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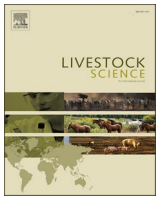

Mild synergistic effects of a dietary source of polyphenols (*Ceratonia siliqua* L.) and vitamin E on light lambs' rumination activity, nutritional status, and gastrointestinal redox-immune markers

Diego Nicolas Bottegal [a,b,*], Sandra Lobón [c,d], Beatriz Serrano-Pérez [a,e], María José Martín-Alonso [a], María Ángeles Latorre [d,f], Javier Álvarez-Rodríguez [a,g]

[a] *Departament de Ciència Animal, Universitat de Lleida, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain*
[b] *Instituto Nacional de Tecnología Agropecuaria (INTA), Rivadavia 1439, Ciudad de Buenos Aires C1033AAE, Argentina*
[c] *Departamento de Ciencia Animal, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), 50059 Zaragoza, Spain*
[d] *Instituto Agroalimentario de Aragón—IA2, CITA-Universidad de Zaragoza, 50013 Zaragoza, Spain*
[e] *AGROTECNIO-CERCA Center, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain*
[f] *Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza-IA2, C/ Miguel Servet 177, 50013 Zaragoza, Spain*
[g] *Departamento de Producción Animal y Ciencia de los Alimentos, Escuela Politécnica Superior, Universidad de Zaragoza, Carretera de Cuarte s/n, 22071 Huesca, Spain*

HIGHLIGHTS

- Combining 20 % of carob pulp and Vit E in lamb diets stimulates the rumination activity.
- Dietary inclusion of carob pulp and Vit E down-regulates jejunal expression of IFN-γ.
- Including 20 % of carob pulp in lamb diets is feasible without harming animal performance.
- Carob pulp modulates the immune response in the gastrointestinal tract.
- Vitamin e improves lamb growth and enhances the redox balance.

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ABSTRACT

This study aimed to evaluate the effect of dietary inclusion of carob pulp (0 vs. 20 % Cp, *Ceratonia siliqua* L.) and vitamin E (Vit E, 40 vs. 300 IU/kg of concentrate) on growth performance, time-budget and eating behaviour, blood metabolites, gastrointestinal and immune markers of fattening light lambs. Seventy-two weaned lambs (females and entire males) were randomly allocated in 12 group pens (6 animals/pen) in a 2 × 2 nutritional factorial design. Growth performance was evaluated, and blood samples were collected twice, whereas behaviour and eating pattern were recorded three times in the fattening period. Gene expression was evaluated by collecting ruminal, jejunal and ileal tissue samples at slaughter. The Cp inclusion did not affect lambs' performance, but the High Vit E improved the lambs' growth. Likewise, both Cp and High Vit E independently increased the haematocrit value while only High Vit E improved the plasma α-tocopherol concentration and decreased the ratio MDA: α-tocopherol, considered a proxy of enhanced redox balance. Providing simultaneously 20 % of Cp and High Vit E boosted rumination activity. Dietary Cp modified the eating pattern which was slightly delayed to the early afternoon, and decreased blood lactate. In the jejunum, High Vit E and 20 %-Cp diets down-regulated IFN-γ expression, while High Vit E down-regulated TNF-α expression. In the ileum, Cp decreased the expression of GPX2, whereas High Vit E down-regulated TGF-β and up-regulated CAT expression. Mild synergistic effects of dietary Cp and Vit E were evident on the nutritional status and gastrointestinal redox-immune markers of concentrate-fed lambs.

* Corresponding author.
E-mail address: diego.bottegal@udl.cat (D.N. Bottegal).

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1. Introduction

The inclusion of agricultural by-products as feeds reinforces the circular economy concept since waste and environmental impact are reduced by either shortening the feed transport from abroad or lowering the surface destined for animal feed (European Commission, 2020). Unlike conventional by-products used as feed (i.e., soybean meal, sunflower meal and others), "non-conventional" ones are mainly derived from processed fruit and vegetables and are characterised by a high content of polyphenols (Correddu et al., 2020). These compounds may have beneficial effects on ruminants, such as reducing enteric CH_4 production as reviewed by Gessner et al. (2017). In the Mediterranean area, Carob pulp (Cp) is an alternative ingredient which is obtained from the fruit of the evergreen Carob tree (*Ceratonia siliqua* L.) once seeds of great value in the human food, pharmaceutical and cosmetic industries are extracted (Zhu et al., 2019). The Cp is characterised by a high content of sugars, mainly sucrose, and fibre (Owen et al., 2003). Hence, Cp represents a readily resource for intensive sheep production systems located in semiarid or arid regions like Spain, where carob is largely produced (Tzatzani and Ouzounidou, 2023).

Carob by-products encompass several classes of polyphenolic substances such as gallic acid, hydrolysable tannins, condensed tannins (CT) or proanthocyanidins, flavonol-glycosides (abundant in quercetin, myricetin and kaempferol derivatives), as well as trace quantities of isoflavonoids and flavanol galloyl esters (Owen et al., 2003; Papagiannopoulos et al., 2004). Among all these compounds, the CT content stands out, since its concentration is around 16–20 % w/w of dry matter (DM) (Saratsi et al., 2020; Silanikove et al., 2006). However, carob-CT content depends on the origin, format, processing of fruit and extraction method applied (Ioannou et al., 2023).

Historically, CT have been recognized for their negative effects on palatability, digestibility, and subsequent animal growth (Mueller-Harvey, 2006; Priolo et al., 2000). In contrast, research indicates that including high doses of Cp (30−35 %) in lambs' diet is feasible without affecting animal performance (Noor-Ehsan Gobindram et al., 2015; Pelegrin-Valls et al., 2022), although the findings are not conclusive about the Cp impact on the gastrointestinal function and health of intensive fattening lambs. Noor-Ehsan Gobindram et al. (2015) demonstrated that feeding lambs a diet containing 35 % Cp increased serum urea and non-esterified fatty acids (NEFAs), suggesting increased catabolism of body reserves. Conversely, Pelegrin-Valls et al. (2022) denoted that 30 % Cp in concentrate-based (as-fed basis) lamb diets did not affect animal behaviour or metabolic status. Nevertheless, Cp modulated immune responses and endogenous antioxidant defences (Pelegrin-Valls et al., 2023).

Concentrate-based diets for light lamb (22–27 kg of body-weight at slaughter) is key for the Spanish livestock industry, aiming to maximize growth performance and obtain a homogenous product with a minimum carcass fat content to meet consumer demands. However, intensive systems expose lambs to stressful situations such as weaning, regrouping and highly concentrated diets, potentially causing acidosis. Accordingly, lambs experience oxidative stress, leading to metabolic and physiological disturbances, including inflammatory conditions (e.g., in the gastrointestinal tract) compromising their welfare (Pelegrin-Valls et al., 2023). Polyphenols, such as tannins, exhibit antioxidant and immune properties, boosting some anti-inflammatory cytokines (e.g. interleukin 10, IL-10) and suppressing pro-inflammatory cytokine (i.e. interferon-γ (IFN-γ), tumour necrosis factor (TNF-α)) in cows (Santillo et al., 2022). Besides scavenging reactive oxygen species (ROS) (Ioannou et al., 2023), polyphenols may protect and regenerate other antioxidants, such as Vitamin (Vit) E, enhancing the redox balance and antioxidant defences (Jacondino et al., 2022).

