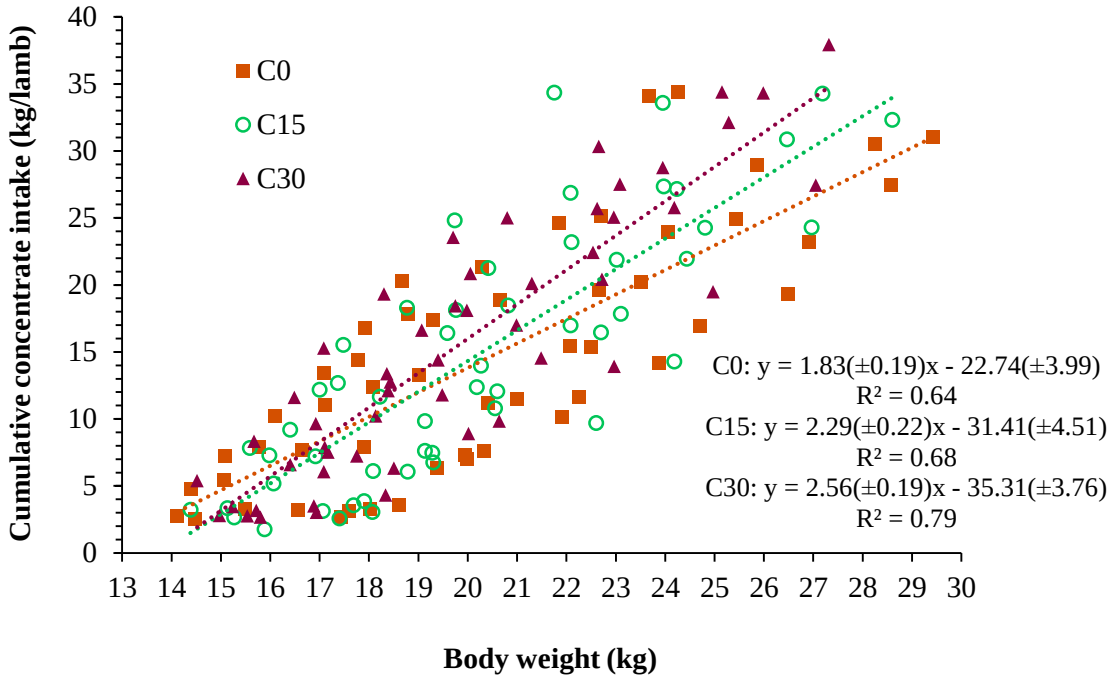

Figure 1. Linear regression of body weight versus cumulative concentrate intake throughout the fattening period according to the level of carob pulp in the diet (C0 = 0 g carob pulp/kg concentrate, C15 = 150 g carob

pulp/kg concentrate and C30 = 300 g carob pulp/kg concentrate).

The nutritional potential of an ingredient has to be examined not only from the point of view of chemical composition, but it is necessary to include additional conditions such as the presence of CT; bioactive components that provide benefits to production animals. Richane et al. (2022) concluded that the inclusion of carob in animal diets needs to be carefully considered because of the possible adverse effects of its CT and because it is deficient in protein. However, if it is balanced by other sources of CP it is an ingredient of high nutritional value for ruminants. Likewise, sainfoin is a legume with a high TC content in its structure and also has a good concentration of CP (Mueller-Harvey et al., 2019). This forage has been used as a dietary basis for ewes during the lactation phase and it has been observed that it does not affect the productive parameters of suckling lambs (Chapter 5; Baila et al., 2022; Lobón et al., 2018).

Although production data are useful to determine whether changes in the diet of lambs are effective or not, it is necessary to obtain accurate data on the availability of nutrients and how they are digested by the animal (Sales and Janssens, 2003). Therefore, nutrient digestibility must be taken into account in the formulation of concentrates for the animals. In global terms, the CP readjustment (CP18/17) reflected a higher digestibility of organic matter (OM) during the growing and finishing phases, as well as a higher digestibility of phosphorus (P) compared to lambs fed with higher CP inclusions (CP20/19) (Chapter 1, Table 7). Similarly, when these nutrients were analysed throughout the day (Chapter 2, Figure 1, 2 and 3), it could be observed that the digestibility of OM and P remained higher during the growing phase with a low level of CP (CP18), but this effect disappeared in the finishing phase. In lambs and kids, it has been shown that the digestibility of OM increases with increasing dietary CP and seems to stabilise around 18% CP (Atti et al., 2004; Haddad et al., 2001). Dietary CP positively influences nutrient digestibility by contributing to the microbial activity and fermentation of OM (Negesse et al., 2001; Riaz et al., 2014). Therefore, it could be concluded that including more than 18% CP in the diet of non-improved breeds of lamb would compromise the digestibility of OM and P.

