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The amino acid profile of Camelina sativa seeds correlates with the strongest immune response in dairy ewes

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ABSTRACT

Camelina sativa is an alternative protein source (with a specific amino acid profile) rich also in bioactive compounds (such as polyunsaturated fatty acids and antioxidants), which have immunomodulatory properties. This study aimed to assess the impact of the dietary inclusion level of Camelina seeds, on the expression levels of key genes involved in ewes' innate immunity. Forty-eight dairy ewes were assigned into four homogenous groups of 12 animals that were fed individually with alfalfa hay, wheat straw, and concentrate. The concentrate of the control group (**CON**) had no Camelina seeds, while in the treated groups, Camelina seeds (**CSs**) were incorporated at 6 (**CS6**), 11 (**CS11**), and 16% (**CS16**) in the concentrates, respectively, as partial substitution of both soybean meal and maize grain. The relative transcript levels of the immune-related genes were determined using a real-time PCR platform. The relative transcript levels of toll-interleukin receptor-domain-containing adapter-inducing interferon-β, tumour necrosis factor receptor-associated factor 3, Interferon regulatory factor 5, and Mitogen-activated protein kinase were upregulated in monocytes of the CS11-fed ewes. Furthermore, in the CS6-fed ewes, the relative transcript levels of Interleukin-1 beta (***IL1B***) were upregulated in monocytes compared to the CON, while those of *IL1B*, Interleukin-8, and Interleukin-10 were upregulated in neutrophils compared to the CON and the CS11-fed ewes. The highest inclusion level of CS (CS16) did not have a negative impact on ewes' innate immunity. The response of monocytes on dietary amino acid (mainly threonine, tyrosine, serine, and lysine) changes related to Camelina inclusion is different from that of neutrophils. The observed responses need to be further investigated.

Implications

The modification in the dietary amino acid profile (mainly of threonine, tyrosine, and serine) related to the dietary inclusion level of Camelina seeds can trigger the expression levels of some genes involved in the immune system. The highest dietary inclusion level of Camelina sativa seeds (16%) did not have a negative impact on the ewes' innate immunity.

Introduction

The intensification of dairy farming systems to achieve optimum milk production is related to higher energy and nutrient demands, as well as stressful conditions that may result in oxidative stress and the so-called immunosuppression in ruminants (Celi, 2011). Consequently, precision in nutritional management is crucial to balance the optimum production and simultaneously maintain a functional immune system by preventing inflammation, tissue damage, and metabolic disorders. Supplementing ruminant diets with feedstuffs rich in bioactive compounds –e.g., polyunsaturated fatty acids (**PUFAs**), amino acids, and antioxidants- is an effective strategy for the enhancement of the immune system and the prevention of immunosuppression (Calder, 2001; Lee and Han 2018). The potential immunoregulatory properties of PUFA, including those of α-linolenic acid, were comprehensively addressed in both animal and human trials, by inhibiting the production of pro-inflammatory cytokines (Calder, 2001). More specifically, an n-3 fatty acid-enriched diet (flaxseed oil rich in α- linolenic acid) inhibited Interleukin-1β (**IL-1β**) and tumour necrosis factor-α (**TNF-α**) synthesis (Caughey et al., 1996). In detail, TNF-α and IL-1β, which

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are the dominant pro-inflammatory cytokines, are regulated by the Nuclear Factor kB (**NF-κB**) and Mitogen-activated protein kinase (***MAPK***) pathways, both being triggered by the Toll-like receptor-4 (***TLR4***) (Honda et al., 2015). On the other hand, PUFA at high dietary doses might negatively affect the immune system since they are prone to oxidation, potentially inducing oxidative stress (Tsiplakou et al., 2017).

Amino acids have also been suggested to trigger ewes' immune responses (Tsiplakou et al., 2018). Significantly lower mRNA relative transcript levels of *TNFA*, *IL1B* and *CXCL16* genes in monocytes, as well as *IL1B* genes in neutrophils, have been reported when ewes consumed rumen-protected amino acids (lysine, methionine) (Tsiplakou et al., 2018).

Camelina further to its high α-linolenic acid content also contains other bioactive compounds such as phenols, tocopherols (specifically γ-tocopherol, accounting for over 90% of the total) and glucosinolates (Matthäus, 2002; Abramovič et al., 2007). The immunostimulatory effect of γ-tocopherol (Reiter et al., 2007), as well as the immunomodulatory effects of vitamin E (as different forms of tocopherols and tocotrienols), had been also investigated in-depth (Lewis et al., 2019). The different immune regulatory response was also observed in different cell types treated with isothiocyanates *in vitro* (Jakubikova et al., 2011). However, scarce information exists regarding the impact of the dietary inclusion of Camelina in innate immunity. Moreover, the different inclusion levels of bioactive compounds, their type, and the source might reveal diverse responses in innate immunity (Mavrommatis et al., 2021; Mitsiopoulou et al., 2021).

The already published studies testing the Camelina effects in ewes had two limitations: they were using ω-3 PUFA (α-linolenic acid) rich forage diets (fresh forage, pasture, or silages) and they were testing a single dose of Camelina seeds (ranging from 70 to 200 g/ewe/day) (Mierlita et al., 2011; Mierlita and Vicas, 2015; Mierlita et al., 2018). Because of this, in those studies, it was difficult to discriminate between the effects of the forage and the Camelina on animals' physiology. Moreover, the use of a single dose of Camelina prevents to clarify the dose–response of the immune system to a PUFA rich feed which potentially can induce oxidative stress (Tsiplakou et al., 2017). Therefore, the aim of this study was to evaluate the impact of different concentrate inclusion levels of Camelina seeds, on the relative transcript levels of selected genes related to innate immunity using forages with low PUFA content.

Material and methods

Experimental design and dietary treatments

Animal handling, housing, and care conformed to the approved protocols by Ethical Committee guidelines of the Department of Animal Sciences of the Agricultural University of Athens, while considering an extended experimental design report, the Bioethical Committee of Faculty of Animal Science (currently known as: Agricultural University of Athens Ethical Committee in Research; FEK 38/A/2-3-2018, eide. aua) approved the experimental protocol under the No. 000007/22-01-2017. The animals were part of the experimental farm of the Research Institute of Animal Science of the ELGO-DIMITRA (Giannitsa, Greece; 40°44′N, 22°27′E), and they were constantly monitored for their health status by a professional veterinarian in charge of the sanitary control of the flock. Moreover, they were under a protocol for the monitoring and control of infections and parasitic diseases (including among the others, pneumonia, and gastrointestinal nematodes). At the beginning of the trial, a faecal egg count (FEC) was performed following the MAFF (1986), and the animals resulted negative to parasitosis (eggs of gastrointestinal nematodes and *Coxidia* were lower than 50 EPG/OPG, respectively). Therefore, it was possible to avoid the application of the deworming protocol with the advantage to avert potential interferences of the antiparasitic molecules with the immune system. During the trial, the health status was monitored daily through the observation of the general behaviour, feed intake and body functions. Forty-eight dairy Chios ewes, of similar age (2–4 years old) and BW (55 ± 6.5 kg), were divided into four homogenous groups (n = 12) considering fat- corrected (6%) milk yield (**FCM**) (1.85 ± 0.3 kg/d) as well as days in milk (67 ± 8 days). Ewes were housed in groups at a common stall for each group and at feeding time, they were transferred to individual pens to achieve individual feeding. Before the official start of the experiment, a seven-day adaptation period was provided for the ewes to get used to the new environment of the individual pens. The whole experiment lasted 60 days. Each group was assigned to one of the following four treatments: **CON**: control diet without the supplementation of Camelina seeds; **CS6**: concentrate containing 6% of Camelina seeds; **CS11**: concentrate containing 11% of Camelina seeds, and **CS16**: concentrate containing 16% of Camelina seeds. In the three CS concentrates, Camelina seeds were included to substitute both soybean meal and maize grain to obtain isoenergetic and isoproteic diets. The negligible variation of CP in diets is related to the fact that Camelina seeds had CP content equal to the average of the two substituted components (Table 1). The high amount of α-linolenic acid in Camelina seeds and the high amount of linoleic acid in maize grain led to a consequent variation in fatty acid profile (Christodoulou et al., 2021). The detailed diet composition (% DM), daily feed intake (g of DM), calculated composition (% DM), nutrient intake (g/day/ewe), and amino acid profile (% concentrate as fed) of the four dietary treatment groups (CON, CS6, CS11, CS16) is provided in Table 1. Also, a detailed description of the concentrate components is provided in Supplementary Table S1. The diets were formulated to meet ewes' maintenance and production requirements (fat-corrected milk yield) using the software nutritional dynamic system (**NDS**) (version 3.9.10.a) which is mainly based on NRC (2007), and consisted of 1.5 kg of alfalfa hay, 0.2 kg of wheat straw, and 1.5 kg of concentrate (per ewe per day). On a daily basis, the forages were offered separately from the concentrates in two equal portions after milking (0700 h and 1700 h). Animals had free access to fresh water.