The Vit E is extensively used in animal nutrition since it improves antioxidant and anti-inflammatory capacity under *in-vivo* conditions (Lashkari et al., 2024). Currently, high doses of Vit E (i.e., 200–300 IU/kg) are used in ruminant diets to increase the α-tocopherol (the most active isomer of Vit E) muscle deposition and subsequently improve oxidative stability and extend meat shelf-life (Calnan et al., 2019). However, a further role in improving gastrointestinal tract health cannot be discarded, since its supplementation might modulate the activity of cytokines such as interleukin which regulate the immune responses (Lee and Han, 2018). Thus, the supplementation of Cp and Vit E can be a nutritional alternative to synergistically cope with intensive rearing conditions in weaned lambs. This study evaluated the effect of the dietary inclusion of Cp (0 vs. 20 %) and supplementation of Vit E (40 vs. 300 IU/kg of feed) on growth performance, time-budget and eating behaviour, blood metabolites and gastrointestinal and immune markers in concentrate-fed light lambs.

2. Materials and methods

2.1. Animals and management

Lambs were raised in the experimental farm of bonÀrea Group (Guissona, Spain), where all procedures followed the Spanish Regulations RD 53/2013 and European Union Directive 2010/63 regarding the protection of experimental animals. In addition, the Ethical Committee of the University of Lleida approved the animal procedures protocol (decision N° CEEA 02-03/21 procedure N° 01).

A total of 72 crossbred (Berberine x Romane x Ripollesa) weaned lambs (half entire males and half females) were used. Animals arrived at facilities with 41.8 ± 5.8 days old and 13.3 ± 1.30 kg of body weight (BW). After arrival, the lambs were allocated into 12 indoor pens with 0.87 m^2 per animal. The animals were distributed into homogeneous groups, balanced by initial weight and sex (3 females and 3 males in each one). There was a 7-day adaptation stage, in which the lambs received a standard commercial concentrate before the 41-day experimental period. The environmental conditions inside the facilities, such as temperature (T, °C) and relative humidity (RH, %), were logged at 10-minute intervals using Elitech RC-51H data loggers (Elitech, London, United Kingdom). Additionally, the Temperature and Humidity Index (THI) was calculated at hourly intervals as THI = (0.8 x T) + (RH/100) x (T − 14.4) + 46.4 (Thom, 1959). During the whole experiment the mean T, RH and THI were 23.8 ± 4.34 °C, 55.0 ± 14.2 % and 70.2 ± 3.6, respectively.

The pens were assigned to four different diets (3 pens/diet), according to a completely randomized design with a 2 x 2 factorial arrangement, with two Cp inclusion levels (0 vs. 200 g Cp/kg DM) and two Vit E doses (40 vs. 300 IU/kg of feed, Low and High, respectively). Thus, the diets were: 1) 0 %Cp−Low Vit E; 2) 0 %Cp−High Vit E; 3) 20 % Cp−Low Vit E; 4) 20 %Cp−High Vit E. The diets were formulated with the same ingredients and the proportions of the ingredients were changed (Table 1) to achieve similar levels of net energy (8.0 MJ net energy for ruminants/kg of DM) and crude protein (174 g of crude protein/kg of DM) (Table 2). The Cp contained 864 g DM/kg with 46.5 g crude protein, 6.0 g ether extract, 34.6 g ash, 169 g neutral-detergent fibre (aNDFom), 153 g acid-detergent fibre (ADFom), and 94.2 g lignin per kg DM. Additionally, the Cp contained 204.3 g of Cp total condensed tannins equivalents/kg DM. Each group was fed *ad libitum* with a pelletized (a diameter of 3.5 mm) concentrate-based diet plus barley straw. Water was always freely available in drinking bowls, one per pen.

2.2. Animal performance

The concentrate and straw were offered and recorded daily on a pen basis, and the amount refused was recorded weekly throughout the experiment. Additionally, all lambs were weighed individually every week. The average daily gain (ADG) by animal was calculated and the feed intake, straw intake, and total and concentrate conversion rate were estimated for each week as well as for the overall trial at the pen level.

At the age of 88 days, and after 4 h fasting period, the lambs were

Table 1
Ingredients of experimental diets, with two levels of carob pulp (Cp, 0 vs. 20 %) and two doses of vitamin E (Vit E, 40 vs. 300 IU/kg).

Ingredients (g/100 of dry matter)	0 % Cp−Low Vit E	0 % Cp−High Vit E	20 % Cp−Low Vit E	20 % Cp−High Vit E
Carob pulp	0		20	
Corn	20.4		26.7	
Barley, 9.6 % crude protein (CP)	36.1		15	
Wheat, 12.9 % CP	5.01		5.0	
Soybean hulls	15		5.0	
Soybean meal 47 % CP	19.8		23.5	
Palm oil	0.20		1.85	
Calcium carbonate	2.54		2.0	
Salt	0.50		0.50	
Vitamin-mineral premix[†]	0.40		0.40	
Vitamin E 50 %, all-rac-α-tocopheryl acetate[‡]	0	0.05	0	0.05

[†] The vitamin-mineral premix contained in each kilogram of feed: 9600 IU of vitamin A, 1920 IU of vitamin D3, 40 IU of all-rac-tocopheryl-acetate (vitamin E), 66 mg of Zn (Zinc Oxide), 60 mg of Mn (Glycine Hydrate Manganese), 3 mg Cu (Cupric Sulfate Pentahydrate), 0.80 mg of I (Potassium Iodide), 0.91 mg of Co (Cobalt Acetate Tetrahydrate), 0.40 mg of Se (Sodium selenite). Antioxidants such as 83 mg Butylated hydroxytoluene (E321) and 10 mg Propyl gallate (E310), binding agents: 200 ppm Sepiolite. [‡]Cuxavit E 50 %, vitamin E (all-rac-α-tocopheryl acetate 285 IU).

slaughtered in the bonÀrea facilities (3 km away from the farm). Pre-slaughter BW and carcass weight (without head) of each lamb were registered, and the carcass dressing percentage was calculated.

2.3. Feed analysis

Feed samples were taken from the feeder throughout the trial, and they were either dried at 60 ºC for proximate analysis or were freeze-dried (Freeze-dryer gamma 2–16 LSC plus, Martin Christ, Osterode am Harz, Germany) for subsequent tocopherols, carotenoids, and polyphenols content analyses. Feed DM (index no 934.01), ash (index no 942.05), ether extract (index no 920.39) and starch (index no 996.11), crude protein (index no 954.01) contents were determined according to AOAC methods (AOAC International, 2019). Neutral-Detergent Fibre (aNDFom, assayed with a heat stable amylase), Acid-Detergent Fibre (ADFom) and Acid-Detergent Lignin analyses were carried out following the sequential procedure of Mertens et al. (2002) using the Ankom 220 fibre analyser (Ankom Technology, Fairport, NY, USA) without sodium sulphite.

The tocopherol isomers (α, γ, and δ-tocopherols), β-carotene and total carotenoids were analysed by liquid chromatography as was previously described by Blanco et al. (2019).

The polyphenols (expressed as tannic acid equivalent) and CT (obtained as the sum of extractable CT, protein-bound CT, and fibre-bound CT) were extracted in the freeze-dried feed samples. The polyphenols were quantified using the Folin–Ciocalteu reaction and the CT by the colorimetric HCl-butanol method, both of which are explained in detail in 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 550 nm for polyphenols and CT content, respectively. In addition, polyphenols content was expressed as tannic acid equivalents. Meanwhile, a standard was extracted and purified from freeze-dried Cp and it was used to quantify the content of CT, which were expressed as CT-equivalent of Cp.

The percentage of fine particles of feed (non-pelletized particles) was determined by shaken 400 g samples through a 2 mm screen for 30 s. Pellet durability was evaluated in a Pfost tester (weight of pellets after 10 min of tumbling x 50 rpm/weight of pellets before tumbling x 100).