On the other hand, in Chapter 3 (Figure 2) it is shown that a high inclusion of carob pulp (C30) in the diet of lambs affects the digestibility of OM and CP from day 50 of age. While P digestibility and ethereal extract digestibility were only lower on days 50 and 80 of age, respectively. These results would be attributed to the CT content of carob pulp and its ability to interact with CP in the gastrointestinal tract (Obeidat et al., 2012; Patra and Saxena, 2011). Similarly, dry matter digestibility of lambs was lower at C30, which results from the interference of CT with nutrients and which has been previously described in lambs and other species (Aissa et al., 2021; Mueller-Harvey, 2006; Obeidat et al., 2012; Pan et al., 2022; Patra and Saxena, 2011). Fibrolytic bacterial populations change according to the diet supplied and the apparent digestibilities of nutrients are associated with these changes (Patra and Saxena, 2011). Furthermore, CT form complexes with protein substances, polysaccharides and minerals ingested in the diet while at the same time, the higher inclusion of CT sources, the lower their digestibility in the intestinal tract (Min and Solaiman, 2018).

However, the formation of tannin-protein complexes is not necessarily negative as it could be protecting proteins, carbohydrates and lipids from oxidative damage during digestion (Cappai et al., 2013; Caprarulo et al., 2021; Mueller-Harvey, 2006; Rodriguez-Solana et al., 2021). The composition of carob CT is mostly prodelphidins (96.7% prodelphidins/3.3% proacianidins) and a high number of gallotannins, which increases the ability of CT to interact with other compounds such as proteins (Mueller-Harvey, 2006; Saratsi et al., 2020). Thus, this complex interaction between CT and nutrients would explain the results observed in faecal CT composition (Chapter 3, Table 5), although further studies should be carried out to confirm these results.

The feedlots animals can lead changes in the feeding pattern of a lamb because of the stimulatory effect of the animal that is eating. This can result in increased feed intake compared to those fed individually (da Chunha Leme et al., 2013). In contrast, some ingredients such as carob pulp have astringent effects and could affect concentrate intake. However, the inclusion of carob pulp in the lamb diet did not affect the balance of daily activities and behaviour of the lambs (Figure 2). A more detailed analysis of the results through a multivariate canonical discriminant analysis showed that the groups fed carob pulp (C15 and C30) spent more time moving around the pen and eating straw. The analysis also showed that lambs in the C30 group spent more time eating concentrate and less time on negative social interactions. Since the overall intake of concentrate and straw did not differ between dietary treatments (Chapter 3, Table 3), it would be plausible that lambs fed carob pulp reduced their intake rhythm compared to the control diet. Similarly, Gobindram et al. (2015) demonstrated that a high inclusion of carob pulp (35%) in the lamb diet does not modify the overall feed intake during the day, but modulates the rhythm of intake. In addition, underfeeding long-cut straw facilitates the stimulation of animals to graze and to play for longer periods of time, thus reducing negative social interactions (Aguayo-Ulloa et al., 2019). Multivariate results would support the hypothesis on the temporal distribution of feeding activity according to the continuous avoidance-tolerance mechanism (Iason and Villalba, 2006). Therefore, lambs fed ad libitum would be self-managing through modulation of feeding behaviour and thus, they would be trying to avoid the toxic effects of secondary metabolites such as plant CT. On the other hand, lambs fed carob pulp seemed more active in the pen, although negative encounters were lower. This would indicate that the modulation of feeding behaviour acquired by the inclusion of carob pulp together with the availability of straw would be generating an environment more suitable for the animals. Increased movement activity of lambs is interpreted as a confidence response to the environment (Averós et al., 2014). Rice et al. (2016) suggested that decreased resting activity may be associated with increased adaptation to feedlot conditions, but not necessarily with changes in peripheral cortisol concentrations, which is a measure of physiological stress and temperament not analysed in this thesis.