Animal performance

BW was measured at the official start of the experiment (day 0) as well as on the 30th and 60th experimental days. Furthermore, individual milk yield, $FCM_{6\%}$, energy-corrected milk yield (**ECM**), fat (%), fat yield, protein (%), protein yield, lactose (%), and lactose yield were determined at the beginning of the experiment, and on the 15th, 30th, 45th, and 60th experimental days (Table 2). A detailed description of the chemical composition analysis procedures and equations that were used for the calculation of $FCM_{6\%}$ and ECM is provided in Supplementary Material S1.

Feed sample analysis

Individual samples of the concentrates, alfalfa hay, and wheat straw were analysed for, DM, and CP according to the AOAC (1984), while neutral detergent fibre (**aNDFom**) and ADF - expressed exclusive of residual ash- following the methods of Van Soest et al (1991). Furthermore, ether extract was determined according to Soxhlet (1879). In addition, the amino acid profile was determined in the concentrates of the fed diets according to a previously published method (Prandi et al., 2021).

Table 1
Diet components (% DM), diet composition (% DM), daily feed intake (g of DM), nutrient intake (g/d/ewe), and amino acid profile (% concentrate as fed) of the four dietary treatments fed to ewes, calculated by the nutritional dynamic system software (NDS, version 3.9.10.a).

	Dietary treatments			
Item	CON	CS6	CS11	CS16
Diet components (% DM)				
Forages				
Wheat straw	6.13	6.13	6.13	6.13
Alfalfa hay	47.22	47.22	47.22	47.22
Concentrates				
Maize grain	16.05	13.95	12.55	11.15
Barley	9.33	9.33	9.33	9.33
Wheat middling	4.67	4.67	4.67	4.67
Sunflower meal	7.46	8.40	8.40	8.40
Soybean meal	7.23	5.60	4.67	3.73
Camelina seeds	0.00	2.80	5.13	7.46
Mineral vitamin premix	1.91	1.91	1.91	1.91
Calculated composition (% DM)				
Ash	7.81	7.82	7.79	7.77
aNDFom	36.78	37.93	38.68	39.42
ADF	25.21	26.12	26.74	27.37
ADL	5.41	5.77	6.02	6.27
NFC	35.72	33.81	32.46	31.12
Starch	17.23	15.86	14.94	14.03
Sugars	3.41	3.40	3.38	3.36
EE	1.65	2.63	3.45	4.27
CP	18.12	18.19	18.23	18.27
Sol CP	5.13	5.30	5.40	5.49
RDP3x	11.73	11.76	11.77	11.78
NEl (Mcal/kg)	1.54	1.52	1.52	1.51
Daily feed intake (g of DM)	2 840.00	2 842.00	2 844.00	2 846.00
Nutrient intake (g/d/ewe)				
aNDFom	1 044.55	1 077.97	1 100.1	1 121.89
ADF	715.96	742.33	760.49	778.95
ADL	153.64	163.98	171.21	178.44
NFC	1 014.45	960.88	923.16	885.68
Starch	489.33	450.74	424.89	399.29
Sugars	96.84	96.63	96.13	95.63
EE	46.86	74.74	98.12	121.52
CP	514.61	516.96	518.46	519.96
Sol CP	145.69	150.63	153.58	156.25
RDP3x	333.13	334.22	334.74	335.26
Amino acid profile (% concentrate as fed)				
Glycine	1.21	1.19	1.17	1.29
Alanine	0.85	0.97	0.96	0.83
Arginine	1.60	1.42	1.39	1.75
Aspartic acid	1.71	2.08	2.04	1.67
Cysteine	0.33	0.39	0.39	0.44
Glutamic acid	3.77	4.39	4.34	3.87
Histidine	0.36	0.30	0.25	0.33
Isoleucine	0.64	0.68	0.64	0.62
Leucine	1.42	1.46	1.34	1.29
Lysine	0.37	0.66	0.61	0.46
Methionine	0.27	0.34	0.33	0.35
Phenylalanine	1.10	0.84	0.72	0.83
Proline	1.15	1.19	1.14	1.15
Serine	1.06	1.00	0.95	1.01
Threonine	0.78	0.76	0.72	0.77
Tyrosine	0.49	0.37	0.32	0.39
Valine	0.75	0.85	0.80	0.81
Lysine/Methionine	1.39	1.93	1.86	1.31
Total	17.88	18.88	18.10	17.88

Abbreviations: CON = control treatment; CS6 = dietary treatment with 6% Camelina seeds; CS11 = dietary treatment with 11% Camelina seeds; CS16 = dietary treatment with 16% Camelina seeds, aNDFom = ash-free neutral detergent fibre treated with amylase; NFC = non-fibre carbohydrates; EE = ether extract; sol CP = soluble CP; RDP3x = rumen degradable protein at 3 times the maintenance intake; NEI = net energy intake.

Blood samples

Blood sample collection for monocyte and neutrophil isolation

Individual blood samples were collected from ewes' jugular vein on the final day of the experiment. The blood sampling was conducted in the morning (0700 h), before feeding, and the blood samples were collected into tubes (BD Vacutainer, Plymouth, UK) containing heparin (17 Units/mL) as an anticoagulant. The tubes were placed in ice and processed for analysis immediately after collection to ensure optimal results.

Cell isolation

Cell isolation was carried out as previously described by Tsiplakou et al. (2018). For the isolation of monocytes and neutrophils was used a centrifugation Histopaque density gradient (Sigma-Aldrich Co., USA). In detail, whole blood was mixed with

Table 2
BW (kg), milk yield (kg/d), fat-corrected milk yield (6%) (kg/d), energy-corrected milk yield (kg/d), fat content (%), fat daily yield (kg/d), protein content (%), protein daily yield (kg/d), lactose content (%), and lactose daily yield (kg/d) of ewes fed the four dietary treatments throughout the experimental period.

	Dietary Treatments (D)					Sampling Time (S)						Effect	
Item	CON	CS6	CS11	CS16	SEM	0	15	30	45	60	SEM	D	S
BW (kg)	55.10	55.30	54.50	54.70	0.18	54.60		55.10		54.80	0.80	0.262	0.326
Milk yield (kg/d)	1.71	1.86	1.87	1.89	0.02	1.973^{A}	1.869^{B}	1.866^{AB}	1.700^{BC}	1.743^{BC}	0.05	0.433	<0.001
$FCM_{(6\%)}$ (kg/d)	1.68	1.79	1.81	1.73	0.02	1.85^{AB}	1.87^{A}	1.74^{BC}	1.68^{C}	1.62^{C}	0.05	0.577	<0.001
ECM (kg/d)	1.47	1.58	1.63	1.58	0.02	1.65^{A}	1.66^{A}	1.56^{B}	1.50^{C}	1.47^{C}	0.36	0.424	<0.001
Fat (%)	5.89^{a}	5.71^{ab}	5.85^{a}	5.35^{b}	0.15	5.56^{B}	6.02^{A}	5.48^{B}	5.98^{A}	5.46^{B}	0.10	0.046	<0.001
Fat yield (kg/d)	0.10	0.11	0.11	0.10	0.001	0.107^{A}	0.112^{A}	0.101^{B}	0.100^{B}	0.094^{C}	0.003	0.511	<0.001
Protein (%)	5.22	5.29	5.44	5.24	0.07	5.29^{AB}	5.38^{A}	5.23^{B}	5.27^{AB}	5.30^{AB}	0.04	0.145	0.010
Protein yield (kg/d)	0.09	0.10	0.10	0.10	0.001	0.103^{A}	0.100^{AB}	0.097^{B}	0.089^{C}	0.092^{C}	0.003	0.267	<0.001
Lactose (%)	4.94	5.00	5.04	5.03	0.05	5.08^{A}	5.00^{BC}	4.97^{BC}	4.95^{C}	5.02^{AB}	0.03	0.456	<0.001
Lactose yield (kg/d)	0.08	0.09	0.10	0.10	0.001	0.10^{A}	0.09^{B}	0.09^{B}	0.08^{C}	0.09^{B}	0.003	0.314	<0.001

Abbreviations: CON = control dietary treatment without the supplementation of Camelina seeds; CS6 = dietary treatment containing 6% of Camelina seeds; CS11 = dietary treatment containing 11% of Camelina seeds; CS16 = dietary treatment containing 16% of Camelina seeds; ^{a,b} = values within a row with different superscripts differ significantly at $P < 0.05$; ^{A,B,C} = values within a row with different superscripts differ significantly at $P < 0.05$; $FCM_{(6\%)}$ = fat-corrected milk yield in 6%; ECM = energy-corrected milk yield.

