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Effect of Dietary Olive Leaf Integration on Qualitative Characteristics of Sheep Cheese During Ripening

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Abstract: Olive leaf by-products may be an important feed source for ruminants in the Mediterranean area, due to their nutritional value and high levels of functional metabolites. Additionally, their use can enhance the environmental and economic sustainability of the productions. To evaluate the effect of olive leaf supplementation on the fatty acid profile of sheep cheese, two farms with Comisana breed sheep with free access to pasture and fedwith 300 g/head/day of concentrate were considered. One farm supplemented the feed with clover hay ad libitum (NOL) and the other farm replaced hay with olive leaves (OLI) in the autumn period. Cheese analyses were performed at 15, 30, and 60 days of ripening. Saturated fatty acids were lower in OLI cheese than NOL cheese, while MUFA and PUFA n-3 and n-6 were higher in OLI cheese. Myristic acid (C14:0) and palmitic acid (C16:0) were lower in OLI cheese compared to NOL (8.31% vs. 8.90% and 21.52% vs. 24.95%, respectively), while oleic acid (C18:1 cis-9) was higher in OLI cheese (20.66% vs. 18.78%). Also, CLA cis-9 trans-11 (0.98% vs. 0.84%), and other isomers were higher in OLI cheese. Health indexes, such as the thrombogenic and atherogenic index, were lower in OLI than in NOL cheese (1.96 vs. 2.38 and 1.69 vs. 2.05, respectively) showing the improvement in the health quality of cheese due to olive leaf integration in directly on farm sheep feeding.

Keywords: olive leaf by-products; sheep cheese; fatty acids; ripening

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1. Introduction

Sheep production in many Mediterranean countries is mainly characterised by extensive and semi-extensive systems, where meadows and pastures are the main components of the diet. Due to their frugality, these animals can also consume by-products, including those rich in fibre and phenolic compounds (tannins), such as olive leaves [1].

Italy is one of the main producers of olive oil in the world and central-southern Italy, where dairy ewes are mainly reared, has the highest concentration of olive groves [2]. Olive oil holds a pivotal role in the Mediterranean diet, recognising its positive impact on human health due to its essential fatty acids and antioxidant content. The production chain of olive oil generates significant waste, including olive pulp, stones, leaves, branches, and other by-products. To estimate the amount of waste generated in oil production, we refer to a study cited in Espeso et al. [2], which documented a leaf production rate of approximately 6.23 kg per litre of oil. These by-products offer opportunities for diverse applications in the pharmaceutical industry because they are rich in different bioactive molecules such as anthocyanins, tannins, flavonoids, and dietary fibre (pectin), but also as livestock diet supplementation [3]. Nowadays it becomes fundamental to valorise the by-products of the food industry, like olive leaves, which contain high levels of functional metabolites, but

also to reduce the environmental impact of livestock farming, highlighting the principle of the "circular economy" as a strategic approach [4].

Furthermore, olive by-products contain elevated levels of polyunsaturated fatty acids such as α-linolenic and linoleic acids as well as being very rich in oleic acid, and their inclusion in small ruminant diet leads to changes in the fatty acid profile of milk, due to their effect on rumen and mammary gland metabolism [5]. Recent studies have investigated the impact of olive leaves on cheese quality in experimental groups of goats [6] and sheep [7]. However, for years, the breeders of small ruminants have supplemented the diets of their animals with olive leaves obtained as by-products from oil mills or as pruning residues, but there are few studies about the impact of olive leaf supplementation on sheep cheese quality, as noted by Bolletta et al. [7]. Even fewer studies have been conducted supplementing the sheep's feed with fresh olive leaves directly on farms, where numerous variables, some of which are random and difficult to measure, can influence the outcomes.

The use of olive leaves, treated in various ways, presents both advantages and disadvantages depending on the method employed. Specifically, dried and ensiled leaves allow for the extended use of this by-product during periods far from the harvest season, prolonging its availability [3]. Additionally, the pelleted form minimises livestock's feed waste, improving the efficiency of its utilisation [7]. Fresh leaves, on the other hand, offer a significant benefit: they preserve the concentration of tocopherols and bioactive compounds [4], and can provide animals with green forage that stimulates ruminal bacterial activity and increases nutritional value [3]. This process promotes the transfer of highly nutritious molecules into the milk, such as trans isomers of oleic acid and conjugated linoleic acids (CLA), which are well-known for their health benefits in both humans and animals. Thus, different treatments and uses of olive oil by-products result in varying outcomes for the production and quality of animal-derived products [8].

The aim of this study was to assess the effects of diet supplementation with more 10% of ration of not dried olive leaf by-product on the quality of sheep's cheese, specifically its fatty acid composition. The milk used in the study was obtained from two farms where animals were traditionally raised on pasture, and one of these used olive leaves instead of hay in the diet of lactating sheep during the autumn season, without introducing any practices beyond those commonly used on the farms.

The possibility of adding the leaves to the ration could be a sustainable solution to valorise this waste biomass and reduce the costs of feeding the animals. Furthermore, the use of fresh olive leaves on a farm located in the same area where olive oil was produced could also reduce transport and processing costs to preserve the product. Evaluating the impact of integrating olive leaves under authentic farming conditions allows for the consideration of random effects occurring in real-life situations involving a significant number of animals. Consequently, this evaluation enables us to define the quality of cheese that could be used on the market as a sustainable product.

2. Materials and Methods

2.1. Animals and Diets

The study was carried out from October to November, involving multiparous Comisana breed sheep from two adjacent farms in central Italy. These farms originally belonged to the same company before being divided into two separate entities, both with identical breeding systems. During the trial period, most of the animals were in the early stage of lactation in both farms.