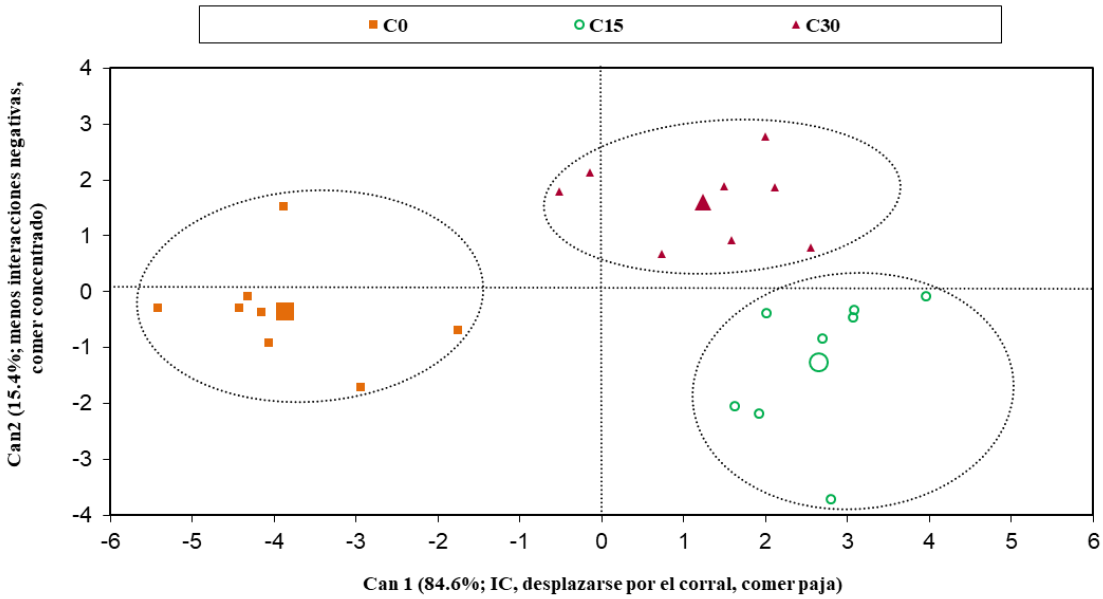

Figure 2. Canonical discriminant analysis between dietary strategies (C0 = 0 g carob pulp/kg concentrate, C15 = 150 g carob pulp/kg concentrate and C30 = 300 g carob pulp/kg concentrate) based on animal performances and behaviours. Function 1 (Can1) accounted for 84.6% of the total variation between dietary treatments and was mainly determined by FCR (r = 0.99), time spent moving around the pen (r = 0.87) and time eating straw (r = 0.67). Function 2 (Can 2) accounted for 15.4% of the variance and positive values were mainly determined by less time spent in negative social interactions (r = -0.99) and more time eating concentrate pellets (r = 0.78).

3. Nutritional metabolism and immune and gastrointestinal antioxidant response.

The study of a lamb's metabolic profile is vital to understand how dietary changes affect more or less its nutritional, oxidative or health status (Silva et al., 2023; Souza et al., 2020). Therefore, blood metabolite analysis is a supporting tool to explain the production data.

Plasma urea and creatinine are two metabolites which allow observation of dietary nitrogen intake and muscle mobilisation (Chapter 1; Bilancio et al., 2014; Gabr et al., 2023; Kaneko et al., 2008). In Chapters 1 and 2, it can be seen how urea and creatinine fluctuate due to dietary CP inclusion levels. The time of the day when sampling was performed during the growing phase did not affect any of the metabolites analysed. In contrast, during the finishing phase, creatinine of the CP17 group was higher at 8:00 a.m. and 12:00 p.m. than at 4:00 p.m. (Chapter 2, Figure 5). The blood parameters of a healthy lamb change during its first 4 months of life. In fact, this variation is usually associated with metabolic changes caused by nutritional, immunological or physiological adaptation factors (Souza et al., 2020). Hence, the increase in plasma urea concentration observed in the CP20/19 groups would be due to the higher protein intake of the diet. Furthermore, lambs of the CP18/17 group would not show deficiency of ingested CP because creatinine

remained stable. On the other hand, the higher plasma creatinine concentration during the first hours of the day would be explained by postprandial variation and protein catabolism, probably reflecting a higher creatinine metabolisation in blood from glomerular filtration before the last sampling (Cruz et al., 2017; Dos Santos et al., 2018; Souza et al., 2020; Vivian et al., 2017).