2.4. Animal behaviour evaluation

On three days of the experiment (54, 68, and 82 days of average age),

Table 2
Chemical composition, expressed on a dry basis (DM), and physical pellet quality of the experimental diets, with two levels of carob pulp (Cp, 0 vs. 20 %) and two doses of vitamin E (Vit E, 40 vs. 300 IU/kg).

Item	0 % Cp−Low Vit E	0 % Cp−High Vit E	20 % Cp−Low Vit E	20 % Cp−High Vit E
Nutrients (g/kg of DM feed)				
Dry matter	890		885	
Gross energy (MJ/kg DM)	17.9		18.8	
Net energy for ruminants (MJ/kg DM)[†]	8.0		8.0	
Crude protein	173		175	
Ether Extract	25.6		33.6	
Total sugars	24.7		96.0	
Neutral-Detergent Fibre	182		178	
Acid-Detergent Fibre	101		80	
Acid-Detergent Lignin	0.88		2.44	
Ash	55.9		63.4	
Calcium	12.8		13.2	
Phosphorus	3.26		4.57	
Starch	458		387	
β-carotene (μg/g)	1.47	1.57	9.65	8.81
Total carotenoids (μg/g)[‡]	4.69	4.71	13.5	15
α-tocopherol (μg/g)	37.3	241	40	283
γ-tocopherol (μg/g)	11.6	11.7	12.9	14
δ- tocopherol (μg/g)	5.16	5.13	4.75	6.38
Polyphenols (g tannic acid eq./kg)	4.35		6.46	
Total condensed tannins (g Cp total CT−eq./kg)	1.43		18.93	
Extractable (% out of total CT)	55.4		1.10	
Protein-bound (% out of total CT)	6.9		69.2	
Fibre-bound (% out of total CT)	37.7		29.7	
Physical pellet quality				
Fine particles (<2 mm) (%)	1.06		1.60	
Durability (%)	98.8		96.7	

[†] Net energy for meat production was estimated as the weighted sum of the net energy value of each ingredient according to the FEDNA Tables (FEDNA, 2019). Total carotenoids represent the sum of Zeaxanthin, Lutein, and β-carotene content.[‡].

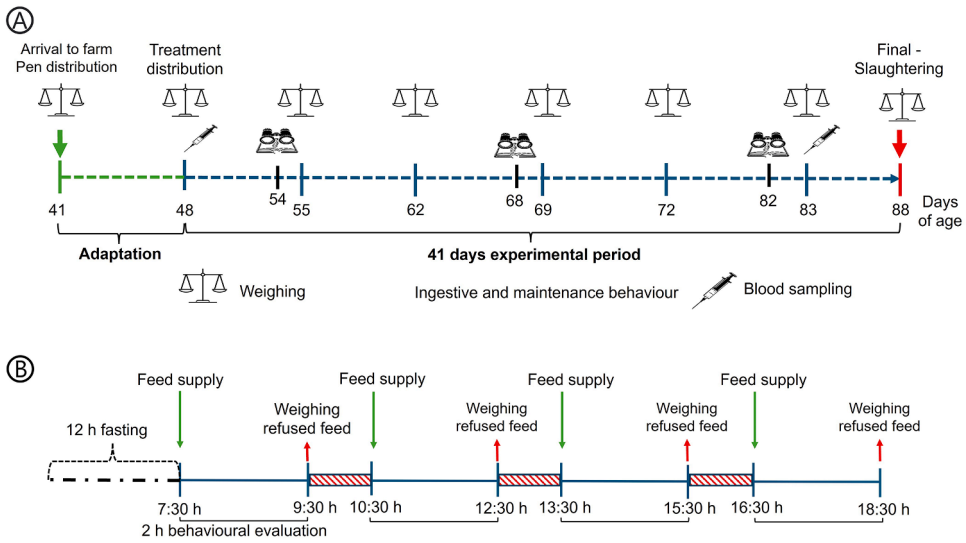

Fig. 1. Experimental design. Timeline A) of the fattening period and the different moments of weighing, behavioural and blood collection. The figure below B) illustrates the protocol used during the behavioural evaluation days.

the feeding behaviour, represented by the daily pattern of feed consumption, and the maintenance time-budget behaviours were evaluated using two methodologies (Fig. 1). Feeding behaviour was assessed at four consumption intervals time (sessions) throughout the evaluation day (i.e., 7:30–9:30 h, 10:30–12:30 h, 13:30–15:30 h, 16:30–18:30 h), by successive weighing of the feed supplied and the remaining amount in the feeder. The concentrate was restricted for 12 h before the measurement. This allowed to study the pattern of concentrate intake by deprived lambs and whether the diets differed in palatability. The feed intake per session was evaluated on a pen basis, and then it was estimated as mean individual intake (total pen intake/number of animals per pen).

Simultaneously to the intake evaluation, the maintenance time-budget behaviours were recorded through the instantaneous focal sampling technique on four animals per pen (two animals per sex), which were individualized with marker spray. On each evaluation day, a trained person carried out direct observations on the selected lambs every 10 min during the four sessions of 2-hour length, which were coincident with those of the intake evaluation. Eight possible behaviours were recorded: (1) eating concentrate (the animal had its head near or inside the feeder), (2) drinking (the animal had its head near or above the trough), (3) ruminating (the animal ruminated in lying or standing position), (4) straw consumption, (5) positive social interactions (which included explorative behaviour, affiliative interactions or shelf-grooming), (6) negative social interactions, (7) inactivity (standing or lying position) and (8) locomotion (the animal was moving over the pen). As a result of low frequency, positive social interaction and locomotion were summarized in a single category as other activities. The behaviour performed by each marked lamb was recorded and expressed as a proportion of the total time evaluated. All behaviours initially outlined in the ethogram were evaluated, however, only one negative social activity was recorded among the 7488 observations conducted, therefore this activity was not considered for the statistical analysis.

2.5. Blood sampling and analyses

At 48 and 83 days of age (at the beginning and one week before the end of the experiment, respectively, see Fig. 1), blood samples were collected by puncture of the jugular vein from four selected animals per pen (two male and two female lambs per pen). The blood was withdrawn by means of BD Vacutainer 1.2 x 38 mm needles and 10 ml Vacutainer containers with EDTA as an anticoagulant (BD® Vacutainer® with EDTA K2 additive 10.08 mg) and before the weekly weighing. Haematocrit was determined manually in duplicate samples after centrifugation of a microcapillary tube filled with whole blood (Bolliger and Everds, 2012). Subsequently, the remaining blood sample was centrifuged (Cencom II angular rotor centrifuge, JP SelectaTM, Spain) at 3000 rpm for 15 min and immediately later, 1.5 ml aliquot of plasma was transferred to Eppendorf tubes and stored at −80 °C until further analyses. The plasma concentration of creatinine, glucose, urea, lactate, cholesterol, triglycerides, and NEFA was determined with an automatic analyser (Olympus AU400, Germany). The triglycerides, NEFA and cholesterol concentration were analysed only at 83 days of lamb age. The protocols and reagents used in this study were standardized by the Biochemical Veterinary Laboratory Service (UAB, Barcelona, Spain). Additionally, the plasma malondialdehyde (MDA), α-tocopherol, and retinol concentrations were analysed in samples at the age of 48 and 83 days. The MDA concentration in plasma was determined using the method described by Yonny et al. (2016) and expressed as the sum of the free MDA and protein-bound MDA, which were separately quantified. Meanwhile, plasma α-tocopherol concentrations were analysed through liquid chromatography following the methodology described by Rufino-Moya et al. (2020).

The plasma α-tocopherol:total lipids ratio is considered a valuable index to identify Vit E deficiencies and it was calculated as the plasma concentration of α-tocopherol (mg/L)/(cholesterol (g/L)+triglycerides (g/L)) (Winbauer et al., 1999). Moreover, two other ratios were calculated to identify relationships between lipid oxidation (expressed as blood MDA concentration) and both the total circulating lipids and the α-tocopherol concentrations .

