

Dietary protected fat and conjugated linoleic acid improves ewe milk fatty acid composition

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Abstract

The effects of protected fats (Optima 100) and conjugated linoleic acid (Endulac®-CLA) supplementation on sheep milk saturated and unsaturated fatty acid composition were investigated. Sheep were divided into four experimental groups (15 ewes/group) including: i) a control group - basal diet without any nutritional supplements; ii) experimental group 1 - basal diet + 12g/sheep/day of the protected source of fats in the feed; iii) group 2 - 12 g of CLA in the feed; iv) group 3 - 12 g of protected fats and CLA in feed. Sixty milk fatty acids were different in milk from treated fat and CLA-treated sheep compared to the control group. The most biologically important fatty acid constituents of milk were identified as butyric, caproic, caprylic, lauric, myristic, palmitic, stearic, arachidonic, behenic, oleic, and linoleic acid (C4 to C18). Ewes that received protected fat or CLA, or both, displayed an increased concentration of oleic acid compared to the control. Both treatments modified milk lipid quality parameters and increased the polyunsaturated/saturated fatty acids ratio (PUFA/SFA), the polyunsaturation index (PI), and the thrombogenic index (TI). Group 3 had similar milk lipid quality parameters as untreated animals. Compared to the CLA and control groups, milk production in the protected fat treatment was higher in Turcana dairy ewes. The inclusion of protected fats and CLA as dietary supplements in lactating ewes modified the milk fatty acid profile, with a concomitant impact on suckling lamb performance and consumer health.

Keywords: Turcana sheep, milk fatty acid profile, conjugated linoleic acid, protected fats, nutrition

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Introduction

The worldwide interest in healthy food products is increasing. Different markets seek foods with fortified nutritional composition obtained via the addition of different biologically active substances and higher contents in unsaturated fatty acids such as conjugated linoleic acid (CLA) and ω -3 fatty acids, but with a reduced amount of saturated fatty acids (Rodríguez *et al.*, 2020). Animal-derived food products encompass a wide umbrella of different healthy and biologically active substances known under the term “nutraceuticals,” many of which are fatty acids (Serra *et al.*, 2018). Currently, sheep products are receiving increased attention because of the feasibility of supplementation with various types of fatty acids, such as α -linolenic acid (C18:3 cis 9, cis 12, cis 15), CLA and vaccenic acid, which have potentially human health benefits (Angeles-Hernandez *et al.*, 2020). In contrast to cow milk, sheep milk is approximately three-fold richer in CLA and vaccenic acid (Gómez-Cortés *et al.*, 2008). Previous

studies have shown that CLA consumption from ruminant products notably enriches the levels of long-chain polyunsaturated fatty acids in tissues of rodents and humans, and might decrease risks associated with chronic diseases, prevent atherosclerosis, decline body fat accumulation, and modulate immune and inflammatory responses (Angeles-Hernandez *et al.*, 2020; Balta *et al.*, 2021). Stearic acid (C18:0) represents approximately 10% of dairy fat. Oleic acid (C18:1 *cis*-9) is the second most abundant fatty acid (15–25%) in milk and widely known for its antiatherogenic activity in the human body (Gómez-Cortés *et al.*, 2018). Oleic acid is one of the principal monounsaturated fatty acids with a distinctive function known to elevate the activity of low-density lipoprotein receptors (LDL-R), minimizing the cholesterol content of the blood serum (Chen & Liu, 2020).

Sheep dietary manipulations can increase the unsaturated fatty acid profile of milk, while decreasing the levels of saturated fatty acids (Pecka-Kielb *et al.*, 2020). High amounts of saturated fatty acids such as lauric (C12:0), myristic (C14:0), and palmitic (C16:0) fatty acids from dairy products were previously linked with putative negative health effects, while butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric acid (C12:0) were connected with potentially healthy biological activity by delaying tumour growth progression, reducing body fat/weight, and exhibiting antibacterial and antiviral capacity (Balthazar *et al.*, 2017; Ptáček *et al.*, 2019). However, other researchers have stated that myristic (C14:0) and palmitic (C16:0) fatty acids are engaged in the post-translational protein modifications, such as N-terminal myristoylation and side-chain palmitoylation, both molecular mechanisms responsible for governing major metabolic functions in the human body (Gómez-Cortés *et al.*, 2018). Myristic acid (C14:0) is also involved in the regulation of human cardiovascular parameters, and a balanced myristic acid consumption from dairy products is suggested to improve long-chain omega-3 (C20:5 *n*-3 and C22:6 *n*-3) fatty acids from phospholipid blood levels, increase HDL-high density lipoprotein content, reduce triacylglycerol levels without affecting low density lipoprotein (LDL) (Gómez-Cortés *et al.*, 2018).

Protected fats or rumen protected fats can advance animal performance and are considered insoluble fats due to their ruminal pH stability, protection against microbial fermentation, and biohydrogenation without impacting the rumen metabolism. Rumen protected fats avoid rumen fermentation and are used by the animal as an energy source following intestinal absorption and may also decrease methane generation from the ruminal microbial ecosystem (Behan *et al.*, 2019). Different seeds, oils, and fatty acids can be utilized as natural sources of protected fats, enhancing milk production and increasing the fat percentage in dairy sheep milk (Bianchi *et al.*, 2018). Fatty acids are essential building molecules for cellular structures, tissues, and organs and are also involved in the production of essential biologically active substances, as well in coordinating the proper function of metabolic processes (Sokoła-Wysoczańska *et al.*, 2018; Balta *et al.*, 2021). These molecules from mammalian milk are essential for the appropriate growth of young animals and are critical to the proper development of the nervous system of growing animals (Flis & Molik, 2021).