In Chapter 3, fortnightly blood sampling was included from the beginning of the study until the animals were slaughtered. In this, it could be observed that urea increased over time, but glucose decreased (Table 6). The interaction between ingested protein and its fermentation in the rumen is closely linked to the rumen microbial populations and the age of the animal (Li et al., 2022). Thus, if blood urea increased with the lifetime of the lamb, this would be an indication that the animals reduced their percentage requirement for CP. Furthermore, this would also demonstrate that the inclusion of CP in lamb diets would not negatively impact on their productivity and it shows that, with a constant supply of CP during fattening, lambs would have an excess of CP at the end of the fattening period. On the other hand, the decreasing dynamics observed in glucose was probably due to an adaptation of the rumen which, as the lamb ages, improves the digestibility of carbohydrates producing volatile fatty acids as a source of energy to the detriment of those that would be digested by intestinal hydrolysis (Giráldez et al., 2021). Moreover, with the growth of the lamb, morphofunctional changes are generated in the liver and the pancreas improves its enzymatic excretion, which helps to maintain glycaemia (Baldwin and Jesse, 1992; Cruz et al., 2017; Souza et al., 2020). The glucose range observed in Chapter 3 between 50-day-old lambs (82.3 mg/dL) and 80-day-old lambs (78.6 mg/dL) is in agreement with the results of Souza et al. (2020) and is interpreted as normal glycaemic indices.

Oxidative stress and its relationship with health and disease in farm animals has been analysed in other studies (Celi, 2011; Lykkesfeldt et al., 2007). Indeed, the most investigated causes of oxidative stress are those involving metabolic and inflammatory pathways and environmental factors (Celi and Gabai, 2015). In this regard, the levels of protein-bound malonaldehyde (MDA) and total MDA in blood of lambs remained constant with the inclusion of up to 150 g/kg of carob pulp, but increased with 300 g/kg of carob pulp (Chapter 4, Table 2). In some studies, it has been shown that diets rich in CT may have the ability to reduce lipid peroxidation by reducing MDA levels (Jerónimo et al., 2012; Soldado et al., 2021; Yonny et al., 2016). Nevertheless, it could be that increased lipids (up to 72 g ethereal extract/kg concentrate) in the feed of C30 lambs to maintain all isoenergetic diets are increasing plasma concentrations of total and protein-bound MDA, regardless of the inclusion of bioactive nutrients such as CT. Wang et al. (2019) also observed that lambs supplemented with high doses of antioxidant ingredients had higher levels of MDA. Therefore, the inclusion of 300 g/kg of carob pulp in lamb diets and the energy compensation of feedstuffs are probably the factors behind the increase in blood MDA. However, lipid variation in the diet did not affect the triglyceride and cholesterol concentrations of the animals (Chapter 3, Table 6).

Metabolites such as iron or total protein are used to assess production parameters, nutrient digestibility and clinical diagnosis. For example, the increased total blood protein observed in fattened lambs during the

period of June and July (considered as a warm period; Chapter 4), would suggest that, in order to maintain the energy requirements, the amino acid catabolism is activated at the expense of protein mobilisation (Slimen et al., 2019). This hypothesis can be supported by the observation of ADG in Chapter 4, where lambs from the cold season obtained a higher ADG than those from the warm season (278 vs. 208 g/day, respectively). Thus, it could be considered that lambs fattened during the summer were at some time of the day under heat stress and indirectly under oxidative stress.