2.6. Sampling, processing of gastrointestinal tissues and quantitative real-time polymerase chain reaction analysis (qPCR)

The entire gastrointestinal tracts of 24 random female lambs were sampled at slaughter ($n = 6$) and immediately placed in an ice tray. For gene expression analysis, a 2-cm length portion of ruminal tissue from the rumen cranial dorsal sac and a 2-cm section were obtained from both the mid jejunum and the ileum, just proximal to the ileocecal junction. Samples were rinsed with phosphate-buffered saline solution, incubated in RNAlater (Invitrogen, Madrid, Spain) for 24 h at 4 °C and finally stored at −80 °C until analysis.

The procedures for RNA extraction, concentration and purity evaluation, DNAse treatment to eliminate contaminating genomic DNA,

complementary DNA synthesis, primer designs, serial dilutions of control copy DNA, and amplification procedures were performed following previous works in the same laboratory (Pelegrin-Valls et al., 2020). Triplicate amplification measurements were averaged to calculate the relative gene amount.

Messenger RNA expression was determined by qPCR for target genes related to immune function and redox status. Transforming growth factor-β1 (TGF-β), TNF-α, nuclear factor kappa β (NFK-β), nuclear factor E2-related factor 2 (NRF2), superoxide dismutase 1 and 2 (SOD1 and SOD2, respectively), Na+/*H*+ exchange isoform 1 (NHE1) and down-regulated in adenoma (DRA) were analysed in the rumen. In jejunum and ileum, IL-10, TGF-β, interferon-γ (IFN-γ), TNF-α, NFK-β, NRF2, peroxisome proliferator activator receptor-γ (PPARG), catalase (CAT), glutathione peroxidase 1 and 2 (GPX1 and GPX2, respectively), SOD1 and SOD2 were evaluated. Primer sequences and sources of the primers are shown in Pelegrin-Valls et al. (2023). Gene normalisation was achieved utilising 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. Data were normalized and analysed by the $2^{-\Delta\Delta Ct}$ method using the real efficiency value obtained for each of the genes studied.

2.7. Statistical analysis

All animals were considered in the dataset since no deaths, wasting syndrome or other problems were registered in the present study.

Data were analysed using the Infostat software (version 2020, Córdoba, Argentina). General linear models were carried out for productive performance considering the Cp, Vit E, sex, and the single interaction between them as fixed effects. The pen was considered the experimental unit for the majority of the productive variables. The ADG was determined individually by linear regression of weekly BW against time. Gastrointestinal gene expression data from females were log-transformed to meet the assumption of normality and homoscedasticity and analysed with the same aforementioned model.

Within each daily session, the feed intake pattern was analysed with a general linear model considering the fixed effects of Cp, Vit E, and age and the single interaction between them. Since there was no effect of age, it was removed from the final model.

Due to the non-normal distribution of behavioural category data, an arcsine transformation was applied to the square roots of the percentages to fit parametric statistical analysis. Behavioural transformed data and plasma metabolites were analysed through mixed models with repeated measurements that included the Cp, Vit E, age, and sex and their interactions as fixed effects, as well as the pen (for behaviour) and the animal (for physiological variables) as a random effect.

The single interactions between fixed effects were included in the models but only were reported when resulted significant. Significant differences were considered when $P \leq 0.05$, and a tendency was denoted when $0.10 > P > 0.05$. The results are presented as least square means and their associated standard errors. Means were separated by the Fisher's least significant difference test when significant fixed effects were detected. Finally, the results of behaviour were converted to the original scale and arithmetic means with their standard errors were calculated.

3. Results

3.1. Animal performance

The effects of Cp, Vit E and sex on performance parameters are presented as main effects because no interactions between them were detected on productive variables ($P > 0.10$). The results of the productive performance of lambs are presented in Table 3. In all dietary treatments, lambs began the experiment with similar BW ($P > 0.05$), although males tended to be heavier ($P = 0.095$) than females (15.4 vs. 14.8 ± 0.26 kg of BW, respectively). At the end of trial, no differences were found ($P > 0.10$) on the majority of productive variable due to Cp or Vit E inclusion. Nevertheless, the lambs fed 300 IU Vit E/kg showed a higher ADG compared to lambs fed 40 IU of Vit E/kg ($P < 0.05$). No differences were found ($P > 0.10$) on any of the weighing days in BW due to the inclusion of the different levels of Cp or Vit E (data not shown).

Table 3
Effects of dietary inclusion of Carob pulp (0 % vs. 20 % Cp) or vitamin E (40 (Low) vs. 300 (High) IU Vit E/kg of concentrate feed) on productive performance variables.

Variable	Carob pulp		Vitamin E		SEM	*P* -value	
	0 %	20 %	Low	High		Cp	Vit E
n	6	6	6	6			
Initial Age (days)	47.7	47.9	48.8	46.7	0.93	0.888	0.175
Initial BW (kg)	15.0	15.1	15.2	14.9	0.26	0.825	0.384
Final BW (kg)	25.2	25.1	24.8	25.5	0.38	0.917	0.172
Within-pen coefficient of variation of final BW (%)[†]	8.89	8.20	8.36	8.74	1.65	0.768	0.870
Average Daily Gain (g BW/d)	248	245	234	260	8.00	0.762	0.032
Daily concentrate Intake (g/d)	860	890	870	890	20.0	0.218	0.382
Daily straw intake (g/d)	108	109	111	106	10.0	0.867	0.496
Total Feed (concentrate and straw) conversion (Feed:Gain)	3.85	4.04	4.13	3.76	0.14	0.378	0.100
Concentrate conversion rate (Feed: Gain)	3.42	3.60	3.66	3.36	0.12	0.323	0.113
Hot Carcass Weight (kg)	11.8	11.8	11.7	12.0	0.24	0.961	0.276
Dressing percentage (%)	46.9	47.1	47.0	47.1	0.38	0.705	0.814

Note: SEM, standard error of means; BW, body weight.
[†] Within-pen coefficient of variation of final BW (100 x standard deviation of final BW/mean final BW). No significant interactions were detected ($P > 0.10$).

Although data was not shown in Table 3, males showed a greater final BW than female lambs ($P < 0.001$, 26.3 vs. 24.0 ± 0.38 kg), higher ADG ($P < 0.001$, 270 vs 224 ± 8.0 g/day) and hot carcass weight ($P < 0.05$, 12.3 vs. 11.4 ± 0.24 kg), although the carcass dressing was similar in both sexes ($P > 0.10$, 47.0 ± 0.38 %).

3.2. Intake and activity time-budget

Feed consumption decreased linearly ($P < 0.001$) from morning to afternoon session regardless of diet. Therefore, 39 % of total feed intake occurred in the first 2 h of feed provision in the morning (7:30–9:30 h), 30 % at midday (10:30–12:30 h), 18 % in the early afternoon (13:30–15:30 h), and the remaining 13 % in the afternoon (16:30–18:30 h), thence it represented on average 291, 227, 135 and 98 ± 10.0 g of feed/lamb, respectively. The effects of Cp and Vit E on the ingestive activity (expressed as % of the total concentrate feed intake) during each session of evaluation throughout the day are shown in Fig. 2. In the early afternoon session, the Cp-animals consumed more than the lambs without Cp ($P = 0.041$, 157 vs. 131 ± 13.3 g of feed/lamb, respectively). During the rest of the day, both groups of animals had similar levels of feed intake ($P > 0.10$). The supplementation of Vit E did not affect the feed daily intake pattern ($P > 0.10$).