Animal studies have demonstrated that dietary supplementation with rumen-protected (RPO) pellets infused with eicosapentaenoic acid (EPA, C20:5 n 3) and docosahexaenoic acid (DHA, C22:6 n 3); pellets containing oils from canola, rice bran, safflower, linseed, flaxseed, marine oils; and protected tuna oil can efficiently enrich the concentrations of omega 3 long-chain (C20) polyunsaturated fatty acids (n 3 LC-PUFA) in sheep milk (Nguyen *et al.*, 2018; Nudda *et al.*, 2020). Moreover, flaxseed oil administration in the sheep diet enriches milk with α -linoleic acid. Dairy ewes and cows supplemented with isomerized CLA-rich poppy seed oil had a reduced fat proportion in the milk and continuing declines of short-chain fatty acids (C4–10) and medium-chain fatty acids (C12–16), which exert atherogenic and thrombogenic effects in humans (Bodkowski *et al.*, 2020) occurred. Conversely, CLA-rich poppy seed oil increased the concentrations of biologically active fatty acids with health-inducing properties, such as CLA isomers (C18:2 *c*9, *t*11) and (C18:2 *t*10, *c*12) and trans-vaccenic acid (C18:1 *t*11) in the milk of both species. The use of CLA as a dietary additive can improve milk quality and value by ascribing additional pro-health properties for humans (Bodkowski *et al.*, 2020). A similar study illustrated that increased levels of C18:3 *n*-3, C18:1 isomers, and CLA isomers from milk from ewes fed with hemp seed cake led to a diminished atherogenic and thrombogenic index (Mierlita, 2018). The enrichment in PUFA concentrations following hemp feeding, which is rich in α -tocopherol, has been shown to prevent lipid oxidation of the raw milk.

A probiotic, *Saccharomyces cerevisiae* yeast product administered to ewes increased the concentrations of pentadecanoic acid (C15:0), palmitoleic acid (C16:1), linoleic acid (C18:2 n 6 t), and α -linolenic acid (C18:3 *n*3) in milk and decreased stearic acid (C18:0), respectively (Mavrommatis *et al.*, 2020). Odd chain saturated fatty acids, such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), are found in low concentrations, approximately 1.5% of the total fat fraction (Gómez-Cortés *et al.*

al., 2018). Odd chain fatty acids are used as biological markers of dairy fat intake and were previously documented to occur through endogenous synthesis through α -oxidation and could exert healthful effects in our body. Several studies have clearly shown an inverse association (Gómez-Cortés *et al.*, 2018).

The objective of the current study was to evaluate the effects of the inclusion of protected fat and CLA as a source of dietary fat on the fatty acid profile of milk and the performance of Turcana ewes during a 62-day experimental period.

Materials and Methods

The experiment was carried out using 60 Turcana sheep (48–52 kg) during the lactation period. The research was carried out on a farm in Buzias, Romania, between March and May, 2019. The experiments were performed according to the legislation in place (Law 471/2002 and Government Ordinance 437/2002) and under the supervision of the National Sanitary Veterinary Agency. The ethics committee of Banat University of Agricultural Sciences and Veterinary Medicine – King Michael I of Romania, approved this work. Recently additional data have been published, which are part of the same experiment and explore the relationship between protected fats and CLA on the bioproductive indices (body weight and total gain, daily weight gain) in ewes and suckling lambs (Grigorescu *et al.*, 2021).

In Romania, the Turcana sheep breed, belonging to the East European Zackel group, represents over 70% of the national flock and accounts for over 6 million head. The characteristics of the breed are: adult body weight of 40–50 kg in the ewes and 70–75 kg in rams, growth rates of 110–160 g/day and 105–115% prolificacy (Voia & Padeanu, 2021). Due to different geographical and rearing conditions, and also due to different selection traits, there is a high heterogeneity within the breed, e.g., the Breaza de Petrosani ecotype has an average prolificacy of 130–140%, whereas Transhumanta de Sibiu has a prolificacy of 103–105% (Budai *et al.*, 2013). The Turcana is a dual-purpose breed, utilized for milk, wool, and meat across Romania. Milk production of ewes is 100 ± 40 L over a 200-day lactation (at first lactation) increasing to 130 ± 40 L in the second lactation. The average milk fat content is 6–7%, with a protein content of 5–6%. From a reproductive perspective, the breed displays a 92–97% fertility, 90–95% lambing rate, and 120–140% prolificacy (Voia & Padeanu, 2021).

A housing area of 1.4 m² was allocated for each sheep. Feeders were equipped with gutters for concentrate and salt was provided. The straw bedding was refreshed daily. The experimental groups were housed in similar environmental conditions, in order to reduce the impact on observed results. All ewes were fed twice/day in hayrack feeders and hook-on sheep trough feeders for the concentrate (Table 3). Lambs were fed with alfalfa hay and concentrate mix in a space separate from the mothers. The microclimatic conditions were held at physiological requirements: average ambient temperature, 12–15 °C; relative humidity, 70–75%; air currents, 0.2 m/s; maximum concentration of CO₂, 0.03%; and the maximum NH₃ concentration, 0.002%. The 60 sheep were randomly divided into four groups (n = 15/group), each of which received a basal diet in order to meet their daily needs according to their physiological condition. The experimental groups were based on the ewe body weight and order of lactation. Ewes in the second lactation were selected (body weights 50 ± 2 kg) and after lambing, lambs were selected and blocked by weight before being randomly assigned to groups.

The sheep were grouped as: the control group (C) received only the basal ration (BR; Tables 2 and 3); experimental group 1 (G1), the basal ration was supplemented with 12 g/sheep/day of the Optima 100 product, containing ruminally-protected fats; experimental group 2 (G2), BR was supplemented by Endulac®-CLA at 12 g/sheep/day; and experimental group 3 (G3) received both Optima 100 (12 g/hd/d) and Endulac®-CLA (12 g/hd/d) (Table 1). The feed source of fats were supplemented in the ewe diets, starting two weeks before lambing to allow ruminal symbionts to establish at the beginning of the experimental period. All animals were weighed using an electronic scale. After lambing, the experiment lasted for 62 days with the sheep's milk production and lamb weight gain being recorded individually in each experimental group. Each lamb was weighed individually at lambing, at 21 days, and 62 days (at weaning). The amount of milk used by suckling lambs at each feeding time point was estimated as previously described (Voia & Padeanu, 2021).