When iron was analysed, it was found that although the inclusion of carob pulp did not affect the levels of this mineral in the blood, a reduction of iron was observed in the lambs of the warm period (Chapter 4, Table 2). Iron is vital for oxygen transport in the blood and preserves lambs' health by playing a key role in the metabolism of other micronutrients and in the antioxidant system (Asadi et al., 2022). Iron levels of around 40 mg/kg should be provided to achieve dietary requirements (Lawlor et al., 1965; Zhang et al., 2018). In this respect, the diets supplied probably provided the minimum requirements as, a vitamin and mineral mix was introduced with a contribution of 21.4 mg/kg of direct iron and, additionally, it is worth to consider the indirect sources of other main ingredients such as maize, barley, wheat and soybean (37, 184, 155 and 201 mg/kg of iron on DM, respectively) (Chapter 3, Table 1; Feedipedia, 2023). Therefore, the iron levels observed in warm season are probably linked to a pathological rather than climatological stressor. In this regard, an active coccidiosis infection can damage the intestinal mucosa and affect iron absorption in the duodenum, leading to a dysregulation of blood iron levels (Koçkaya and Özşensoy, 2016).

The homeostasis of the animals might have been altered during the warm season as a lower faecal consistency and an increase of coccidial oocysts in the faeces of the 65-day-old lambs were observed (Chapter 4, Figure 1B; Table 3). On the one hand, it is possible that the higher coccidial excretion of the warm season group was due to optimal temperature and density conditions for oocyst development (Chartier and Paraud, 2012; Froyet, 1990; Taylor, 2009;). On the other hand, the inclusion of carob pulp as a source of CT in lamb diet would regulate coccidial infection as already argued in other studies (Burke et al., 2013; Saratsis et al., 2016) and, certainly for this reason, the C0 group excreted a higher oocyst count than lambs fed by carob pulp (C15 and C30 at 3 weeks after the start of fattening). In Chapter 5 (Table 2) oocyst excretion in the faeces of suckling lambs on the day of slaughter was also analysed and it was observed that maternal feeding of sainfoin (source of CT) tended to protect their lambs from coccidial excretion. Consequently, CT ingestion via maternal diet would have an immunoregulatory effect on the intestinal environment of the lambs.

Dietary nutrient imbalance, heat stress and inflammation are some factors that can lead to reactive oxygen species formation (Celi and Gabai, 2015). The redox balance is key to ruminant immunity and health (McGrath et al., 2018), so it is necessary to understand the role of the factors which modulate cytokine production during a gastrointestinal inflammatory response or oxidative stress. Therefore, it is essential to include the observation of gene expression to the other variables analysed during the application of feeding strategies (Celi et al., 2019; McGrath et al., 2018; Sun et al., 2015). Gut homeostasis depends on the balance

between pro- and anti-inflammatory cytokines which, on the one hand, act by regulating the tolerance of gut microbiota and dietary antigens (Kogut and Arsenault, 2016; Maynard et al., 2012). However, when the immune system is challenged by a pathogen or during a stressful situation such as dietary changes, proinflammatory cytokines are released that decrease protein deposition in muscle and consequently affect ADG (Johnson, 2012). In Chapter 1, cytokines related to the immune balance of the digestive tract such as tumour necrosis factor α (TNF-α; pro-inflammatory), interleukin 10 and transforming growth factor β1 (IL10 and TGF-β1; anti-inflammatory) in the rumen and ileum without showing any significant difference between CP20/19 and CP18/17 groups, suggesting that reducing CP to 17% in lamb diets does not interfere with the gastrointestinal tolerogenic environment.

The CT-rich ingredients have antioxidant activity in ruminants and probably promote antioxidant and regulatory enzyme mechanisms in the gastrointestinal tract (Patra et al., 2019; Soldado et al., 2021). Intensive fattening conditions would be expected to generate gastrointestinal disorders and heat stress would aggravate inflammation and oxidative stress, probably due to hypoxia in these tissues (Koch et al., 2021). To control free radical production, it is possible that the antioxidant mechanism of dietary CT is involved in the capacity to scavenge free radicals, as well as in a defence promoted by antioxidant enzymes (Soldado et al., 2021). However, to reach a homeostasis status between oxidative stress and antioxidant capacity of CT, a synergistic modulation between the immune response and antioxidant defences could be established (Figure 3A). Otherwise, there would be oxidative stress and the possibility of increased susceptibility to disease if endogenous and exogenous antioxidant defences do not have sufficient capacity to reverse the situation (Figure 3B). On the other hand, most of the CT and their metabolites are bound. These xenobiotic compounds for the animal organism are transformed and metabolised by the action of gut microbiota, suffering a complex intraluminal transformation derived from digestive and absorption processes (Koudoufio et al., 2020; Zhang et al., 2021). This suggests that different sections of the digestive tract could alter the effects of CT on the expression of antioxidant enzymes or transcription factors.