Dietary inclusion of Cp or Vit E supplementation did not show effects on any of the behaviours recorded (Table 4). Nevertheless, the age factor affected the eating concentrate, eating straw, inactive state, and rumination time. Lambs spent more time eating concentrate ($P < 0.05$) at 54 days of age (beginning of the trial) compared to 68 days of age, while at

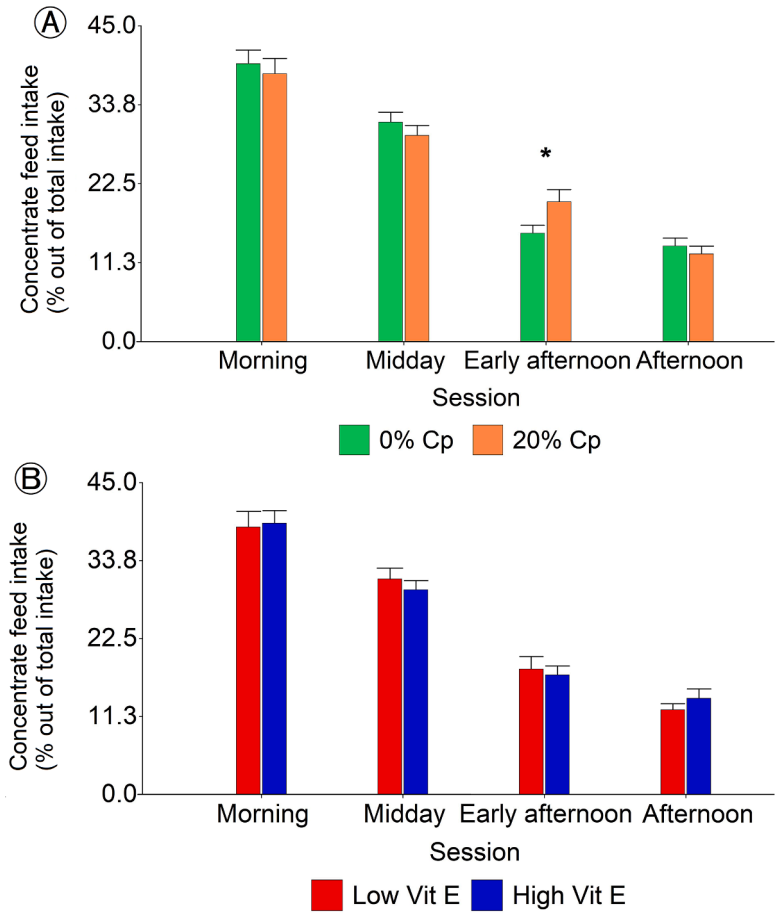

Fig. 2. Effects of dietary inclusion of carob pulp (Cp, A) and vitamin E (40 vs. 300 IU Vit E, Low and High, respectively, B) on the concentrate feed intake in each session: morning (7:30–9:30 h), midday (10:30–12:30 h), early afternoon (13:30–15:30 h) and afternoon (16:30–18:30 h). *Indicates significant differences ($P < 0.05$) between diets in that specific session.

Table 4
Activity time-budget (proportion out of total time recorded in each activity) in light lambs feeding diets with Cp (0 vs. 20 %) and Vitamin E (40 vs. 300 UI/kg of concentrate feed, Low and High, respectively) at three ages during the fattening period.

	Carob pulp		Vit E		Age (d)			SEM	*P*-value		
Behaviour (%)	0	20	Low	High	54	68	82		Cp	Vit E	Age
Inactive	52.8	52.4	52.7	52.5	57.2^{a}	47.1^{b}	53.5^{a}	1.76	0.897	0.915	<0.001
Eating concentrate	17.3	15.7	16.7	16.3	18.0^{a}	15.2^{b}	16.3^{ab}	1.10	0.350	0.89	0.026
Ruminating	13.1	14.3	13.4	14.0	10.1^{b}	15.5^{a}	15.5^{a}	1.14	0.531	0.707	<0.001
Eating straw	9.68	9.24	9.59	9.30	7.14^{b}	14.2^{a}	7.00^{b}	1.09	0.615	0.864	<0.001
Other activities[†]	6.50	7.00	6.49	7.04	6.50	7.00	6.72	0.943	0.748	0.776	0.998
Drinking	0.62	1.36	1.12	0.86	1.07	1.00	0.98	0.470	0.277	0.813	0.503

Notes: SEM, Standard error of means ($n = 6$). Interactions between Cp and Vit E were tested in the model and were significant only in rumination activity (shown in Fig. 3).

[†] Other activities include positive behaviours and locomotion activity.

the end of the experiment, they showed intermediate values. At the age of 68 days, the lambs spent more time eating straw and were more active ($P < 0.001$) compared to the other two evaluation days. Regarding rumination, it showed the lowest value ($P < 0.001$) at the beginning of the experiment. Furthermore, the interaction between Cp and Vit E only affected ($P = 0.039$) the rumination activity (Fig. 3). The lambs fed the 20 %Cp-High Vit E diet dedicated more time to rumination compared to those fed 0 %Cp-High Vit E and 20 %Cp-Low Vit E diets, while lambs fed 0 %Cp-Low Vit E diet presented intermediate values.

3.3. Metabolic profile

The effects on the physiological variables of the dietary treatments and age are shown in Table 5, except for the interaction between Vit E and age detected on the α-tocopherol content which is shown in Fig. 4. The average haematocrit value increased in lambs fed 20 % Cp compared to 0 % Cp ($P = 0.002$), as well as in lambs fed High Vit E ($P = 0.004$) compared to Low Vit E. The inclusion of 20 % Cp tended ($P = 0.067$) to increase the plasma glucose and decreased ($P = 0.046$) the lactate concentration compared to lambs without Cp. High Vit E diet tended ($P = 0.073$) to increase the MDA levels compared to Low Vit E. Blood urea concentration was greater in females than in male lambs (35.1 vs. 30.2 ± 1.24 mg/dL; $P = 0.02$). No other effects of sex were found on metabolic profile variables.

The interactions between factors were only detected between Vit E and age ($P < 0.001$) on the α-tocopherol concentration (Fig. 4). At the

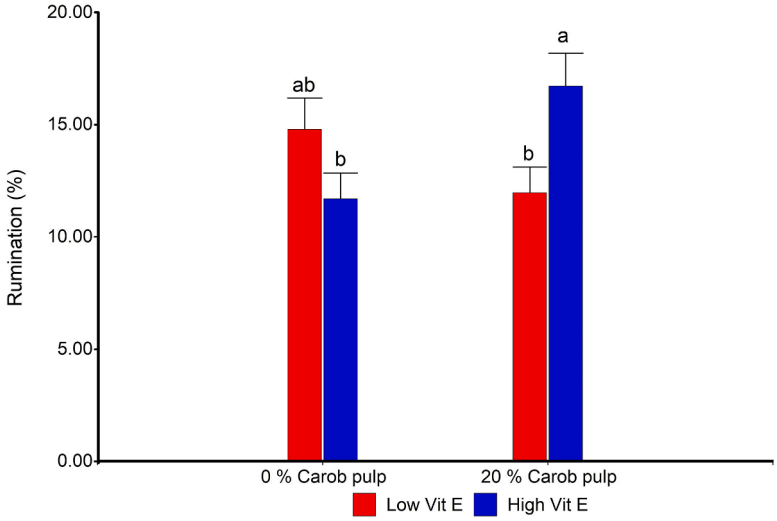

Fig. 3. Effect of dietary carob pulp inclusion (0 vs. 20 %) and Vitamin E (40 vs. 300 IU/kg of concentrate, Low and High, respectively) on rumination time (proportion out of total time budget, %). Different letters indicate differences between groups ($P < 0.05$).