Table 1 Experimental design for 60 Turcana sheep (48–52 kg) during the lactation period

Specification	C	Group 1	Group 2	Group 3
n	15	15	15	15
BR	According to INRA (2007; 2018) (Agabriel, 2013; Noziere <i>et al.</i> , 2018)			
Supplemented BR	-	+12 g/sheep/day Optima 100	+12 g/sheep/day Endulac® CLA	+12 g/sheep/day Optima 100 +12 g/sheep/day Endulac® CLA

Note: BR – basic diet; Optima 100 – protected fats; Endulac® CLA – conjugated linoleic acid

Ruminally-protected fats were included in the form of Optima 100, a source of protected fats that avoid ruminal degradation (Asianagro Agungjaya, Indonesia). Optima 100 is designed as a dietary energy source based on refined palm oil that is fractionated, non-hydrogenated, free of trans fatty acids. This product contains saturated fatty acids such as C14:0 Myristic acid with a proportion of 0–3%; palmitic acid C16:0 of ~80%; stearic acid C18:0, 5–10%; and C18:1 oleic acid, 8–12%. The CLA source (Endulac®; BASF Group, Ludwigshafen, Germany) is an energy source that consists of refined and hydrogenated rapeseed oil and CLA derived from refined sunflower oil, safflower, and wheat gluten hydrolysates. The CLA-containing product also contains at least 93% crude fat and 6.5% crude ash. To date, there are no data in the literature concerning the dietary administration of these two ingredients to sheep; research has been reported on dairy cows (Oyebade *et al.*, 2020; Rahbar *et al.*, 2021).

The two experimental feed additives were incorporated into the concentrate mixture supplemented with calcium carbonate to support proper homogenisation with the ration. The hay was administered in hayracks and the concentrate was provided in feed troughs (Table 2). The feed ratio and dry matter consumed by the sheep, raw chemical composition of the feed, average daily net energy consumption for sheep in each experimental group, and the composition of the feed concentrate is presented in Table 3.

Table 2 Chemical composition of the feed ingredients used in the sheep diets

Raw material	DM	CP	EE	CF	Crude ash
Corn silage (%)	35.09	1.83	0.91	9.39	2.17
Triticale (%)	89.54	11.37	1.32	3.41	2.08
Barley (%)	89.12	10.31	1.55	5.54	2.11
Sunflower meal (%)	88.92	35.52	1.78	29.22	6.33
Alfalfa hay (%)	90.46	15.86	1.46	31.17	8.37

DM-dry matter, CP- crude protein, EE- ether extract, CF- crude fibre

The extraction and methylation of fatty acids was performed as previously described (Liu *et al.*, 2019). Briefly, raw milk (15 mL) was centrifuged at 4 °C for 20 min (3000 rpm, Hermle Labortechnik, Wehingen, Germany); 0.1 g crude fat from the top fat layer was weighed into a 5-mL glass vial and directly subjected to methylation using 3 ml of a 20% boron trifluoride (BF₃) methanolic solution (Sigma-Aldrich Chemie GmbH, München, Germany). Derivatization was done for one hour at 80 °C in the ultrasonic bath (FALC Instruments, Treviglio, Italy). After the samples were cooled, 2.5 ml of 10% sodium chloride solution was added, and the extraction of methyl esters was done using 2 ml of hexane. The organic layer was separated using centrifugation at 3000 rpm for 15 min (Hermle Labortechnik, Wehingen, Germany) and the hexane fraction (1 µl) was used for Gas chromatography–mass spectrometry analysis of fatty acids.

Table 3 Basic diet for a 50-kg ewe with one suckling lamb

Ingredients (kg)	
Corn silage	1.80
Alfalfa hay	0.90
Concentrate feed mixture	0.40
Salt	0.009
Nutritive value	
DM (kg)	1.78
UFL	1.46
PDIN (g)	154.85
PDIE (g)	151.52
Ca (g)	27.27
P (g)	7.03
Salt (g)	13
Concentrate feed composition	
Triticale (%)	34
Barley (%)	35
Sunflower meal (%)	28
Limestone (%)	1
Calcium monophosphate (MCP) (%)	1
Salt (%)	1
Nutritive value of the concentrate feed mixture (for 1 kg)	
DM (kg)	0.86
UFL	0.81
PDIN (g)	114.41
PDIE (g)	93.3
Ca (g)	7.41
P (g)	7.38
Salt (g)	10

DM, dry matter; UFL, forage unit for lactation, according to INRA 2018 (INRA, Noziere, Sauvant, & Delaby, 2018); PDIN, intestinal digestible protein for nitrogen, according to INRA 2007; PDIE, intestinal digestible protein for energy, according to INRA 2007 (Agabriel, 2013); Ca, calcium; P, phosphorus

The fatty acids composition was analysed as fatty acid methyl esters (FAMES). The hexane fraction (1 µl) obtained as above, was injected into GCMS-QP2010 (Shimadzu, Kyoto, Japan). Helium was used as carrier gas with a flow rate of 1 ml/min and a linear velocity of 37.8 cm/s. The splitting ratio was 1:10, and the injection port temperature was set to 250 °C. The temperature of the ion source and the GC-MS interface was 210 °C and 255 °C. The chromatographic column used was a polar AT-WAX column with the characteristics: 30 m x 0.32 mm x 1 µm (Agilent, Santa Clara, USA). The temperature gradient was initially at 140 °C for 10 min, then the temperature was increased by 7 °C/min to 250 °C and maintained for 10 min (the total analysis time of a sample was 35.71 min). Methyl esters were identified using the NIST 05 spectrum library, and quantification was performed using the area standardization method. The percentage (%) of the various compounds was determined by relating the peak area corresponding to a given compound to the total area of 260 of all chromatogram peaks (Pop *et al.*, 2020). The fatty acids analysis was done by the Interdisciplinary Research Platform (PCI) belonging to the Banat University of Agricultural Sciences and Veterinary Medicine, "King Michael I of Romania," from Timisoara.