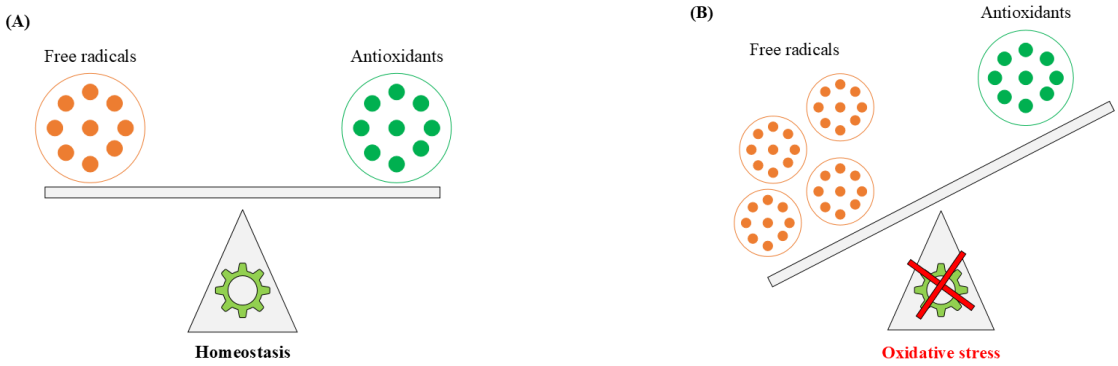

Figure 3. Representation of homeostasis between free radicals produced by oxidative stress and the antioxidant capacity of the animal (A). Development of oxidative stress when the animal's endogenous or

exogenous antioxidant capacity cannot reverse the continuous input of free radicals (B).

According to Ighodaro and Akinloye (2018), the first line of antioxidant defence occurs with the activation of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) enzymes. On the other hand, peroxisome proliferator-activated receptor γ (PPARG), nuclear factor kappa β (NF-kB) and transcription factor (NRF2), regulate inflammation and antioxidant defences (Bionaz et al., 2013; Shi et al., 2020; Surai et al., 2021). In particular, NRF2 interferes with oxidative homeostasis by being activated or inhibited with the onset or disappearance of stress (Königsberg, 2007; Shi et al., 2020), while PPARG and NF-kB are involved in anti-inflammatory and inflammatory gene activation pathways, respectively, as well as in the homeostatic maintenance of oxidative stress (Bhatt and Ghosh, 2014; Bionaz et al., 2013; Surai and Earle-Payne, 2022). Inclusion of CT-rich ingredients could inhibit inflammatory cytokines and modulate antioxidant defences through down-regulation of NF-kB and up-regulation of NRF2 activity/expression (Koudoufio et al., 2021; Rajput et al., 2019; Stefanson et al., 2014).

Pro- and anti-inflammatory gene expression (TNF-α and TGF-β1, respectively) in the jejunum of suckling lambs was lower when dams were fed sainfoin without polyethylene glycol (i.e. allowing the effects of CT to be expressed). Likewise, a decrease in GPX1 and GPX4 enzymes could be observed in the same study group and tissue, although CAT and GPX2 expression increased in the ileum (Chapter 5, Figure 1A). According to Peck et al. (2017), the cellular composition of gut is not uniform across anatomical and functional regions of the gastrointestinal tract. Therefore, the different gene expression between jejunum and ileum would be associated with microbial diversity and different nutritional and environmental exposures of immune cells throughout the gut (Peck et al., 2017). Moreover, these results would indicate that the transfer of some polyphenols through the dams' milk could have contributed to modulate the immune response and endogenous antioxidant defences of the jejunum (Yahfoufi et al., 2018). Similarly, it could be considered that including sainfoin in dams' diets would favour the stimulation of hydroperoxide detoxifying metabolism in suckling lambs by increasing CAT and GPX2 expression in the ileum. Analyses of lamb meat have shown similar results where the inclusion of grape seeds (source of CT) showed a linear increase in CAT antioxidant activity (Mu et al., 2020). On the other hand, several authors have concluded that enrichment of dairy goat diets with CT sources increases GPX2 expression in mammary gland (Tian et al., 2019; Zhang et al., 2023).