Table 5
Effect of the inclusion de carob pulp (Cp, 0 vs. 20 %), two doses of vitamin E (40 (Low) vs. 300 (High) IU/kg of concentrate feed) and the age (48 and 83 days) of fattening light lambs on plasma metabolites.

	Carob pulp		Vit E		Age		SEM	*P*-value		
Item	0 %	20 %	Low	High	48	83		Cp	Vit E	Age
Haematocrit, %	36.6	39.2	36.7	39.1	38.0	37.8	0.007	0.002	0.004	0.760
Creatinine, mg/dL	0.67	0.66	0.66	0.67	0.64	0.69	0.011	0.574	0.620	<0.001
Glucose, mg/dL	95.5	100	98.0	97.9	103	93.1	2.26	0.067	0.957	<0.001
Urea, mg/dL	32.8	32.4	32.3	33.0	28.4	36.9	1.42	0.849	0.740	<0.001
Lactate, mmol/L	3.09	2.47	2.71	2.86	2.97	2.59	0.211	0.046	0.623	0.151
α-tocopherol, μg/mL	0.94	0.86	0.65	1.15	1.08	0.72	0.061	0.379	<0.001	<0.001
γ-tocopherol, ng/mL	27.1	27.3	27.5	26.9	26.0	28.3	2.14	0.921	0.775	0.281
δ-tocopherol, ng/mL	5.18	5.30	4.94	5.54	5.88	4.6	0.514	0.863	0.409	0.005
Retinol, μg/mL	0.39	0.4	0.39	0.4	0.35	0.44	0.013	0.676	0.660	<0.001
Malondialdehyde, μM	6.94	7.09	6.90	7.13	7.15	6.88	0.092	0.217	0.073	0.003

Notes: SEM, Standard error of means ($n = 12$). Interactions between Cp x age and Vit E x age were tested in the model but were not significant ($P > 0.10$).

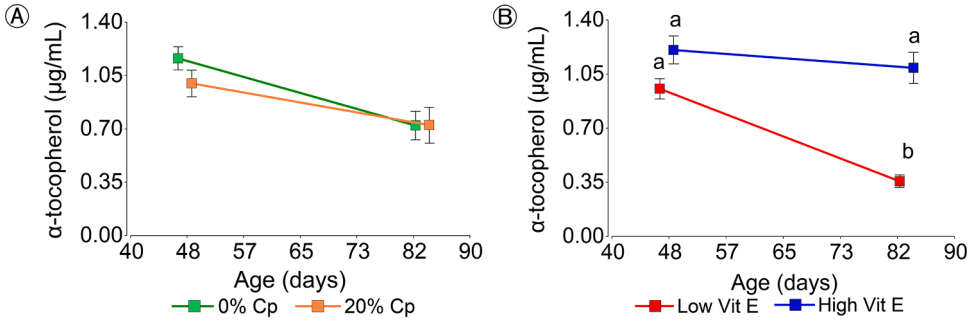

Fig. 4. Effect of the supplementation of carob pulp (0 vs. 20 % Cp, A) and vitamin E (40 and 300 IU Vit E/kg of concentrate, Low and High, respectively, B) on the evolution of α-tocopherol blood concentration throughout the fattening period of light lambs. Different letters indicate significant differences between groups ($P < 0.05$).

end of the study, lambs fed Low Vit E diet showed a decrease in the plasma α-tocopherol concentration, while in lambs fed High Vit E, the α-tocopherol remained stable.

At the end of the fattening period, triglycerides, cholesterol and NEFAs were unaffected by any of the factors studied ($P > 0.10$, Table 6). However, the ratio α-tocopherol:lipids (cholesterol + triglycerides, mg/g) and MDA:α-tocopherol (μM/mg) showed effect of the Vit E doses ($P < 0.001$), while the ratio MDA:lipids (μM/g) tended ($P = 0.068$) to be lower in the High Vit E group. The Cp or sex did not affect these variables ($P > 0.10$).

3.4. Gastrointestinal gene expression analysis

There was only a tendency for lower NFK-β expression ($P = 0.08$) in the rumen of lambs fed the Cp diet. Vitamin E supplement did not affect any target gene expression in the ruminal tissue ($P > 0.10$).

In jejunal tissues, an interaction between Cp and Vit E affected the expression of IFN-γ and IL-10. Gene expression of IFN-γ was the lowest

Table 6
Effect of the inclusion de carob pulp (Cp, 0 vs. 20 %), two doses of vitamin E (40 vs. 300 IU/kg of concentrate feed, Low and High, respectively) on plasma concentration of plasma lipids compounds of fattening lambs at the end of the experiment.

Item	Carob pulp		Vitamin E			*P*-value	
	0 %	20 %	Low	High	SEM	Cp	Vit E
Triglycerides (mg/dL)	15.6	14.4	14.6	15.5	0.83	0.319	0.462
Cholesterol (mg/dL)	41.6	45.6	41.3	45.9	2.25	0.193	0.141
NEFAs (mmol/L)	0.16	0.20	0.15	0.20	0.031	0.265	0.237
α-tocopherol:lipids (mg/g)[†]	1.25	1.14	0.64	1.75	0.084	0.404	<0.001
MDA:lipids (μM/g)	12.01	12.2	12.93	11.29	0.66	0.827	0.068
MDA:α-tocopherol (μM/mg)	14.24	15.05	21.77	7.53	1.31	0.674	<0.001

Notes: SEM, standard error of means ($n = 12$); MDA, malondialdehyde. Interactions between Cp and Vit E were tested in the model but were not significant ($P > 0.10$).
[†] Blood lipids were considered as the sum of triglycerides and cholesterol concentrations.