The nutritional quality of the fat from the sheep milk was assessed based on the ratio of fatty acids on the basis of qualitative lipid health indices. The identified fatty acids were grouped in the following order:

Sum of saturated fatty acids (SFA):

$$\Sigma\text{SFA} = \text{C6:0} + \text{C8:0} + \text{C10:0} + \text{C12:0} + \text{C14:0} + \text{C14:0}, 12\text{-CH}_3 + \text{C15:0} + \text{C16:0} + \text{C16:0}, 14\text{-CH}_3 + \text{C17:0} + \text{C18:0}$$

Sum of monounsaturated fatty acids (MUFA):

$$\Sigma\text{MUFA} = \text{C16:1} + \text{C18:1cis-9}$$

Sum of poly-unsaturated fatty acids (PUFA):

$$\Sigma\text{PUFA} = \text{C18:2 (omega-6)} + \text{C18:3 (omega-3)}$$

HFA: hypercholesterolaemic fatty acids (C12:0 + C14:0 + C16:0)

hFA: hypocholesterolaemic fatty acids (C18:1 + polyunsaturated FA)

The polyunsaturation index (**PI**) was calculated according to the formula of Timmons *et al.* (2001):

$$\text{PI} = \text{C18:2 n-6} + (\text{C18:3 n-3} \times 2)$$

In order to highlight the relevance for human health, the atherogenic (**AI**) and thrombogenic (**TI**) Index of lipids was calculated according to the equations proposed by Ulbricht & Southgate (1991):

$$\mathbf{AI} = (\text{C12:0} + \text{C16:0} + 4 \times \text{C14:0}) / [\Sigma\text{MUFA} + \Sigma(n-6) + \Sigma(n-3)]$$

$$\mathbf{TI} = (\text{C14:0} + \text{C16:0} + \text{C18:0}) / [0,5 \times \Sigma\text{MUFA} + 0,5 \times \Sigma(n-6) + 3 \times \Sigma(n-3) + \Sigma(n-3) / \Sigma(n-6)]$$

The ratio of hypocholesterolaemic to hypercholesterolaemic fatty acids was calculated using the equation proposed by Fernandez *et al.* (2007):

$$\mathbf{h / H} \text{ (hypocholesterolaemic / hypercholesterolaemic)} = (\text{C18:1} + \text{PUFA}) / (\text{C12:0} + \text{C14:0} + \text{C16:0})$$

The Health Promotion Index (**HPI**) was calculated according to the formula proposed by Chen *et al.* (2004):

$$\mathbf{HPI} = (n-3 \text{ PUFA} + n-6 \text{ PUFA} + \text{MUFA}) / [\text{C12:0} + (4 \times \text{C14:0}) + \text{C16:0}]$$

All the statistical analyses were performed using IBM-SPSS 23.0 Software (analysis of variance and Tukey test), the Shapiro–Wilk test was used for testing normality, and graphical representation was performed using GraphPad software. Data are represented as mean \pm standard deviation. Significance was assigned at *P*-value < 0.001, 0.01, and 0.05.

Results

The impact of ruminally-protected fat and CLA on the sheep milk fat composition was determined (Table 4). The addition of separate dietary sources of protected fat and CLA in the ration caused shifts in the milk FA profile. The concurrent inclusion of the two nutritional supplements to the feed of ewes did not yield significant changes in these fatty acids. All dietary treatments reduced the total sum of ΣSFA in milk compared with the control group. Total levels of ΣMUFA showed an increasing tendency G3, which received Optima 100 product and Endulac®CLA. In the case of the ΣPUFA sum, the G3 group was similar to the control group, while G1 and G2 showed a nearly three-fold increase.

Regarding the specificity of each fatty acid in sheep milk, all groups represented fluctuations in FAs profiles compared to the control group. The dietary intervention with protected fat source, Optima 100, and Endulac® CLA resulted in a lesser increase in C6:0, C8, C17:0, and C18:0. Dietary treatment with protected fat in G2 induced a marked decline in C12:0, C14:0, and C15:0 compared with the control. CLA treatment in the G1 group appeared to influence milk FA profile by decreasing C:14 and C:15. Combined (Endulac® CLA and Optima 100) dietary exposure in the G3 group was shown to lessen only the levels of C:14 in milk, whereas the rest of the fatty acids were similar to the control.

Table 4 The fatty acid content of Turcana sheep milk in Buzias, Romania

Treatments	Control*	Group1*	Group 2*	Group 3*	P-value
C6:0, caproic acid (%)	8.795±0.480 ^a	9.4225±2.753 ^a	8.2125±1.724 ^a	9.4875±1.380 ^a	0.720
C8:0, caprylic acid (%)	6.5325±1.788 ^a	7.9450±2.332 ^a	7.2725±1.725 ^a	5.0325±0.498 ^a	0.153
C10:0, decanoic acid (%)	12.4325±5.496 ^a	12.4150±3.884 ^a	12.6975±3.815 ^a	6.9975±0.894 ^a	0.166
C12:0, lauric acid (%)	7.470±0.442 ^a	11.125±1.317 ^a	4.715±1.801 ^a	6.5125±1.133 ^a	0.595
C14:0, myristic acid (%)	15.270±0.835 ^a	8.8225±2.367 ^b	10.065±3.125 ^{b,c}	14.990±2.677 ^{a,c}	0.004
C14:0, 12-CH ₃ , 12-metil myristic acid (%)	0.4975±0.073 ^a	0.405±0.165 ^a	0.365±0.097 ^a	0.560±0.094 ^a	0.112
C15:0 pentadecanoic acid (%)	1.2425±0.146 ^a	0.8225±0.232 ^b	0.7500±0.094 ^b	1.3350±0.096 ^b	0.000
C16:0, palmitic acid (%)	20.310±0.695 ^a	20.225±1.658 ^a	21.1275±2.216 ^a	20.755±0.723 ^a	0.808
C16:0, 14 -CH ₃ 14- methyl palmitic acid (%)	0.260±0.113 ^a	0.335±0.127 ^a	0.230±0.124 ^a	0.265±0.017 ^a	0.565
C17:0 heptadecanoic acid (%)	0.795±0.323 ^a	1.105±0.337 ^a	1.140±0.408 ^a	0.6575±0.290 ^a	0.183
C18:0, stearic acid (%)	6.2675±2.718 ^a	9.4725±1.838 ^a	9.185±2.767 ^a	8.985±1.971 ^a	0.244
ΣSFA (%)	79.87±4.308^a	75.17±5.546^a	75.76±6.038^a	75.58±3.730^a	0.529
C10:1,4, decanoic acid (%)	0.515±0.191 ^a	0.1375±0.112 ^b	0.1325±0.096 ^b	0.605±0.114 ^a	0.000
caproic acid (dec-9-enoic acid) (%)					
C16:1 CIS 9 palmitoleic acid (omega 7) (%)	2.9475±0.435 ^a	2.490±0.482 ^a	2.670±0.722 ^a	2.885±0.108 ^a	0.552
C18:1 CIS 9- oleic acid (%)	14.6625±3.787 ^a	17.280±5.012 ^a	16.3975±5.787 ^a	19.240±3.717 ^a	0.587
(omega 9) (%)					
ΣMUFA (%)	17.61±4.183^a	19.77±5.002^a	19.07±5.250^a	22.13±3.691^a	0.582
C18:2 CIS 9, 12- linoleic acid (omega 6) (%)	1.1725±0.192 ^a	2.1950±0.400 ^b	2.2225±0.466 ^b	1.2050±0.185 ^c	0.001
C18:3 CIS, 9, 12, 15 linolenic acid (omega 3) (%)	0.4150±0.564 ^a	2.2475±0.828 ^b	2.4575±0.897 ^b	0.4200±0.268 ^c	0.002
ΣPUFA (%)	1.59±0.731^a	4.44±0.976^b	4.68±1.161^b	1.63±0.055^a	0.000
Total (%)	99.07±0.417^a	99.38±0.369^a	99.51±0.128^a	99.33±0.836^a	0.323