an equal volume of Hanks' balanced salt solution (Sigma-Aldrich, USA) and three parts of diluted blood layered on two parts of Histopaque. Samples were centrifuged for 40 minutes at 4 °C at 500*g*. After centrifugation, between the Histopaque-1077 and the blood plasma, a whitish-yellow thin layer containing the mononuclear cells became visible. The plasma was rejected. The monocytes were withdrawn into a new tube after extracting the supernatant blood plasma. The red cells were lysed by adding up to 20 mL endotoxin-free ultrapure water in a 50 mL falcon tube and with vigorous handshaking for 1 minute to isolate the neutrophil cells. Afterwards, 10 mL of 2.7% w/v NaCl was added to resuspend cells into an isotonic solution (0.9% NaCl). These cells were washed several times by repeating the previous step, using the same quantity of endotoxin-free ultrapure water, and were centrifuged for 5 minutes at 4 °C at 1 000*g* until a white and consistent cell precipitate was visible at the bottom of the tube. In the sequel, the final cell suspensions were cultured in 1 mL of growth medium RPMI (RPMI-1640 containing 2 mM L-glutamine; Biosera) (being incubated at 37 °C) and then centrifuged at 1 000*g* for 5 minutes at 4 °C. Finally, the resulting cell pellets were again washed at least twice in 0.5 mL of PBS and centrifuged at 700 rpm for 1 minute at 4 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from 5×10^6 cells of the 48 individual blood samples using TRIzol™ (Invitrogen, California, USA). In detail, the isolated cells were lysed in 0.5 mL TRIzol™. The homogenised samples were incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex and then were shaken vigorously. For the homogenisation, 0.1 mL of chloroform was used, followed by vigorous tube shaking for 2–3 minutes at room temperature. Samples were then centrifuged at 12 000*g* for 15 minutes at 4 °C. The mixture was separated into a lower red phenol–chloroform phase, interphase, and a colourless upper aqueous phase, which is 50% of the total volume, and there the RNA remains. The aqueous phase was removed at a 45° angle with care to avoid drawing any of the interphase or organic layer into the pipette during this step. The aqueous phase was then placed in a new Eppendorf tube. Afterwards, for the RNA precipitation, 5–10 μg of RNase-free glycogen as a carrier was added to the aqueous phase. For the homogenisation, 0.25 mL of 100% isopropanol was added to the aqueous phase, followed by incubation at room temperature for 10 minutes, and then by centrifugation at 12 000*g* for 10 minutes at 4 °C. For the RNA wash, the supernatant was then removed from the tube, leaving only the RNA pellet. The pellet was washed with 0.5 mL of 75% ethanol. Samples were then vortexed briefly, the tubes were centrifuged at 7 500*g* for 5 minutes at 4 °C, and the wash was discarded. Afterwards, RNA was resuspended in 30 μL of endotoxin-free ultrapure water.

Five thousand ng (5 000) of RNA was treated with 2 units of Turbo DNase (Invitrogen, California, USA) following the manufacturer's protocol. Samples were incubated in a heat block at 37 °C for 30 minutes. The treated RNA was then washed twice using an equal volume of 24:23:1 phenol: chloroform: isoamyl alcohol RNA stabilised solution and centrifuged at 13 000 *rpm* for 5 minutes at 4 °C. The supernatant was transferred into a new Eppendorf tube, and then, 1/10 × volume 3 M acetic acid solution was added following 2.5 × volume 100% absolute ethanol. After a vigorous vortex shaking, the RNAs were precipitated overnight at −40 °C. Afterwards, the RNA was precipitated after centrifugation at 13 000 *rpm* for 30 minutes at 4 °C. The RNA pellet was then washed with 100 μL 75% ethanol and precipitated at 13000 *rpm* for 10 minutes at 4 °C. The pure RNA was resuspended in 21 μL endotoxin-free ultrapure water.

Both quantity and quality of the extracted RNA were evaluated both pre- and post-DNase treatment spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); the quantity was measured in ng/μL, while the purity was determined based on the A260/A280 and A260/A23 ratios. Besides, the RNA integrity was evaluated with agarose gel (3%) after isolation and after DNase treatment of the samples. Discrete bands were monitored in all samples representing 28 s and 18 s ribosomal RNAs, respectively, showing little or no RNA hydrolysis. The absence of a possible genomic DNA contamination was confirmed by PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene).

Precisely, 500 ng of pure RNA was used and reversely transcribed per cDNA synthesis by using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Japan) following the manufacturer's protocol, using a mix of random hexamers and oligo-dT primers, following incubation at 37 °C for 15 minutes and enzyme deactivation at 85 °C for 16 seconds. Finally, cDNAs were diluted at 1:10 and the final PCR template was obtained.

Primer design

The primer pairs have been used in our previous studies (Tsiplakou et al. 2018; 2020). Briefly, the primers were designed to be specific for *Ovis aries* according to their coding sequence (CDS by GenBank) using the PerlPrimer software. Geneious software (Biomatters, New Zealand) was used to design the primers.

Additionally, dissociation curves were generated, and the amplification products were subjected to agarose gel electrophoresis to confirm the production of a single amplicon per reaction.

Normalisation

Calculations regarding the relative transcript levels of the studied genes were explained by Tsiplakou et al. (2018). In detail, the relative expression levels of the studied genes were calculated as follows:

$(1 + E)^{-\Delta Ct}$, where ΔCt is the difference between the geometric mean of the two-reference gene's Cts and the Ct of the target gene. Furthermore, the primer efficiency is the mean of each amplicon's efficiency per primer, which was calculated by employing the linear regression method on the log (fluorescence) per cycle number (ΔRn) using the LinReg PCR software (Ramakers et al., 2003).

The mean of each amplicon's efficiency per primer (the primer efficiency) was calculated by employing the linear regression method on the log (fluorescence) per cycle number (ΔRn) using the LinReg PCR software (Ramakers et al., 2003). To normalise the cDNA template concentrations, we used GAPDH and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) as housekeeping genes. The selection of these housekeeping genes was based on Vorachek et al. (2013) who reported these genes as the most stable in sheep phagocytic cells.

Real-time quantitative PCR

The relative transcript mRNA levels of the studied genes were quantified by a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix (Applied Biosystems, Austin, TX, USA), gene-specific primers at a final concentration of 0.2 μM each (forward and reverse), and 1 μl of each cDNA as template. The PCR program was programmed for 40 cycles. Template denaturation was carried out for 15 min at 95 °C, followed by 15 seconds at 95 °C and 1 min at 58 to 64 °C based on specific primer annealing temperature (Table 3).

Statistical analysis

The statistical analysis was conducted using the IBM SPSS Statistics for Windows (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, version 24.0. Armonk, NY).

A general linear model for repeated measures ANOVA was used to assess the effect of the dietary treatment between the four groups. A total of 144 observations (twelve ewes × four dietary groups × three sampling times) were obtained for BW, while 240 observations (twelve ewes × four dietary groups × five sampling times) were obtained for milk yield and chemical composition. The dietary treatments (**D**) (CON, CS6, CS11, and CS16) were defined as the fixed factor and the sampling time (**S**) (0, 30, and 60 days for BW and 0, 15, 30, 45, and 60 days for milk yield) as the repeated measure for BW, milk yield, and milk chemical composition, while their interactions (D × S) were also assessed, according to the following model:

$$Y_{ijkl} = \mu + D_i + S_j + A_k + (D \times S)_{ij} + e_{ijkl}$$

where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of dietary treatment (i = 4; CON, CS6, CS11, and CS16), S_j the effect of sampling time (j = 3; 0, 30, and 60 days for BW, and j = 5; 0, 15, 30, 45, and 60 days for milk yield and milk chemical composition), A_k is the animal's random effect, $(D \times s)_{ij}$ is the interaction between the dietary treatments and sampling time, and e_{ijkl} are the residual errors.

Furthermore, one way ANOVA analysis was performed to compare the effect of the dietary treatment (CON vs CS6 vs CS11 vs CS16) in the expression of the targeted genes according to the model:

$$Y_{ijk} = \mu + D_i + e_{ijk}.$$

where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of the dietary treatment (i = 4), and e_{ijk} the residual error.