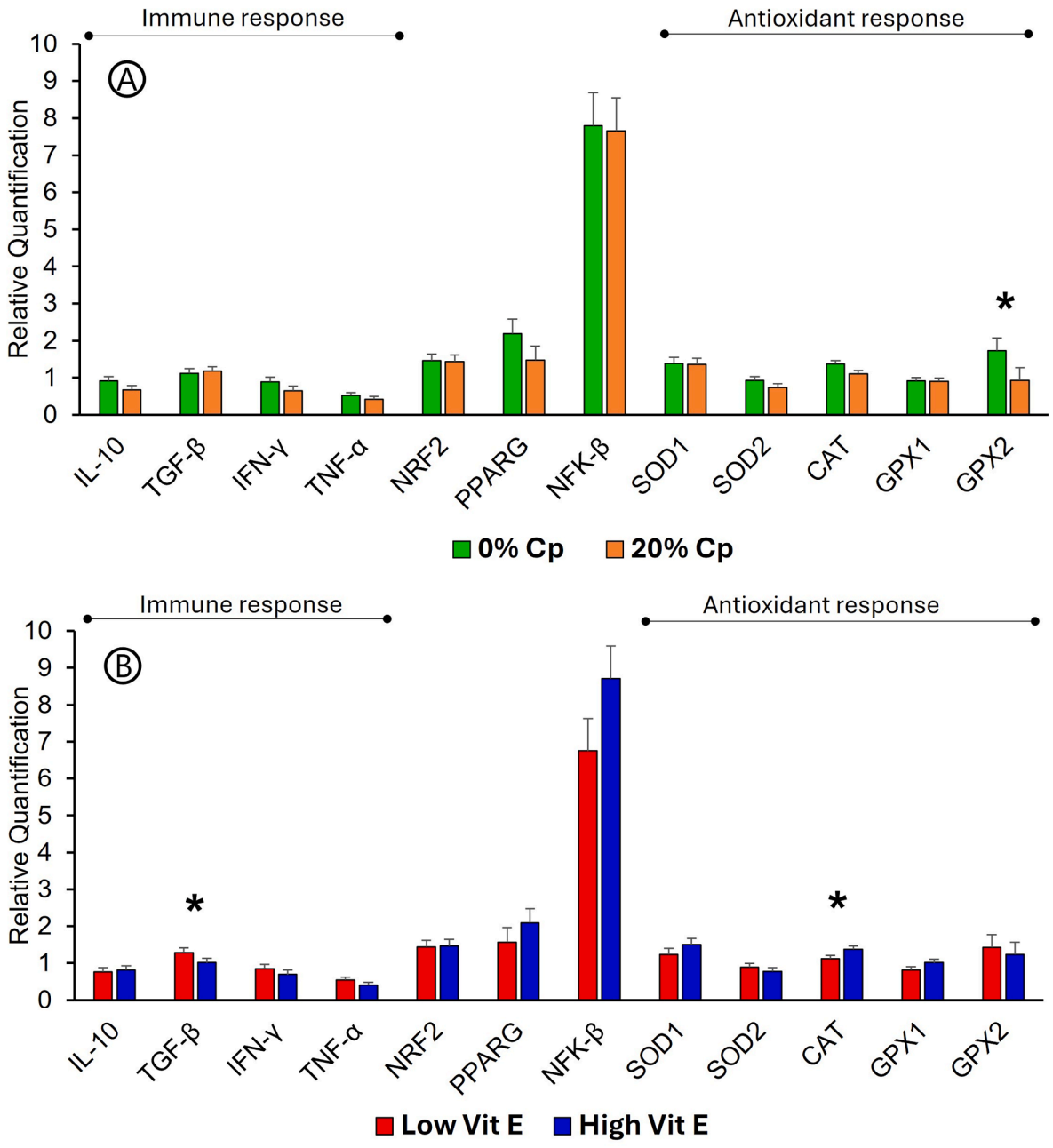

Fig. 5. Relative quantification of gene expression in the ileum tissue of fattening lambs supplemented with carob pulp (0 vs. 20 % Cp, A) and vitamin E (40 vs. 300 UI/ kg of concentrate, Low and High, respectively, B). *Indicates differences ($P < 0.05$) between diets above pair bars.

($P < 0.05$) when the lambs fed the 20 %Cp-High Vit E diet, while expression of IL-10 was the highest ($P < 0.05$) when the lambs were fed 20 %Cp-Low Vit E diet. Additionally, there was a Vit E effect on TNF-α expression, which was lower ($P < 0.05$) in High than in Low Vit E diets.

In ileal tissues, gene expression of GPX2 was lower in 20 %-Cp diets ($P < 0.05$) whereas no other genes were affected by the Cp inclusion in this tissue. Meanwhile, High Vit E decreased TGF-β and increased CAT expression (Fig. 5).

4. Discussion

The polyphenols are considered potent antioxidants since they may scavenge ROS by accepting unpaired electrons to form stable phenoxyl radicals (Landete, 2013) to promote enzymes' antioxidant defences and regenerate α-tocopherol through reduction of the α-tocopheryl radical (Mazur-kuśnirek et al., 2019; Rice-Evans et al., 1996). However, phenolic compounds are poorly absorbed in the gastrointestinal tract or, even if absorbed, are extensively bio-transformed by classic detoxification pathways (Gessner et al., 2017; Luciano et al., 2017). Thus, the inclusion of polyphenol-rich ingredients may be considered a tool to protect some vitamins during digestion and absorption processes in the intestines (Lauridsen et al., 2021).

4.1. Interactions between dietary carob pulp and vitamin E effects

The interplay between Cp and Vit E increased the rumination time and affected the gene expression of key cytokines that regulate the pro and anti-inflammatory immune response (IFN-γ and IL-10) in the jejunal tissue. Increasing rumination activity may be considered a proxy of digestive welfare because of buffering capacity to alleviate low pH of the rumen contents, especially when lambs are fed concentrates with high starch levels (Monjezi et al., 2022). However, in this study, there were no differences across diets in straw intake nor in time eating straw. The NDF content of the concentrates was similar, which may suggest that lambs did not have to adjust their sorting behaviour in favour of physically effective NDF. Nonetheless, several studies have showed that supplementing polyphenols in cows fed grain-rich diets increases both the rumination and the chewing activity (Daddam et al., 2023; Kröger et al., 2017). Wu et al. (2023) even found that the Vit E supplementation may alleviate the acidosis condition and protecting the bacterial membrane in the rumen from a free radical attack, improving rumen fermentation. Thus, the observed differences in the rumination time-budget may be related to a synchronic supply of high Cp and Vit E.

In agreement with previous studies, the supplementation of Cp (rich in CT) helped to modulate gut immune responses (Pelegrin-Valls et al., 2023). A decreased transcription of IFN-γ in jejunum may reflect a lower pro-inflammatory condition of the small intestine (Frutos et al., 2018) which suggests a beneficial synergic role between Cp's polyphenols and the α-tocopherols in the control of tolerance mechanisms. Additionally, the increment in the expression of IL-10 in jejunum from lambs fed 20 % Cp and Low Vit E may suggest an anti-inflammatory activity of Cp's polyphenols but not of High Vit E level. Increased production of the anti-inflammatory cytokine IL-10 exerts a protective effect against diverse inflammatory conditions, either by inhibiting tissue damage or limiting pro-inflammatory cytokines such as TNF-α, release by T cells and antigen-presenting cells (Atta et al., 2023). Similar to the current results, Ciliberti et al. (2024) observed an increase of blood IL-10 in lambs supplemented with hazelnut skin, a by-product rich in CT, indicating an anti-inflammatory effect. Therefore, as oxidative stress and inflammation are interdependent, it is possible that 20 % of Cp inclusion modulates lymphocyte T regulatory function and hence IL-10 secretion in jejunal tissues without an involvement of Vit E.

The rest of variables are discussed by considering dietary Cp and Vit E as independent factors.

4.2. Effects of carob pulp inclusion

While including non-conventional by-products in animal feed offers potential sustainability benefits, their incorporation is often limited by the presence of anti-nutritional factors and consequently a lower growth rate (Mueller-Harvey, 2006). Nevertheless, including 20 % of Cp in fattening lambs' diets did not affect either the feed intake, growth performance or carcass traits, which means it was a moderate level of inclusion, considering also that diets were balanced including protein and oils sources. Regardless the treatment, animals, showed elevated ADG (247 ± 8 g/day) and kept a high level of feed intake (878 ± 20 g/day). Previously, and under identical rearing conditions, Pelegrin-Valls et al. (2022) assessed the impact of feeding lambs two different levels of Cp (15 and 30 %) and detected only a higher feed conversion rate in the lambs receiving Cp compared to the control group, but the growth rate was similar in all treatments. Also, Priolo et al. (1998) replaced barley with 20 % of Cp and observed that animals fed Cp had similar final BW but higher feed intake, thus a worse conversion rate compared to the control group. Nevertheless, other studies found a decrease in the digestibility of nutrients and hence ADG and feed efficiency when lambs (Priolo et al., 2000) or kids (Silanikove et al., 2006) were fed with around 50 % Cp. At such high levels, tannins bind to both proteins and bacterial enzymes and therefore reduce their activity, with consequent slower rates of feed digestion (Priolo et al., 2000). Herein, no anti-nutritional effects were found, since Cp-lambs consumed 17 g of CT/day, which is considered a low amount. A level of up to 60 g of CT/day does not induce any toxic syndrome (Silanikove et al., 2006). When sheep were fed at high feeding levels, the metabolic cost of CT intake was reduced (Méndez-Ortiz et al., 2018), in line with the present outcomes. In addition, unlike most of the references mentioned, in this study the animals received concentrated diets with lower levels of dietary fibre.