ΣSFA - sum of saturated fatty acids [C6:0 + C8:0 + C10:0 + C12:0 + C14:0 + C14:0, 12-CH₃ + C15:0 + C16:0 + C16:0, 14-CH₃ + C17:0 + C18:0]; ΣMUFA -sum of monounsaturated fatty acids [C16:1 + C18:1cis-9] ; ΣPUFA - sum of poly-unsaturated fatty acids [C18:2 (omega-6) + C18:3 (omega-3)]. Statistically significant differences among experimental groups for each performance-related parameter obtained using the Tukey test are indicated by different lowercase letters.

There were no differences between the lipid quality parameters of group G3 and the control group, whereas there were substantial increases in G1 and G2 (Table 5). However, compared to the control group, the dietary treatments in G1 and G2 increased ($P < 0.01$) the PUFA/SFA ratio and PI (Figure 1). A decrease ($P < 0.01$) in HFA and TI was also observed in G1, G2, and G3, compared to the control. Overall, the parameters of health-related lipids in ewe milk in G3 were similar to the control and G1 treatments.

Table 5 Lipid health quality parameters in Turcana sheep milk in Buzias, Romania

Lipid Health Indices	Control*	Group1*	Group 2*	Group 3*	P-value
HFA	43.81±0.613 ^a	33.98±2.220 ^b	36.50±5.097 ^{b,c}	43.08±3.184 ^{a,c}	0.002
hFA	16.27±3.594 ^a	21.72±5.541 ^a	21.08±6.543 ^a	19.82±3.826 ^a	0.471
PUFA/SFA	0.020±0.008 ^a	0.060±0.016 ^b	0.063±0.019 ^b	0.021±0.001 ^a	0.001
MUFA/SFA	0.223±0.064 ^a	0.268±0.086 ^a	0.257±0.092 ^a	0.295±0.062 ^a	0.628
UFA/SFA	0.243±0.063 ^a	0.328±0.097 ^a	0.320±0.106 ^a	0.299±0.066 ^a	0.477
n-6/n-3	5.842±4.321 ^a	1.051±0.313 ^a	0.976±0.281 ^a	3.315±1.885 ^a	0.050
h/H	0.372±0.084 ^a	0.643±0.168 ^a	0.601±0.248 ^a	0.456±0.117 ^a	0.151
PI	2.048±1.256 ^a	6.690±1.767 ^b	7.138±2.023 ^b	1.835±0.358 ^a	0.000
AI	4.911±1.042 ^a	2.682±0.930 ^a	3.091±1.373 ^a	3.932±1.207 ^a	0.076
TI	3.964±0.743 ^a	2.154±0.415 ^b	2.241±0.600 ^b	3.546±0.280 ^a	0.001
HPI	0.210±0.041 ^a	0.401±0.111 ^a	0.373±0.151 ^a	0.270±0.068 ^a	0.069

*mean ± standard deviation; statistically significant differences among experimental groups for each performance-related parameter obtained using the Tukey test are indicated by different lowercase letters.

HFA - hypercholesterolaemic fatty acids [(C12: 0 + C14: 0 + C16: 0)]; hFA - hypocholesterolaemic fatty acids [(C18: 1 + PUFA)]; PUFA- polyunsaturated FA; SFA - saturated FA; MUFA - monounsaturated FA; UFA - unsaturated FA; h/H - hypocholesterolaemic / hypercholesterolaemic ratio [(C18: 1 + PUFA) / (C12: 0 + C14: 0 + C16: 0)]; PI - the polyunsaturation index [C18: 2 n-6 + (C18: 3 n-3 × 2)]; AI - atherogenic index [(C12:0 + C16:0 + 4 × C14:0) / [ΣMUFA + Σ(n-6) + Σ(n-3)]]; TI - thrombogenic index [(C14:0+C16:0+C18:0) / [0.5 × ΣMUFA+0.5 × Σ(n-6)+3 × Σ(n-3)+Σ(n-3) / Σ(n-6)]]; HPI - the health promotion index [(n-3 PUFA + n-6 PUFA + MUFA) / [C12:0 + (4 × C14:0) + C16:0]]