A total of 48 variables (twelve ewes × four dietary groups × one sampling time) were emerged obtained for the relative transcript levels of the genes. Dataset homogeneity was evaluated through Lavene's test. Furthermore, the Shapiro-Wilk test was used to test the normality of our dataset. Posthoc analysis was carried out considering the Tukey multiple range test for the data that did not violate the homogeneity and normality tests. For the data that violated these criteria, the Games-Howell test was considered. The significance threshold for these tests was set at 0.05.

Discriminant analyses were also conducted on the pooled data of the targeted genes' relative transcript levels to clarify how the variables, may distinguish, and classify among the four dietary treatment groups as well as in the different cell types (monocytes

Table 3
Sequences, amplicon size, and annealing temperature of the primers that were designed to be specific for *Ovis aries*, and were used in real-time qPCR.

Gene	Acc. No.	bp	Tm (°C)	Forward primer 5′–3′	Reverse primer 5′–3′
TLR4	XM_042242671	70	60	ATGAACCACTCCACTCGCTC	TCTTGCTCCTTAGAGGCCGT
TNFA	NM_001024860	70	60	GGGAGACACAAACTAAGGGCT	AACCTGCAGTTCAGCTCCG
NFκB	XM_042251202	70	60	AAGCTGTGGTGGAGGACTTG	ACAGAGTTACCCAAGCGGTC
IL1β	NM_001009465	70	60	TGGATAGCCCATGTGTGCTG	CAGAACACCACTTCTCGGCT
IL2	NM_001009806	70	60	AAATCCCGAGAACCTCAAGCT	TGTAGCGTTAACCTTGGGCA
IL6	NM_001009392	61	60	CAGCAAGGAGACACTGGCAGA	TCCATCTTTTTCCTCCATTTTTGG
IL8	NM_001009401	71	60	CCTGCTCTCTGCAGCTCTGTG	TGCATTGGCATCGAAGTTCTG
IL10	NM_001009327	100	60	CTGGGGGAGAAGCTGAAGAC	CTCTCTTCACCTGCTCCACC
CCL5	XM_027975305	61	61	CAAGTGCTCCATGGCAGCAG	GTTGGCGCACACCTGACG
CXCL16	XM_015098600	70	60	GTGCCTGTGTTGTCCCTCTT	GCTTGCACACCACGTAGAGT
TRIF	XM_042250120	106	61	GCACGTCTAGCCTGCTTAC	TTGCGGGCCCGCAGCATCT
TRAF3	XM_027957435	100	60	TAACTGCTGCATTCGCTCCA	GGAACACAAAGCTGGGGTTG
MYD88	NM_001166183	99	58	ACAGACAAACTATCGGCTGA	CACCTCTTCTCAATGAGTTCA
CHUK	XM_027960582	70	60	TGCAGGGAAAGAGGCAGAAA	GACCGAGCAGAACTCTGTGT
JUND	XM_027969415	100	60	ACGCAGTTCCTCTTTCCCAA	CCAGCTGGTTTTGCTTGTGT
MAPK	XM_027956867	100	60	GCAACGACCACATCTGCTAC	AGGTTGGAAGGCTTGAGGTC
IRF3	XM_004015378	92	64	CCAGAGGCTGGGGCACTGCC	CCTTCGGGACCTCGCCGTCA
IRF5	XM_042248808	100	60	ACATCCCCAGTGAGAAGCAG	ATGGCATACAGATCCTGGCC
STAT3	XM_015098788	142	58	TGTGACACCAACGACCTG	CATGTCGAACGTGAGGGA
HMOX1	XM_027967703	100	60	GAGCTGACCCGAGAAGGTTT	AGACGGGGTTCTCCTTGTTG
GAPDH	NM_001190390	75	60	AAAGGCCATCACCATCTTCCA	ACCACGTACTCAGCACCAGCAT
YWHAZ	NM_001267887	70	60	TGTTCTATTGTGCCTAGTACACTGT	CATCAAGACTCACTGCCTCCC

Abbreviations: Acc. No. = accession number; bp = amplicon size; Tm = primers annealing temperature.

vs neutrophils). The variables entered in the different discriminant analyses were the studied gene transcription levels listed in Table 3. Stepwise discriminant analysis was used to assess which variables contribute the most to the discrimination of the variables between the groups. Wilk's Lambda (λ) criterion was considered to determine how each function separates into groups. Twenty variables for monocytes and twenty for neutrophils as well as forty for both monocytes and neutrophils were added to develop three district models to discriminate the forty-eight individual samples of the dietary groups (n = 12 per dietary group). An additional discriminant analysis was conducted to investigate the simultaneous effect of the different cell types (monocytes vs neutrophils) on the dietary groups and their distinguish grade. Regarding the latter, twenty variables (gene expressions) separated into eight groups (four dietary groups per cell type) were added to develop a district model to discriminate the ninety-six individual samples of the dietary groups (n = 12 per dietary group × 4 dietary groups × 2 cell types). A visual interpretation of the distinguished datasets from the discriminant analyses are provided in Fig. 1. Discriminant analysis was also conducted to evaluate the classification of the four experimental concentrates based on their amino acids' concentrations (listed in Table 1) and this discrimination is presented in Fig. 2. Furthermore, Pearson's correlation was also performed to correlate the studied gene relative transcript levels in both monocytes and neutrophils. The Pearson's correlation results are presented in heat map graphs for each cell type (Fig. 3). We also determined the correlation between the relative transcript levels of the studied genes in monocytes and neutrophils and individual dietary components, including the amino acid profile of the concentrates. The codes for all the statistical methods that were followed in our study are provided in Supplementary Material S2.

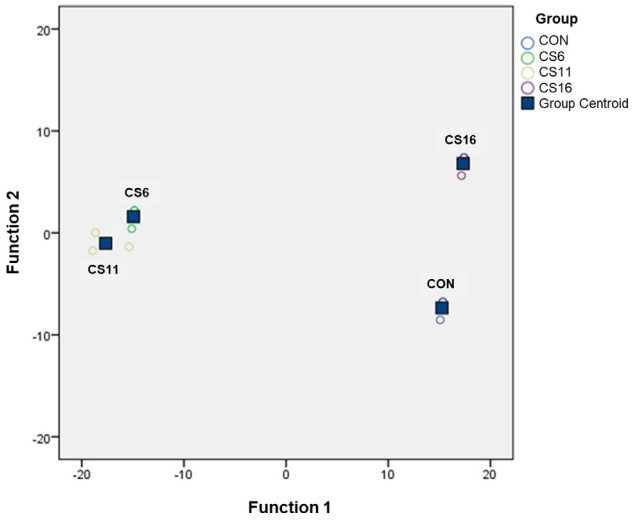

Fig. 2. Discriminant plot separating the four concentrates offered to the ewes based on their amino acid profile. Abbreviations: CON = control dietary treatment; CS6 = dietary treatment with 6% Camelina seeds; CS11 = dietary treatment with 11% Camelina seeds; CS16 = dietary treatment with 16% Camelina seeds.