One of the typical negative effects of dietary CT is the reduction of voluntary feed intake (Priolo et al., 2002). Indeed, Noor-Ehsan Gobindram et al. (2015) observed that high levels of Cp (24–35 %) in diets decrease the proportion of the ingestion during the morning (9–10:30 h) after feed supply, in comparison to a control group. While 20 % of Cp is considered a high inclusion level, it appears insufficient to induce palatability aversion. This is likely because the high sugar content in Cp overcomes the potential astringent effects of CT, as reported in other studies (Priolo et al., 2000). In fact, Costes-Thiré et al. (2018) suggested that a conditioning period is important to facilitate the learning process and allow memorization of the sensorial and post-ingestive characteristics of the feed containing CT. This could explain the observed increase in early afternoon eating behaviour by the 20 %Cp-lambs compared to the control group, which aligns with prior research (Noor-Ehsan Gobindram et al., 2015).

The physiological effects of carob in lambs have been previously evaluated. For instance, the inclusion of 35 % of Cp in a forage-based diet increased both serum NEFA and urea levels while caused a reduction in blood cholesterol (Noor-Ehsan Gobindram et al., 2015). However, the current outcomes are in accordance with a previous experiment in a concentrate-based diet where the key metabolites related to nutritional status were not impaired by including up to 30 % of Cp (Pelegrin-Valls et al., 2022). Additionally, the haematocrit values (red blood cell count), which at low level may be a proxy of anaemia risk due to iron deficiency in growing lambs (Crilly and Plate, 2022) was increased in the diet containing 20 % Cp. Secondly, blood lactate, which would highlight metabolic acidosis (Constable, 2022), tended to be lower in the animals fed Cp. Despite Cp's diets containing approximately 30 % more ether extract than 0 %Cp-diets, the animals consuming feed with Cp did not exhibit discernible variations in plasma lipid concentrations compared to lambs without Cp, which suggest that lipogenic pathways were not altered by CT.

Finally, gene expression of antioxidant and immune markers in gastrointestinal tissues were not deeply affected by Cp inclusion, since

only a down-regulation of GPX2 gene expression in the ileum was observed in the lambs fed with 20 % of Cp in the concentrate. Among the gastrointestinal glutathione peroxidases, GPX2 is considered the most important for removing peroxides in the intestine (Florian et al., 2001). In addition, the ileum is an abundant section of lymphoid tissue (Lie et al., 2005) which consequently produces free radicals such as hydrogen peroxide. Thus, animals fed diets with 0 % Cp had to increase endogenous antioxidant defences in the ileum. In this line, Pelegrin-Valls et al. (2023) observed a lower expression of SOD and CAT when lambs fed Cp, since CT acts by hindering the local production of ROS thanks to its ability to eliminate them.

4.3. Effects of high dose of vitamin E

The current experimental approach aimed at evaluating the effect of Vit E supplementation on lamb performances and physiology. Therefore, a negative control without any dietary Vit E source was not planned to avoid any metabolic deficiency. No differences in the growth performance were expected when providing supra-nutritional doses of Vit E to fattening lambs (Bellés et al., 2019). However, in beef bulls, a high dose of Vit E (105 IU Vit E/kg of feed for 100 days) improved ADG and decreased feed conversion ratio, as well as increased serum CAT concentration (Luan et al., 2023), which would support the higher growth in lambs fed with 300 IU/kg of concentrate of the present study. However, the feed conversion ratio and the time-budget behaviours, including eating patterns, were not altered by Vit E supplementation.

The blood nutritional status of lambs showed minor differences across Vit E diets, as only the haematocrit values and α-tocopherol concentration were increased by High Vit E. These results agree with De Wolf et al. (2014), who detected a protective effect of Vit E supplementation on haematocrit values when healthy lambs were challenged with experimental gastrointestinal nematode infections, which may reflect a role of Vit E to improve the haematological status of the animals.

Interestingly, while the High Vit E did not lower plasma MDA (a marker of lipid peroxidation), it did decrease the ratio of MDA to blood lipids and α-tocopherol. These findings suggest that in animals fed supra-nutritional doses of Vit E, the amount of MDA formed per unit of blood lipids or α-tocopherols is lower compared to the Low Vit E-group. Similarly, Luciano et al. (2017) did not observe a reduction of plasma MDA nor of total antioxidant capacity as a consequence of increasing dietary Vit E. Although Luan et. al. (2023) found no effect of Vit E on MDA serum content, they observed an increasing antioxidant enzyme activity (e.g., SOD and CAT) due to the Vit E supplementation in finishing bulls diets, which may be in agreement with the up-regulation of CAT in the ileum tissue of the current lambs fed High Vit E.

It is known that Vit E attenuates lipid peroxidation and protein oxidation and increases antioxidant defence mechanisms in the small intestine (Shirpoor et al., 2007). In this study, dietary Vit E supplementation did not have antioxidant effects in the jejunum tissue, probably because this part of the small intestine is leading most of the nutrient absorption and is not challenged by high levels of ROS. However, in the ileum tissue, the High Vit E provoked an up-regulation of CAT expression, which may indicate greater gut health in this group of lambs, since the deficiency in gene and protein expression of this antioxidant contributes to the pathophysiology of intestinal diseases (Iborra et al., 2022). In addition, some beneficial bacteria such as *Lactobacilli* can transform toxic O_2- to less active H_2O_2, and hence stimulate the CAT activity which controls the transformation of H_2O_2 to water and oxygen, alleviating intestinal inflammation (Tomusiak-Plebanek et al., 2018)

Interestingly, dietary Vit E supplementation had carry-over effects on the small intestine by down-regulating certain immune function markers in small intestine tissues, both TNF-α in the jejunum and TGF-β in the ileum. These two cytokines may modulate pro- and anti-inflammatory responses, respectively, to keep the intestinal barrier integrity (Miner-Williams and Moughan, 2016) suggesting that the small intestine was less challenged when High doses of Vit E were provided.

5. Conclusion

Mild synergistic effects of dietary Cp and Vit E were evident in the nutritional status and gastrointestinal redox immune markers of concentrate-fed light lambs, since providing simultaneously 20 % of Cp and High Vit E (300 IU/kg of the concentrate) in lambs boosted rumination activity and down-regulated IFN-γ expression in the jejunum. When supplemented independently, carob pulp inclusion did not affect animal performance, whereas High Vit E improved the lambs' growth. However, dietary Cp modified the eating pattern, which was slightly delayed to the early afternoon, and decreased blood lactate. High Vit E improved the plasma α-tocopherol concentration decreasing the ratio MDA:α-tocopherol, which may indicate an enhanced redox balance. High Vit E exhibited tissue-specific effects immune and antioxidant effects. High Vit E down-regulated the expression of jejunal TNF-α and ileal TGF-β, while up-regulated ileal CAT expression, suggesting modulation of immune response and endogenous antioxidant defences. In the ileum, the Cp inclusion down-regulated the GPX2 expression, likely due to the ability of CT to scavenge free radicals.

Animal welfare statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes. Additionally, all procedures received the approval of the Ethical Committee of the University of Lleida (decision N° CEEA 02–03/21 procedure N° 01).

Ethical statement

The Ethical Committee for Animal Experimentation of the University of Lleida approved the protocol used in this study (CEEA 02-03/21-procedure 01). All animals were raised, managed and slaughtered under the Spanish Animal Protection Regulations RD 53/2013 and EU Directive 2010/63 for animal experiments.

CRediT authorship contribution statement

Diego Nicolas Bottegal: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sandra Lobón:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Beatriz Serrano-Pérez:** Writing – review & editing, Validation, Software, Methodology, Investigation. **María José Martín-Alonso:** Writing – review & editing, Software, Methodology. **María Ángeles Latorre:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Javier Álvarez-Rodríguez:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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