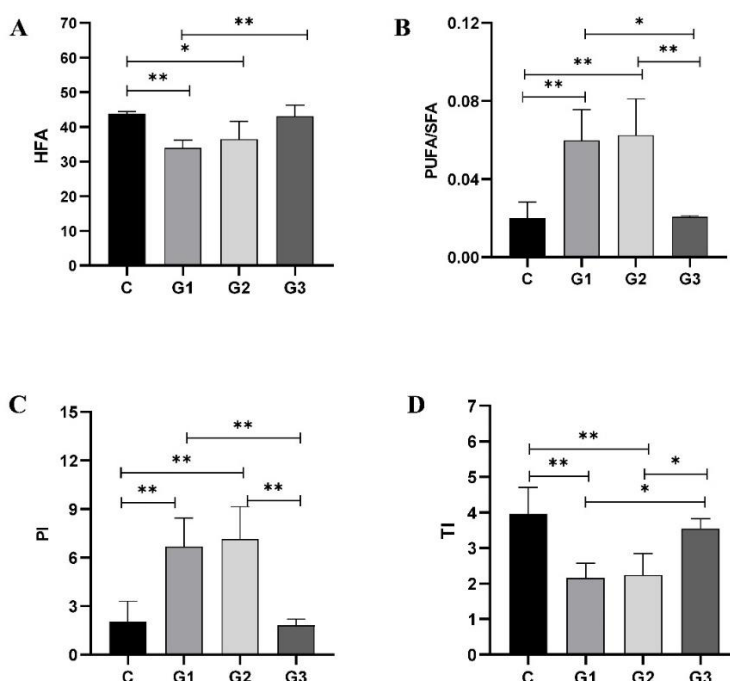


Figure 1. Differences between lipid health indices of Turcana sheep milk ($n = 15/\text{group}$) according to different dietary experimental treatments represented as: C = control group; G1 = 12 g/sheep/day of protected fat/Optima 100 product; G2 = Endulac®-CLA at 12 g/sheep/day; G3 = 12 g/sheep/day of Optima 100 product and Endulac® CLA; **A**) HFA: hypercholesterolaemic fatty acids (C12: 0 + C14: 0 + C16: 0); **B**) PUFA/SFA: polyunsaturated/saturated fatty acids ratio; **C**) PI - the polyunsaturation index [C18: 2 n-6 + (C18: 3 n-3 × 2)]; **D**) TI - thrombogenic index [(C14:0+C16:0+C18:0) / [0.5 × ΣMUFA+0.5 × Σ(n-6)+3 × Σ(n-3)+Σ(n-3) / Σ(n-6)]]; The variables were analysed using the Tukey test and are marked as: * significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$; ns - not significant

The effects of dietary sources of protected fat and CLA on the saturated and the unsaturated fatty acid composition of sheep milk are shown in Figures 2 and 3. The experimental groups, G1 and G2, which received the basal diet supplemented with protected fat and CLA, respectively, displayed a substantially decreased saturated fatty acid profile than the control group and G3. A decreasing trend was observed for myristic acid (C14:0) and pentadecanoic acid (C15:0) (Figure 2A, B). G3 (basal diet + Optima 100 and Endulac®-CLA) had an unchanged saturated fatty acid pattern that was very similar to the control group, possibly due to antagonistic effects of both supplements on the milk SFA profile.

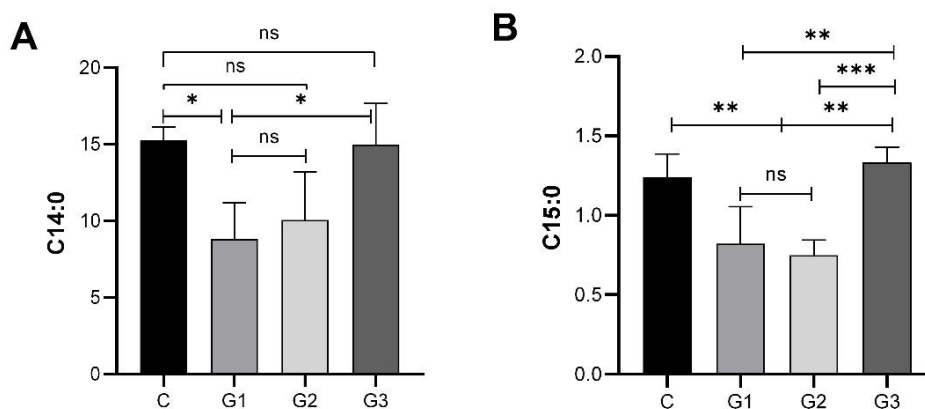


Figure 2. Saturated fatty acid profiles from the different dietary treatments after 60 days. Treatments: C = control group; G1 = 12 g/sheep/day of protected fat/ Optima 100 product; G2 = CLA at 12 g/sheep/day; G3 = 12 g/sheep/day of Optima 100 product and Endulac®-CLA; A) Myristic acid (C14:0); B) Pentadecanoic acid (C15:0). Mean values \pm standard deviation ($n = 10$); statistically significant differences among experimental groups for each saturated fatty acid variable obtained using the Tukey test are marked as: * significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$; ns - not significant

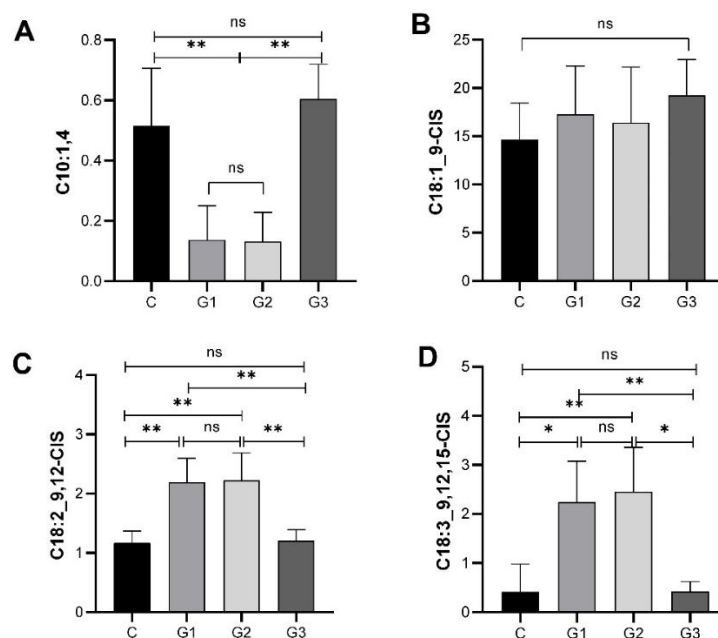


Figure 3. Unsaturated fatty acid profiles of Turcana sheep milk after 60 d on different dietary treatments. Treatments: C = control group; G1 = 12 g/sheep/day of protected fat/ Optima 100 product; G2 = Endulac®- CLA at 12 g/sheep/day; G3 = 12 g/sheep/day of Optima 100 product and CLA; A) Decanoic acid (C10:1,4); B) Oleic acid (C18:1 9-CIS); C) Linoleic acid (C18:2 9, 12-CIS); D) Linolenic acid (C18:3 9, 12, 15 CIS). Mean values \pm standard deviation ($n = 10$); statistically significant differences among experimental groups for each unsaturated fatty acid variable obtained using the Tukey test are marked as: * significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$; ns - not significant