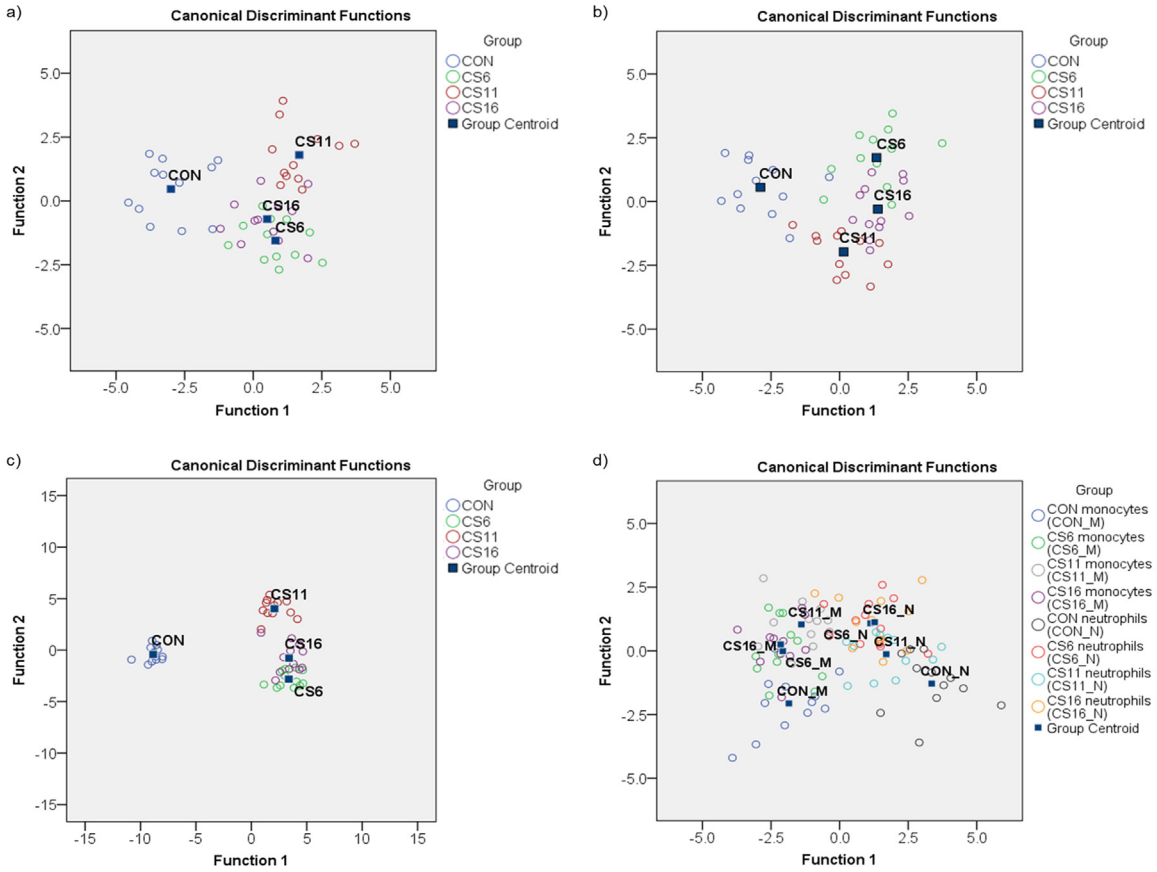

Fig. 1. Discriminant plot separating the four dietary treatments based on genes' relative transcript levels of: (a) ewes' monocytes, (b) ewes' neutrophils, (c) monocytes and neutrophils pooled data, and (d) ewes' monocytes vs neutrophils. Abbreviations: CON = control dietary treatment; CS6 = dietary treatment with 6% Camelina seeds; CS11 = dietary treatment with 11% Camelina seeds; CS16 = dietary treatment with 16% Camelina seeds; CON_M = CON monocytes; CS6_M = CS6 monocytes; CS11_M = CS11 monocytes; CS16_M = CS16 monocytes; CON_N = CON neutrophils; CS6_N = CS6 neutrophils; CS11_N = CS11 neutrophils; CS16_N = CS16 neutrophils.

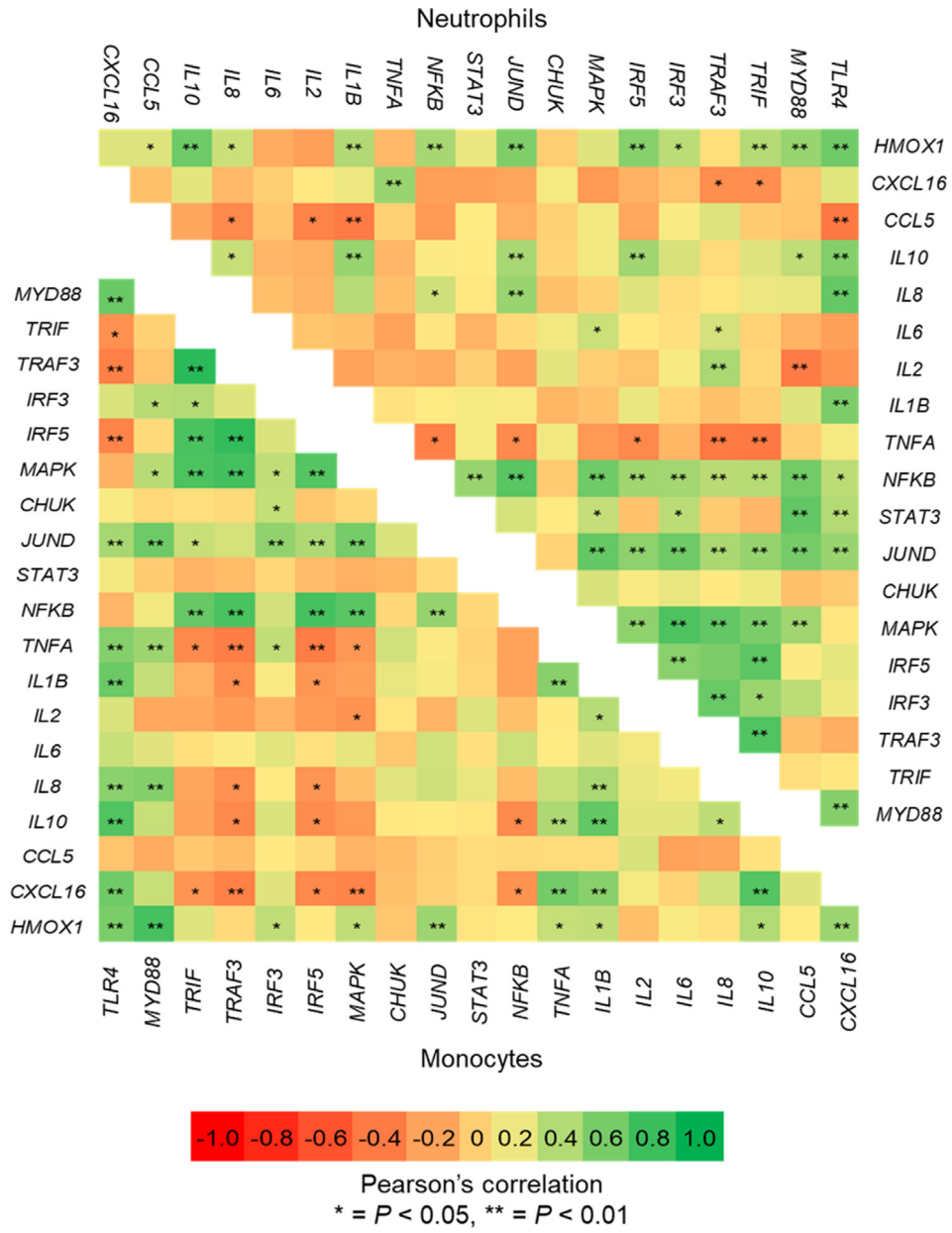

Fig. 3. Pearson's correlation heatmap between genes' relative transcript levels in ewes' monocytes and neutrophils.

Results

Animal performance

During the whole adaptation and experimental periods, no sign of diseases were detected on the animals. Supplementing Camelina seeds in the three different studied levels did not affect BW, milk yield, $FCM_{(6\%)}$, and ECM (Table 2). Regarding milk chemical composition, milk fat (%) was significantly reduced ($P = 0.046$) in the CS16 compared to the CON and the CS11. Considering sampling time, milk fat (%) was significantly increased ($P < 0.001$) on the 15th and 45th experimental days, protein (%) significantly increased ($P = 0.010$) on the 15th experimental day compared to day 0, and lactose (%) were significantly reduced ($P < 0.001$) in the three middle sampling points compared to day 0. However, all returned to initial levels by the end of the experiment. Furthermore, milk yield ($P < 0.001$), milk fat yield ($P < 0.001$), and protein yield ($P < 0.001$) were significantly decreased during the trial. Also, there was a significant interaction in the effect of the dietary treatment and sampling point in milk fat (%) ($P < 0.001$), milk protein ($P = 0.010$), milk protein (%) ($P = 0.008$), lactose (%) ($P = 0.011$), and lactose yield ($P = 0.039$) (Table 2; Supplementary Fig. S1).

Discriminant analysis

Discriminant analysis was conducted on the pooled data of monocytes and neutrophils as well as on their combinations' gene relative transcript levels (Fig. 1) to investigate to which extent the samples can be discriminated based on the dietary experimental group (CON, CS6, CS11, CS16). Given the dietary treatment and entering twenty independent variables in the model, 85.4% of the samples were classified into the correct group when variables (gene expressions) regarding monocytes and neutrophils were considered separately. Besides, 97.9% of the samples were classified into the correct group when forty variables (gene expressions), regarding monocytes and neutrophils, were considered together in

the analysis. More specifically, in monocytes, function 1 explains 62.3% of the variance, and function 2 explains 31.1%, and Wilks' Lambda resulted in 0.06 for function 1 ($P = 0.003$), and in 0.267 for function 2 ($P = 0.120$). Furthermore, considering the stepwise method, *NF-KB*, Signal transducer and activator of transcription 3 (***STAT3***), and *TLR4* were the variables that contributed the most. As for neutrophils, function 1 explains 55.5% of the variance, while function 2 explains 35.2%. Wilks' Lambda resulted in 0.047 for function 1 ($P < 0.05$), and in 0.203 for function 2 ($P = 0.077$). In addition, *STAT3* and Interleukin-1B (***IL1B***) were the main contributing variables through the stepwise method. Accounting for both monocytes and neutrophils, 75.4% of the variance was explained in function 1, and 17.7% in function 2. Finally, Wilks' Lambda resulted in 0.001 at function 1 ($P < 0.001$), and 0.035 ($P = 0.221$) at function 2. In addition, 75.0% of the samples were classified into the correct group for the discrimination of the twenty variables (gene expressions) in the different cell types (monocytes vs neutrophils) of each dietary group. Function 1 explains 53.9% of the variance, while functions 2, 3, 4, 5, 6, and 7 explain 16.3, 15.7, 7.9, 3.1, 2.4, and 0.8% of the variance, respectively. Wilk's Lambda resulted in 0.014 at function 1 ($P < 0.001$), and 0.076 ($P < 0.001$), 0.175 ($P < 0.001$), 0.392 ($P = 0.238$), 0.636 ($P = 0.885$), 0.794 ($P = 0.946$), 0.943 ($P = 0.989$) for functions 2 to 7, respectively. In addition, C-X-C motif chemokine 5 (***CCL5***), *IL1B*, tumour necrosis factor alpha (***TNFA***), *STAT3*, Interferon regulatory factor 5 (***IRF5***) were the main contributing variables through the stepwise method.