With regard to the inclusion of carob pulp, the increase in SOD2 gene expression observed in the rumen (Chapter 4, Figure 3A) would be indicative that the inclusion of carob pulp leads to improvements in the rumen epithelium by decreasing parakeratosis and increasing oxidative defences. Furthermore, it is important to reflect that intestinal tissue triggers an inflammatory response to structural changes caused by pathological damage. Indeed, in figure 3A of Chapter 4, it can be seen how the gene expression analysis performed in the ileum of lambs fed with carob pulp showed a down-regulation of the mediators NRF2 and PPARG, as well as the antioxidant enzymes CAT and SOD1. This would indicate that free radical uptake by the CT present in C15 and C30 diets possibly impaired the local production of reactive oxygen species and prevented the

mobilisation of intrinsic defences. The absence of CT in the C0 may have resulted in an increased susceptibility to coccidial infection in lambs as they showed an increased thickness of the muscular layer of the ileum, probably associated with the increased presence of coccidia in the faeces. The cycle of *Eimeria* spp. begins after coccidial invasion of the intestinal villi, which destroys crypts and promotes cellular infiltration (Khodakaram and Hashemnia, 2017). In addition, literature references describe several species of Eimeria spp. in sheep production, although one of the most common is *Eimeria ovinoidalis* (Carrau et al., 2018; Diaz et al., 2010), being the one showing tropism for ileal tissue (Bangoura and Bardsley, 2020). Probably, intestinal tropism would explain the absence of gene expression in the jejunum. Thus, it would demonstrate that lambs of the C0 group needed to activate inflammatory regulatory pathways and the antioxidant response due to parasitosis.

The exposure of animals to high daytime temperatures can generate heat stress that favours the creation of reactive oxygen species (Slimen et al., 2019). Heat stress negatively impacts on the gastrointestinal tract and can disrupt the functional barrier through damage generated from reactive oxygen species and intestinal hypoxia (Chauhan et al., 2021). Therefore, during the fattening of both batches (cold and warm season), daily temperature and relative humidity data were collected to determine the temperature-humidity index (THI). The results showed that Toll-like receptor 2 (TLR2) and TGF-β1 (Chapter 4, Figure 3B) increased in the rumen of lambs under severe heat stress (average THI of 31 and 31.9 in June and July, respectively). The up-regulation of TGF-β1 most likely expressed a cellular regulation of growth (Foley et al., 2012) as a ruminal alteration was also observed in the warm season lambs (Chapter 4, Table 4). On the other hand, TLR2 is considered a regulator of inflammation and it has been linked to the growth of bacterial populations in high concentrate diets and heat stress (Liu et al., 2015; Malmuthuge et al., 2012; Sophia et al., 2016). Thus, gene expression of this receptor is probably linked to various stressors. In the jejunum, GPX2 was decreased in heat-stressed animals, which would suggest a reduction in peroxidation capacity. Likewise, an increase in TGF-β1 expression was also observed, perhaps to counterbalance the increase in TNF-α as a pathway to regulate inflammation from heat effects, as heat stress has been described to affect immune status (Al-Dawood, 2017).

In the ileum tissue, NRF2, PPARG and NF-kB, as well as the enzymes SOD1, SOD2 and CAT, were up-regulated under summer fattening conditions (Chapter 4, Figure 3B). In this part of the gastrointestinal tract, Peyer's plaques extend from the mucosa to the submucosa (Celi et al., 2017). Therefore, with an increase of ambient temperature, the blood flow would be moved from the intestine to the periphery, leading to a decrease in nutrient absorption and may contribute to alterations of the intestinal architecture (Cantet et al., 2021). Consequently, activation of NF-kB signalling pathways as a key regulator in inflammatory responses (Li and Verma, 2002) can be expected to be conditioned by increased body heat and accumulation of reactive oxygen species (Cantet et al., 2021; Lingappan, 2018). Furthermore, NRF2 would be mediating an oxidative stress response to a possible impairment of the intestinal barrier, with the involvement of antioxidant enzymes

and PPARG in an attempt to moderate the effects of heat stress (Alemu et al., 2018; Dubuquoy et al., 2002). Further studies would be needed to improve understanding of the immunological pathways of lamb adaptation to heat stress and the involvement of antioxidant-powered feeds. Chauhan et al. (2014) observed that supplementation with vitamin E and selenium as potent antioxidants reduced the effects of heat stress.