A major decreasing trend was observed for decanoic acid (C10:1, 4), which showed an approximately three-fold reduction (Figure 3, A). The fortification of sheep diets by respective supplementation of Optima 100 (G1) and CLA (G2) led to a substantial elevation of the unsaturated fatty acid profile of milk, especially in linoleic acid (C18:2 9, 12-CIS) and linolenic acid (C18:3 9, 12, 15 CIS) (Figure 3C, D). The oleic acid (C18:1 9-CIS) fraction and saturated fatty acid content of the milk was similar among groups (Figure 3, B). The saturated fatty acid milk content remained similar after the animals received Optima 100 and CLA; G3 had similar contents of linoleic acid (C18:2 9, 12-CIS) and linolenic acid (C18:3 9, 12, 15 CIS) to the control group. In contrast to the control and G3, the greatest increases in linolenic acid (C18:3 9, 12, 15 CIS) with an approximately five-fold increase, was detected in G1 and G2; linoleic acid (C18:2 9, 12-CIS) content was increased approximately two-fold.

The impact of the protected fat and CLA dietary inclusion on the performance parameters of ewes are displayed in Table 6. None of the experimental treatments caused any significant differences in performance parameters such as feed intake, NEL, and BW change (including initial and final body weight). However, performance indices responsible for milk production varied substantially. The most valuable elevation over the control was in G1, which was subjected to 12 g/sheep/day of protected fat source, followed by G3, which received 12 g/sheep/day of protected fat and CLA. Substantial increases in overall milk production (L), including milk production per day/period, and feed/milk/daily intake were found in G1 and G3.

Table 6 Effect of the addition of protected fat and conjugated linoleic acid to the performance of dairy sheep

Specification	Control	Group1	Group 2	Group 3	P-value
Number of sheep (n)	15	15	15	15	-
Feed intake, kg DM/sheep/day	1.78	1.8	1.79	1.77	-
NEL (MJ/day)	10.57	11.59	11.08	10.5	-
Initial BW (kg)	49.2 ^a	49.5 ^a	49.3 ^a	49.6 ^a	0.057
Final BW (kg)	49.6 ^a	51.2 ^a	49.8 ^a	51.1 ^a	0.065
BW change(kg)	0.4 ^a	0.7 ^a	0.5 ^a	0.5 ^a	0.058
Milk production (L)	73.813 ^a	96.923 ^b	79.066 ^a	89.116 ^c	0.000
Milk production/day/period (L)	1.18 ^a	1.56 ^b	1.27 ^a	1.44 ^c	0.000
Feed/milk(daily intake/daily milk)	1.5	1.15	1.40	1.22	-

Mean values are reported for (n = 15) animals per group. Statistically significant differences among experimental groups for each performance-related parameter obtained using the Tukey test are indicated by different lowercase letters. DM, dry matter; NEL, net energy for lactation according to INRA (2018), Noziere *et al.* (2018); BW, body weight.

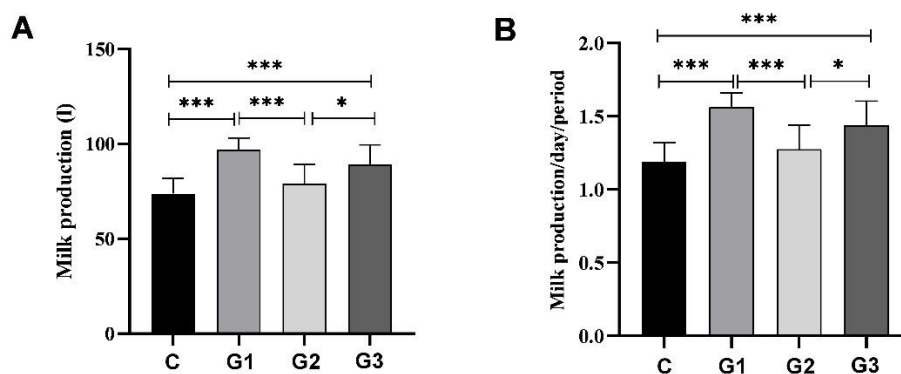


Figure 4. Evaluation of milk production parameters according to different dietary treatments after 60 days. Treatments: C = control group; G1 = 12 g/sheep/day of protected fats/ Optima 100 product; G2 = CLA/Endulac®-CLA at 12 g/sheep/day; G3 = 12 g/sheep/day of protected fats/Optima 100 product and CLA/Endulac®-CLA; A) Milk production (L) B) Milk production/day/period (L); Statistically significant differences among experimental groups obtained using the Tukey test are marked as : * significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$; ns - not significant

Discussion

Changes in animal diets can markedly decrease the saturated fatty acid content and increase the presence of bioactive compounds in milk (Serafeimidou *et al.*, 2013). In the present study, we described the impact of ruminally-protected fats (Optima 100) and CLA (Endulac®- CLA sources) on the fatty acid composition of ewe milk. Using similar approaches, Mierliță (2018) tested the addition of hempseed and hempseed cake in the diets of Turcana dairy ewes and observed a significant reduction in C14:0 and elevation of C18:2 n-6 in milk (Mierlita, 2018). However, our observations indicated increased concentrations of C15:0 and lower C18:1 9-CIS concentrations. The above study showed that compared to the control, hemp feeding increased the content of PUFA, such as n-3 and C9: t11 CLA in milk and improved the hypocholesterolaemic-hypercholesterolaemic ratio. Other similar findings demonstrated that hempseed (180 g/day), and hemp seed cake (480 g/day) dietary supplementation increased C18:1, C18:3 omega-3, MUFA, and PUFA, and drastically declined SFA in Turcana sheep milk (Mierlita, 2018). The authors specified that the increase in C18:1 isomers after hemp incorporation was achieved due to the increase in the ruminal biohydrogenation process. Increases in C12:0, C14:0, and C16:0 FA profiles are considered atherogenic, whilst C14:0 and C16:0 are thrombogenic factors (Ulbricht & Southgate, 1991; Mierlita, 2018).