Discriminant analysis was also conducted in the amino acid profile of the four experimental concentrates to determine their discrimination level (Fig. 2). More specifically, function 1 explained 91.1% of the values, while function 2 explained the remaining 8.9%. Furthermore, Wilk's λ was resulted in < 0.001 ($P < 0.001$) for function 1, and in 0.025 for function 2 ($P < 0.001$).

Gene expression

Supplementing ewe concentrate with 6% of Camelina seeds (CS6) resulted in the upregulation of the *TLR4* in monocytes ($P = 0.019$) and neutrophils ($P = 0.002$) compared to the CS11 (Table 4 and Table 5, respectively). Furthermore, in monocytes, the relative transcript levels of the myeloid differentiation primary response 88 (***MYD88***) were not significantly affected, whilst in neutrophils, they were downregulated in the CS-fed ewes ($P = 0.005$). However, this downregulation was statistically significant ($P = 0.005$) for the CS11 group compared to the CON (Table 4 and Table 5, respectively).

In monocytes, TIR-domain-containing adapter-inducing interferon-β (***TRIF***) and TNF receptor-associated factor 3 (***TRAF3***) relative transcript levels were significantly upregulated ($P = 0.005$ and $P = 0.010$, respectively) in the CS11 group compared to the CON and CS6. However, the *TRAF3* relative transcript levels were not significantly affected in neutrophils (Table 4 and Table 5, respectively). Similarly, *MAPK* relative transcript levels were significantly upregulated ($P = 0.044$) in monocytes of the CS11, also with no significant differences being reported in neutrophils (Table 4 and Table 5, respectively). In the same direction, in monocytes, the relative transcript levels of the *IRF5* were significantly upregulated ($P = 0.002$) in the CS11, but they were not significantly regulated in neutrophils (Table 4 and Table 5, respectively). Moreover, the relative transcript levels of *STAT3* were significantly downregulated ($P = 0.003$) in neutrophils of the CS11 and CS16, while in monocytes, the effect was not significant (Table 4 and Table 5, respectively).

The relative transcript level of the *IL1B* was significantly upregulated in the monocytes of the CS6 ($P = 0.015$) and the neutrophils of the CS6- and CS16-fed ewes ($P = 0.001$; Table 4 and Table 5, respectively). Also, in neutrophils of the CS6 ewes, the relative transcript levels of the Interleukin-10 (***IL10***) were significantly upregulated compared to the CON and the CS11 ($P = 0.007$), with no significant effect being reported in monocytes (Table 4 and Table 5, respectively). In addition, Interleukin-8 (***IL8***) relative transcript level was significantly upregulated in neutrophils of the CS6 compared to the CON and CS11. In contrast, supplementing ewes' diets with Camelina seeds did not significantly affect the relative transcript levels of the Interleukin-2 (***IL2***), Interleukin-6 (***IL6***), *TNFA* in both monocytes and neutrophils.

Likewise, we did not report any significant differences in the relative transcript levels of the Interferon regulatory factor 3 (***IRF3***), conserved helix-loop-helix ubiquitous kinase (***CHUK***), Transcription factor JunD (***JUND***), *NF-kB*, C-X-C motif chemokine ligand 16 (***CXCL16***), and Haem Oxygenase 1 (***HMOX1***) (Table 4 and Table 5, respectively). Still, the relative transcript levels of *CCL5* were downregulated in both monocytes and neutrophils of the CS-fed ewes, but this downregulation was not significant (Table 4 and Table 5, respectively).

Correlations among parameters

Positive correlations between the mRNA expression levels of the *TLR4* and *MYD88* genes in monocytes ($r = 0.625$; $P < 0.001$) and neutrophils ($r = 0.550$; $P < 0.001$) as well as between the mRNA expression levels of the *TRIF* and *TRAF3* genes in monocytes ($r = 0.851$; $P < 0.001$) and neutrophils ($r = 0.728$; $P < 0.001$) were found (Fig. 3). The mRNA expression level of the *MAPK* gene was positively correlated with the *MyD88* in monocytes ($r = 0.342$; $P = 0.025$; Fig. 3). In neutrophils, the mRNA expression level of the *STAT3* was also significantly correlated with both the mRNA expression level of the *TLR4* ($r = 0.393$; $P = 0.006$) and *MYD88* ($r = 0.670$; $P < 0.001$; Fig. 3). In both monocytes and neutrophils, the mRNA expression level of the *IL1B* gene was positively correlated with the *TLR4* ($r = 0.621$; $P < 0.001$ and $r = 0.584$; $P < 0.001$, respectively; Fig. 3).

Positive correlations were found between the mRNA expression levels of the *IL10* and *TLR4* genes ($r = 0.714$; $P < 0.001$) in monocytes, and with the *TLR4* ($r = 0.559$; $P < 0.001$) and *MYD88* genes ($r = 0.354$; $P = 0.015$; Fig. 3) in neutrophils. In neutrophils, the mRNA expression levels of the *MYD88* and *IL2* genes were negatively correlated ($r = -0.395$; $P = 0.006$). Furthermore, in monocytes, *TNFA* was positively correlated with the *TLR4* ($r = 0.527$; $P < 0.001$) and the *MYD88* ($r = 0.437$; $P = 0.003$; Fig. 3). The same was observed for the mRNA expression level of the *IL8* gene, while in neutrophils, it was positively correlated only with the *TLR4* ($r = 0.505$; $P < 0.001$; Fig. 3).

The study of the relations between the relative transcript levels of the studied genes in monocytes and neutrophils and the individual dietary components highlighted significant correlations only with specific amino acids of the concentrates.

A negative correlation between the mRNA expression level of *MAPK* gene in monocytes and the threonine contents of concentrates was found ($r = -0.971$; $P = 0.029$). The opposite was observed between the relative transcript level of *IL2* gene in monocytes and the tyrosine contents of concentrates ($r = 0.973$; $P = 0.027$). Moreover, a trend for a negative correlation was shown between threonine and the mRNA expression levels of *IRF5* gene ($r = -0.924$; $P = 0.076$). In addition, a positive correlation between the relative transcript level of *MYD88* gene in neutrophils and the serine ($r = 0.966$; $P = 0.037$) and tyrosine (r = 0.949; $P = 0.050$) contents of concentrates respectively was found.

A negative correlation was found between the relative transcript level of the *TRIF* gene in monocytes and threonine contents of the concentrates ($r = -0.951$; $P = 0.049$). Similarly, negative correlations were observed between the relative transcript level of the

Table 4
Relative transcript levels of ewes' monocytes immune-related genes of the four dietary treatments.