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IX. CONCLUSIONS

1. A 2% reduction of crude protein (CP) in the diet of intensively reared light lambs (from 20/19% to 18/17% CP at growing and finishing, respectively) did not impair productive performance, reduced blood urea without affecting oxidative status and gene expression of proinflammatory and anti-inflammatory cytokines, and improved the apparent digestibility of organic matter (OM) and phosphorus (P).
2. A single sample of faeces and blood taken in the early morning would be useful to assess apparent digestibility of OM, CP and P, as well as urea and creatinine in ad libitum fed light lambs, as no interaction between sampling time and dietary CP level was observed. However, the estimation of CP digestibility may be more affected by dietary CP in the morning (8:00 a.m.) during the growing period.
3. The inclusion of up to 300 g/kg carob pulp in lamb concentrates does not negatively affect daily gains, daily behaviour activities and peripheral markers of energy and protein metabolism, but reduces the digestibility of dietary nutrients. On the other hand, the feed conversion ratio of the concentrate increases from the inclusion of 150 g/kg of carob pulp.
4. In the rumen, the inclusion of carob pulp (150 and 300 g/kg) in lamb diets improves the thickness of living layers of the papilla epithelium and reduces the darkness of the epithelium colour associated with keratin accumulation and a lower concentration of vacuoles. In addition, increased expression of radical scavenging enzymes such as SOD2 was observed. Moreover, the inclusion of carob pulp in the diet (up to 300 g/kg) also reduced the severity of coccidiosis, improved faecal consistency and helps to modulate the immune response and endogenous antioxidant defences in the ileum. The warm season had negative effects on lamb growth, rumen histological parameters and coccidiosis resistance. The cytokines that regulate inflammation and antioxidant defence system increased in warm-season lambs, modulating the pro-inflammatory and oxidative effects associated with the warm season.
5. Condensed tannins from fresh sainfoin in the diet of ewes tend to reduce the excretion of coccidial oocysts in their suckling lambs. Maternal diets containing fresh sainfoin do not affect the histopathological analysis of the jejunum and ileum of their kids. However, the superoxide radical scavenging activity of condensed tannins in the dams' diets causes down-regulation of TNFA, TGFB, GPX1 and GPX4 in jejunum tissue, while increasing the H_2O_2-degrading capacity of CAT and GPX2 in ileum tissues of lambs whose dams receive sainfoin in the diet with functional condensed tannins. This response evidences complex and compartment/region-dependent mechanisms mediating small intestinal health in suckling lambs.

X. ANNEX

Thesis publications

Pelegrin-Valls, J., Serrano-Pérez, B., Villalba, D., Martín-Alonso, M.J., Bertolín, J.R., Joy, M. and Álvarez- Rodríguez, J. **2020**. Effect of dietary crude protein on productive efficiency, nutrient digestibility, blood metabolites and gastrointestinal immune markers in light lambs. Animals, 10, 328. https://doi.org/10.3390/ani10020328.

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Pelegrin-Valls, J., Álvarez- Rodríguez, J., Martín-Alonso, M.J., Ramírez, G.A., Baila, C., Lobón, 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. https://doi.org/10.3390/agriculture12101694.

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Conference contributions and research divulgation

Pelegrin-Valls, J., Villalba, D., Molina, E., Serrano-Pérez, B., Espinal, J., Joy, M. y Álvarez-Rodríguez, J. **2019**. Efecto del nivel de proteína bruta en el pienso de cebo de corderos sobre sus resultados productivos. En: XVIII Jornadas AIDA sobre Producción Animal.

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Pelegrin-Valls, J., Martín-Alonso, M.J., Bertolín, J.R., Miralles, M., Villalba, D., Álvarez-Rodríguez, J. y Serrano-Pérez, B. **2019**. Effect of crude protein level in lamb feed on circulating malondialdehyde and cytokine expression in rumen and ileum. En: XXIV International Congress of FeMeSPRum.

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Pelegrin-Valls, J. **2022**. Nivel de inclusión óptimo de pulpa de algarroba en corderos de engorde. En: Jornada de Transferencia Tecnológica sobre "Taninos condensados y vitamina E en la dieta para mejorar la vida útil de la carne de cerdo y cordero". Organización: Generalitat de Catalunya.