East Friesian sheep that received isomerized poppy seed oil enriched with conjugated dienes of linoleic acid (CLA) over 4 w had a marked decline in the concentration of saturated fatty acids (e.g., C:12, C:14, and C:16) and an increase in the content of biologically active, pro-healthy fatty acids such as C18:1 t11; C18:2 c9,t11; and C18:2 t10,c12, including the total content of MUFAs and PUFAs in milk (Bodkowski *et al.*, 2020). The authors have described a similar observed spectrum of variations in milk fatty acid profiles in Polish Holstein–Friesian cows (Bodkowski *et al.*, 2020). It was mentioned that East Friesian sheep, and Polish Holstein–Friesian cows displayed substantial declines in overall fat content, SCFAs, MCFAs, and AI in the milk (Bodkowski *et al.*, 2020). Simultaneously, the milk Σ CLA, and C18:0 showed a tendency to increase, compared with the control animals (Bodkowski *et al.*, 2020).

Other studies have provided evidence that protected fat can be used to raise the beneficial fraction of unsaturated fatty acids and drop the content of unwanted saturated fatty acids in meat (Behan *et al.*, 2021). An example was illustrated in Dorper sheep supplemented with rumen bypass fats such as prilled fat or prilled fat combined with lecithin and calcium soap of palm fatty acids, which partially reduced C14:0 and C15:0 concentrations and increased C18:1 9-CIS in the *longissimus dorsi et lumborum* muscle and liver (Behan *et al.*, 2021). Ruminally-protected fat, such as that derived from palm oil, was responsible for positively affecting the weight gain and body performance of lactating Lacaune dairy sheep at lower concentrations of 20 g/kg (Bianchi *et al.*, 2018). The authors observed that a higher concentration (40 and 60 g/kg) of ruminally-protected fat from palm oil produced the worst body weight and body condition score. In addition, a higher concentration of palm oil was shown to enrich milk with a higher fat fraction but decreased the content of lactose, protein, and solid non-fats. In contrast to our findings, a recent study reported that Awassi and Awassi x East Friesian cross-breeds ewes supplemented with rumen-protected pellets fortified by EPA and DHA had a substantially lower proportion of C18:1 9-CIS. EPA and DHA-protected pellets were concluded to efficiently raise n3 LC-PUFA, such as EPA, DHA, and DPA (Nguyen *et al.*, 2018). The rich spectrum of PUFAs in milk products is desirable for modern consumers due to the enriched nutritional quality. However, in some cases, increasing the levels of milk PUFAs can make dairy products prone to oxidation (Mierlita, 2018).

Dairy ewes receiving diets supplemented with wheat-based pellets infused with flaxseed and safflower pellets had a lower mean concentration of Σ SFA (Nguyen *et al.*, 2018). Compared to our study, the values of Σ MUFA were slightly improved after lactating ewes received canola oil and flaxseed pellets, while rice bran and rumen-protected pellet supplementation appeared to reduce the Σ MUFA from milk (Nguyen *et al.*, 2018). In the case of Σ PUFA of milk, the study showed similar increases after supplementation with rice bran, flaxseed, and safflower-enriched, wheat-based pellets. Analogous substantial increases were observed in the milk Σ PUFA content, especially for the groups that received safflower, flaxseed, and rumen-protected pellets. In contrast to our findings, the fatty acid profile of C18:0 appeared to cause a reduction after dietary treatment with wheat-based pellets infused with canola oil, rice bran, safflower, and rumen-protected pellets, while our results showed a gain. In the case of the C14:0 profile, the authors reported elevations after following dietary supplementation with canola oil, rice bran, safflower and rumen-protected pellets, while our milk C14:0 profile tended to decrease following protected fat and CLA supplementation (Nguyen *et al.*, 2018). Flaxseed feed supplementation to Sarda ewes presented similar reductions in C12:0, C14:0, SFA, and n-6/n-3 ratio

and increases in C18:0, Σ MUFA, PUFA, and n-3 FA % compared to our results (Serra *et al.*, 2018). In our study, both dietary treatments regulated the lipid health profile of milk by considerably decreasing the levels of HFA, AI, and TI and increasing PI and PUFA/SFA ratios compared with the control milk. Others have reported that hemp provoked a reduction in lipid health parameters, such as HFA and n6/n-3 FA, as well increased the hFA, h/H, PUFA/SFA, and MUFA/SFA ratios (Mierlita, 2018). Our findings were similar to Hilali *et al.* (2018), who showed that the dietary supplementation of Awassi sheep with cottonseed cake improved milk lipid quality parameters by elevating n-6 FA and reducing TI and AI levels (Hilali *et al.*, 2018). In a previous study, pellets infused with flaxseed oil showed reduced AI and TI indices, which is in line with our experimental observations (Nguyen *et al.*, 2018). The authors showed that the PUFA/SFA ratio increased after the intake of pellets infused with flaxseed oil, safflower, and rumen-protected pellets by lactating ewes (Nguyen *et al.*, 2018). In the current study, the most efficient treatment to impact sheep performance parameters was related to the dietary administration of protected fat, which resulted in the increase of milk yield of ewes from G1, probably due to the increased dietary energy availability.

Conclusions

The current study investigated the impact of sources of dietary fat addition on the quality of sheep milk, an effect previously observed in dairy cow's milk. Furthermore, our study did not detect any significant differences in body weight indices between the dietary treatments. The absence of an effect of body weight was previously demonstrated and it was concluded that providing rumen-protected fats demonstrated no impact on animal performance. However, earlier studies concluded that concentrations of 20 g.kg⁻¹ of protected fats from palm oil caused positive outcomes on weight gain and body condition; higher concentrations of 60 g.kg⁻¹ enhanced milk production and fat composition in lactating Lacaune ewes. The inclusion of protected fats and CLA in Turcana ewe diets substantially improved the quantity of milk and the fatty acid profile of the milk with a direct impact on suckling lamb performance as well as indices of consumer health.

Author Contributions

Conceptualization, L.S., N.C., D.S., T.C.; Data curation, L.S., N.C.; Formal analysis, I.B., L.S., E.S.; Laboratory analysis, E.A., A.M., I.P.; Investigation, A.G., C.J., S.V.; Statistical Processing, E.S., S.V.; Methodology, A.G., I.B., C.J., T.C.; Resources, L.S.; Writing – original draft, I.B., N.C., L.S.; Writing – review & editing, I.B., N.C., L.S., D.S.

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Conflicts of 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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