	Dietary treatment					
Gene	CON	CS6	CS11	CS16	SEM	*P*
TLR4	0.18^{ab}	0.28^{a}	0.15^{b}	0.16^{ab}	0.005	0.019
MYD88	0.57	0.64	0.78	0.46	0.111	0.272
TRIF	0.01^{b}	0.01^{b}	0.02^{a}	0.01^{ab}	0.001	0.005
TRAF3	0.09^{b}	0.09^{b}	0.15^{a}	0.10^{ab}	0.002	0.010
IRF3	0.02	0.02	0.03	0.02	0.000	0.316
IRF5	0.30^{b}	0.30^{b}	0.51^{a}	0.35^{b}	0.007	0.002
MAPK	0.26^{b}	0.27^{ab}	0.37^{a}	0.27^{ab}	0.004	0.005
CHUK	0.07	0.07	0.07	0.08	0.000	0.571
JUND	0.19	0.23	0.24	0.20	0.035	0.368
STAT3	0.20	0.20	0.21	0.19	0.002	0.356
NFKB	0.34	0.33^{t}	0.42^{t}	0.34	0.040	0.079
TNFA	0.08	0.07	0.08	0.08	0.002	0.977
IL1B	0.02^{b}	0.06^{a}	0.03^{ab}	0.05^{ab}	0.001	0.015
IL2	0.003^{t}	0.002	0.001^{t}	0.002	0.000	0.086
IL6	0.001	0.001	0.001	0.001	0.000	0.666
IL8	0.17	0.24	0.13	0.17	0.013	0.849
IL10	0.01	0.02	0.01	0.01	0.000	0.259
CCL5	0.55^{t}	0.41	0.47	0.33^{t}	0.009	0.078
CXCL16	0.04	0.04	0.03	0.03	0.001	0.684
HMOX1	0.09	0.10	0.10	0.06	0.002	0.180

Abbreviations: CON = control dietary treatment; CS6 = dietary treatment with 6% Camelina seeds; CS11 = dietary treatment with 11% Camelina seeds; CS16 = dietary treatment with 16% Camelina seeds; ^{a,b,c} = values within a row with different superscripts differ significantly at $P < 0.05$; ^{t} = tendency towards statistical significance with values ranging between 0.05 and 0.10 ($0.05 < t < 0.10$).

Table 5
Relative transcript levels of ewes' neutrophils immune-related genes of the four dietary treatments.

	Dietary treatment					
Gene	CON	CS6	CS11	CS16	SEM	*P*
TLR4	0.27^{ab}	0.36^{a}	0.14^{b}	0.28^{ab}	0.006	0.005
MYD88	1.28^{a}	0.96^{ab}	0.57^{b}	0.84^{ab}	0.021	0.005
TRIF	0.01	0.01	0.01	0.01	0.000	0.500
TRAF3	0.05	0.07	0.09	0.07	0.002	0.260
IRF3	0.02	0.02	0.02	0.02	0.000	0.924
IRF5	0.17	0.20	0.23	0.23	0.012	0.328
MAPK	0.34	0.30	0.30	0.28	0.003	0.397
CHUK	0.07	0.07	0.08	0.07	0.001	0.124
JUND	0.19	0.23	0.15	0.16	0.005	0.292
STAT3	0.30^{a}	0.25^{ab}	0.21^{b}	0.24^{b}	0.009	0.003
NFKB	0.37	0.36	0.33	0.34	0.036	0.709
TNFA	0.17	0.12	0.15	0.16	0.002	0.102
IL1B	0.03^{b}	0.09^{a}	0.03^{b}	0.08^{a}	0.003	<0.001
IL2	0.003^{t}	0.004	0.006^{t}	0.002	0.000	0.062
IL6	0.001	0.001	0.002	0.001	0.000	0.681
IL8	0.08^{b}	0.33^{a}	0.06^{b}	0.20^{ab}	0.010	0.017
IL10	0.011^{b}	0.024^{a}	0.011^{b}	0.018^{ab}	0.001	0.007
CCL5	1.33	1.08	1.18	0.84	0.221	0.122
CXCL16	0.04	0.04	0.04	0.05	0.001	0.510
HMOX1	0.08	0.09	0.06	0.07	0.002	0.366

Abbreviations: CON = control dietary treatment; CS6 = dietary treatment with 6% Camelina seeds; CS11 = dietary treatment with 11% Camelina seeds; CS16 = dietary treatment with 16% Camelina seeds; ^{a,b,c} = values within a row with different superscripts differ significantly at $P < 0.05$; ^{t} = tendency towards statistical significance with values ranging between 0.05 and 0.10 ($0.05 < t < 0.10$).

TRAF3 gene in neutrophils and the content of the amino acid tyrosine ($r = -0.973$; $P = 0.027$) and serine ($r = -0.996$; $P = 0.004$) of the concentrates. A trend for a positive correlation between isoleucine content of concentrates and mRNA expression level of *IL10* gene ($r = 0.927$; $P = 0.073$) and between valine content of concentrates and mRNA expression level of *IL1B* gene ($r = 0.932$; $P = 0.068$) was found in monocytes.

Discussion

The supplementation of the ewes' diet with the three studied levels of Camelina seeds did not affect the overall animal performance. However, the reported decrease in milk fat (%) in the highest inclusion level of the Camelina seed group (CS16) may be attributed to the highest dietary PUFA content. This outcome was previously discussed (Christodoulou et al., 2021). The observed fluctuations in milk yield and milk chemical composition through the eight-week experimental period are consistent with those reported in a study evaluating the Chios ewe's milk yield and composition (Ploumi et al., 1998).

Ewes were in good health status throughout the experimental period and were free of infective and parasitic diseases such as pneumonia and gastrointestinal parasites (Ingham et al., 2008; Aboshady et al., 2020). Therefore, it is assumed that any trigger of their immune system was mainly dependent on dietary treat-

ment. Moreover, it should be highlighted that the observed reactions in the immune system did not occur in all groups, and this strengthens the assumption that no interferences of infections were leading to the results obtained.

When comparing the relative transcript levels of the MAPK on the CS11-fed ewes with the CON one, it appears that the MAPK cascade was activated, in this case, through the TRAF3-TRIF pathway described by Häcker et al. (2006) and Oganesyan et al. (2006). The *MAPK* family genes regulate several cellular processes including inflammation, cell growth, migration, proliferation, differentiation, and apoptosis (Cuadrado and Nebreda, 2010; Yue and López, 2020). This family genes have the same post-translational mechanism of kinase stimulation. The latter involves the phosphorylation of both tyrosine and threonine, as already indicated in various mammalian cells (Ferrell and Bhatt, 1997). More specifically, it has been proven that threonine affects the phosphorylation of extracellular signal-regulated kinases (**ERKs**), p38, and Jun N-terminal kinases (**JNKs**) (MAPK cascade) in embryonic stem cells of mice *in vitro* (Ryu and Han, 2011). Additionally, a recent review indicates threonine as a nutritional modulator of the intestinal immune system in swine and poultry via complex signalling networking including MAPK (Tang et al., 2021). Moreover, the activated receptors of tyrosine kinases were observed to be involved in the activation of the MAPK pathway (Kranenburg et al., 1999). Taking into account that MAPK kinase (involved in MAPK pathway regulation) is a serine/threonine kinase, the lower contents of threonine, tyrosine, and serine in the concentrates of the CS11-fed ewes might partially explain the activation of MAPK metabolic pathway in their monocytes. Indeed, the negative correlation between the mRNA expression level of the *MAPK* gene in monocytes and the threonine contents of the concentrates implies their close relationship. In the MAPK signalling pathway, the *NFKB* is the downstream target, which affects the cytokine gene expression (Qi et al., 2014). However, in this study, the upregulated tendency in the mRNA expression level of the *NFKB* gene in the monocytes of CS11-fed ewes did not promote the expression of inflammatory cytokines except for the *IL2*, which tended to downregulate. The role of IL2 in the inflammatory process is complex and involves pro-inflammatory as well as regulatory aspects, while dysregulation has been related to inflammation in the rumen of high grain-fed cows (Zhang et al., 2016) and to various immune system diseases in humans (Hanisch and Quirion, 1995, Lal and Bromberg, 2009). In the immune cells, the induction of the IL2 is regulated by cell signals, which are stimulated by the IL2 receptor β-subunit. It has been proven that threonine, tyrosine, and serine phosphorylation sites have a regulatory role also in the capacity of IL2 receptor β-subunit, and consequently in the mRNA expression level of the *IL2*, in multiple cell types *in vitro* including primary human lymphocytes (Ruiz-Medina et al., 2015). Moreover, threonine deficiency impaired the intestinal inflammatory response of fish, which were infected with bacteria by enhancing the expression of pro-inflammatory cytokines (*IL1B*, *IL6*, *IL8*) through the NF-κB signalling cascade (Habte-Tsion et al., 2015; Dong et al., 2017). As mentioned earlier, the concentrate of the CS11-fed ewes had the lowest contents of threonine, tyrosine, and serine, thus, more research is needed to define their role in the immune system of ewes as well as their optimal inclusion level in their diets. Moreover, the positive correlation between the relative transcript level of the *IL2* gene in monocytes and the tyrosine contents of the concentrates which was observed indicates the regulatory role of this amino acid in the *IL2* expression.

There is a close interaction between IRF5 and NF-κB. Indeed, it has been shown in humans that IRF5 forms a protein complex with NF-κB RelA (Krausgruber et al., 2010). Moreover, the IRF:RelA cistrome is best explained by the presence of consensus NF-kB as demonstrated using both *in vivo* and *in vitro* discovery analysis in macrophages (Saliba et al., 2014). IRF transcriptionally regulates type I interferon (IFN)-dependent genes, while also modulates cell cycle progression and cell death (Barnes et al., 2001; Lazear et al., 2013). Serine/threonine phosphorylation regulates *IRF5* transcriptional activity (Chen et al., 2008). Indeed, in many cell models, transiently expressed *IRF5* was phosphorylated by serine/threonine kinases (Foreman et al., 2012). Evidence linked also *IRF5* expression with tyrosine phosphorylation (Hu and Barnes, 2009). In detail, it has been shown that activated IRF5 is associated with the lack of tyrosine phosphorylation, suggesting that this is probably related to the suppression of IRF5 transactivation. Again, as previously reported, the concentrate of CS11-fed ewes had the lowest threonine, tyrosine, and serine content, which might explain the upregulated mRNA expression level of the *IRF5* gene in their monocytes. This hypothesis is supported by the trend for a negative correlation found between threonine and the mRNA expression levels of the *IRF5* gene. In several cases, the MAPK cascade activation can be regulated through the MyD88-dependent pathway (Akira et al., 2006) as confirmed by the positive correlations observed between the mRNA expression levels of *MYD88* and *MAPK* genes in both monocytes and neutrophils. However, the mRNA expression level of the *MyD88* was downregulated in the neutrophils of the CS11-fed ewes. The positive correlations between the relative transcript level of the *MYD88* gene in neutrophils and the serine and tyrosine contents of concentrates unveil the involvement of these amino acids in the mRNA expression level of the *MYD88* gene. Moreover, the significant positive correlation between the mRNA expression level of the *MYD88* and *TLR4* genes in the neutrophils and monocytes of ewes confirms their close relationship. The TLRs have an important role in innate immunity since are the first line of defence against pathogens (Qian and Cao, 2013) although they can also be activated by lipid overload (Tynan et al., 2012) and oxidative stress (McCarty, 2013). TLR activation induces *IL1* gene expression. Thus, our results concerning the mRNA expression level of the *TLR4*, *MYD*88, and *IL1B* genes might show the absence of any microbial pathogen in the CS11-fed ewes, implying the possible regulation role of amino acids in their innate immunity.

The amino acids can also regulate the mRNA expression level of the *STAT3* gene. The excess of amino acids activated *STAT3* transcriptional activity in human hepatic cells *in vitro* (Kim et al., 2009). It has been proven that not only tyrosine (705) (Rébé et al., 2013) but also serine (Kim et al., 2009) phosphorylation are involved in STAT3 activation (Rébé et al., 2013). Again, the concentrate of the CS11-fed ewes had the lowest tyrosine content, which might partially explain our results.

Feeding trials have provided strong evidence that not only the total protein intake but the availability of specific dietary amino acids such as glutamine, glutamate, arginine, and possibly methionine, cysteine, and threonine should be considered. Although the requirements for protein to support immunity are well defined (Ruth and Field, 2013), only recently, the potential use of individual dietary amino acids to optimise immune functions has started to be investigated. Nonetheless, the amino acid requirements in ewes' diets have not been defined.

The amino acids might also affect ewes' innate immunity directly through their interaction with isothiocyanates (**ITCs**) which are the main metabolic products of Camelina glucosinolates in the rumen (Sun, 2020). Indeed, it has been found that ITCs react with lysine and cysteine and form immunogenic complexes in skin cells *in vitro* (Karlsson et al., 2016). The ITCs' action might be both time and concentrate depended as already demonstrated in animal and human models of gastric cancer *in vitro* (Rabben et al., 2021). Moreover, the same researchers found that phenethyl-isothiocyanate (which belongs to the ITC family) altered the intracellular glutamine/glutamate ratio, providing a positive link

between ITCs and amino acid metabolism. It should be highlighted here that the lysine content in the concentrates of both CS11- and CS6-fed ewes was higher than those of CS16 and CON ones.

Furthermore, multiple analyses of phosphorylation revealed changes in MAPK activation and increased phosphorylation of c-JNN in both MM.1S and OPM-1 multiple myeloma cells *in vitro*, after treatment with ITCs (Jakubikova et al., 2011). However, a sustained increase in the phosphorylation of c-JUN (at 2, 6, and 12 h) and p38 (at 2 and 6 h) -MAPK cascade- was found only in the OPM-1 cells (multiple myeloma), treated with phenylethyl isothiocyanate *in vitro* (Jakubikova et al., 2011). Additionally, no effects were found in the phosphorylation status of NF-κB p65 and STAT3 in the multiple myeloma MM.15 cells treated with ITCs by the same researchers (Jakubikova et al., 2011). It seems that each type of cell (like monocytes vs neutrophils of our study) reveals different regulatory immune mechanisms to respond to amino acids and ITC availability. Moreover, the type of ITCs and their synergistic action might have also a different impact on innate immunity. Indeed, allyl isothiocyanate or sulforaphane when added separately in A549 lung cancer cells reduced the expression level of phosphorylated *STAT3* (p- STAT3) in a dose-dependent manner while did not modify the mRNA expression level of the *STAT3* (Rakariyatham et al., 2019). On the contrary, when both allyl isothiocyanate and sulforaphane were added simultaneously in the A459 lung cancer cells *in vitro*, a decrease in the phosphorylated STAT3 was observed (Rakariyatham et al., 2019). Thus, more research is needed to clarify the synergistic role of amino acids and ITCs on innate immunity as well as their optimum inclusion levels in ewes' diets. Moreover, more sampling times could be addressed in future studies to confirm the relationship between the variation of dietary amino acid profile and the expression of genes related to immunity. Indeed, the amino acid profile of the four concentrates or the interaction of the amino acids with the ITCs, rather than the ITCs alone, seems to better explain the results of this study. More specifically, the CS6 and CS11 concentrates had a similar amino acid profile, while the amino acid profile of the CS16 concentrate was closer to that of the CON. Therefore, no differences in the transcriptomic gene levels were observed between the CON- and the CS16-fed ewes. However, leucine was the only amino acid that was lower in the concentrate of the CS16-fed ewes compared to the CON one and might justify the reported upregulated trend in the relative transcript levels of the *IL1B* in both monocytes and neutrophils in the CS16-fed ewes. Interestingly, leucine deficiency has been also associated with upregulated mRNA expression levels of *IL1B*, *IL8*, and *TNFA* in fish gills (Jiang et al., 2017).

Moreover, differences were demonstrated also among the CS-fed groups. In detail, in monocytes of the CS11-fed ewes, the significant upregulation in the relative transcript levels of the *TRIF*, *TRAF3*, and *MAPK* genes compared with the CS6- and CS16-fed ewes may further support our assumption regarding the role of amino acids regulating gene expression. Threonine, tyrosine, and serine were in fact lower in the CS11 concentrate. The negative correlations between the relative transcript level of *TRIF* gene in monocytes and threonine contents of the concentrates as well as between the relative transcript level of *TRAF3* gene in the neutrophils and the tyrosine and serine contents of the concentrates respectively strengthen the above hypothesis. Regarding neutrophils, the relative transcript levels of the *TLR4* as well as those of the *IL1B*, *IL8*, and *IL10* were significantly upregulated in the CS6-fed ewes compared to the CS11. Comprehensively, high levels of branched-chain amino acids (BCAA; isoleucine, leucine, and valine) were reported to activate the NF-kB signalling pathway (Ye et al., 2020). Hence, the upregulated relative transcript levels of the interleukins that were reported in the CS6-fed ewes might be attributed to the higher contents of isoleucine, leucine, and valine that were reported in the amino acid profile of this specific concentrate. Indeed, a trend for a positive correlation was found between isoleucine content of the concentrates and mRNA expression level of the *IL10* and between valine content of concentrates and mRNA expression level of the *IL1B* gene in monocytes.

Conclusion

In conclusion, the highest dietary inclusion level of Camelina sativa seeds (CS16) and the related dietary amino acid profile changes did not negatively trigger the expression of the selected immune genes. The dietary amino acid profile (especially tyrosine, threonine, serine, and lysine) and not the increase in ITC content related to the Camelina inclusion appeared to exert a stronger impact on the mRNA expression levels of the selected genes involved in ewes' immune system. More research is needed to define the role of amino acids in ewes' innate immunity at both protein and mRNA expression levels. Finally, monocytes and neutrophils responded differently to the changes in the dietary amino acid profile, indicating cell differences, which need further investigation.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2022.100621.

Ethics approval

Animal handling, housing, and care followed the Ethical Committee guidelines of the Department of Animal Science of the Agricultural University of Athens (EU 63/2010; Council of the European Union 2010).

Data and model availability statement

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

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Declaration of interest

None.

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