The two farms were part of the regional 'Milkability Programme', provided by the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana (IZSLT) "Mariano Aleandri" (National reference centre for the quality of milk and sheep and goat milk products (C.Re.L.D.O.C.)), which monitors dairy species using the Lactocorder® electronic milk meter (WMB, Balgach, Switzerland) to record individual milk yield, mechanical milking time and other parameters characteristic [9,10]. The average individual milk yield recorded be-

fore the start of the experimental period for daily milking sessions was 0.76 ± 0.19 kg/day vs. 0.78 ± 0.16 kg/day for sheep, with no differences between the two farms (p = 0.736).

Microbiological analysis (EC Regulation 853/04) and milk quality analysis (Italian Ministerial Decree 26 August 2021—Compulsory declarations in the sheep and goat dairy sector) were carried out periodically for bulk tank milk, from at least two consecutive milkings, in the laboratories of the IZSLT (accredited by Accredia, according to the standard of the ISO/IEC 17025:2005 [11]). In the control carried out before the beginning of the experimental period, the total bacterial count was 329 vs. 567 ufc/mL of milk, comparing the two farms. In the same control, milk quality parameters were similar between the two farms with a fat content of 7.64% vs. 7.65% and a protein content of 6.52% vs. 6.69%. From 9:00 am to 4:00 pm, the animals from both farms had free access to the same pasture, constituted of 60% of Leguminosae (*Medicago sativa*) and 40% of Gramineae (*Lolium multiflorum*). The pasture was integrated in the manger with 300 g/head/day of concentrate (barley and protein peas 70:30%). One farm supplemented the feed with clover hay ad libitum without olive leaves (NOL), while the other farm replaced hay with olive leaves (OLI) ad libitum in the autumn period. The olive leaf by-products were obtained from an olive mill near the two farms; they came from organic farms and were not treated with chemical agents. About 300 kg of leaves were delivered from the olive mill every three days for approximately 75 sheep and fed to the animals daily with a residue of about 5% (principally composed of twigs), showing good palatability. Instead, the farm that did not use olive leaves consumed a round bale of hay (360 kg) every 5 days for approximately 90 sheep. Fresh leaves can contribute to the ration in a greater proportion as they have a higher nutritional value than dried products.

Samples were collected from the pasture every 15 days in four different areas of about 2.5 m^2 (two for the part of pasture where each farm resides), the areas were fenced off to prevent access by the animals. The concentrate, clover hay, and olive leaves were collected twice. All feed samples were dried at 65 °C in a ventilation oven, milled through a 1 mm screen, and stored until chemical analysis. Analyses were conducted to determine the dry matter (DM, method 934.01), crude protein (CP, method 984.13), ether extract (EE, method 920.39) and ash (method 942.05) according to the AOAC (2006) [12]. The concentrations of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed using the method of Van Soest et al. [13]. The fatty acid profile was determined by the lipid extract as described in detail in the cheese analyses section.

Chemical composition of diet components is reported in Table 1.

Table 1. Chemical composition (g/100 g of dry matter) and fatty acids composition (percentage of total FAME [1]) of diet components.

Parameters	Barley Grain	Protein Peas	Pasture	Clover Hay	Olive Leaves
Dry Matter [2] %	88.2	88.2	22.7	84.3	57.9
Crude protein %	12.7	25.3	15.3	17.1	11.1
Ether Extract %	2.15	2.94	3.17	1.82	5.31
Ash %	2.48	3.19	8.12	12.2	6.12
NDF [3] %	21.7	13.6	58.9	51.1	38.1
ADF [3] %	8.53	9.41	35.7	34.8	24.8
ADL [3] %	1.6	1.52	6.51	7.85	6.9
Fatty acids					
14:00	1.19	1.36	1.06	3.12	2.41
16:00	18.45	14.27	11.94	28.94	20.3
16:01	1.04	0.73	0.69	2.07	0.85

Table 1. *Cont.*

Parameters	Barley Grain	Protein Peas	Pasture	Clover Hay	Olive Leaves
18:00	2.51	8.93	2.34	6.18	5.16
18:1 *cis*-9	18.32	16.56	3.13	17.94	21.76
18:2 n-6	50.13	48.94	14.48	15.29	8.77
18:3 n-3	5.24	6.87	63.29	22.84	37.06

[1] FAME = Fatty acids methyl ester; [2] percentage on original substance; [3] fibre components NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL= acid detergent lignin.

2.2. Cheese Production and Sampling

One of the two farms houses the company's dairy, where both farms deliver their milk. The milk was collected twice a day from the two farms and processed each day.

The proximate composition and main classes of fatty acids of the cheese at 60 days of maturation produced before the diet diversification were reported in Supplementary Table S1, and they did not show significant differences between the two farms.

For this study, three manufacturing processes were considered. The milk from each farm was processed separately on the first day of the week for three consecutive weeks. The cheese from the two farms was made separately approximately 20 days after the introduction of olive leaves into the diet.

For each batch, approximately 70 L of raw milk were processed daily. The milk was heated to 45 °C for 10 min, cooled to 38 °C, and then vegetable rennet was added (aqueous solution at 1% *v*/*v*), without using starter cultures. Vegetable coagulant was obtained by the maceration of dried flowers of *Cynara cardunculus* L. (50 g/100 L of milk) in tap water at room temperature overnight and subsequently filtered. Curd formation occurred within approximately 40–50 min. The curd was then ground for 3–5 min into pieces as small as wheat grains and left to settle for 10 min. Subsequently, the curd was transferred into molds and manually pressed. The cheese forms were allowed to dry at room temperature for about 10 h, during which they were regularly inverted. The cheese was then salted in brine for 24 h and placed in climate-controlled cells (12 ± 1 °C; 80%, respectively, for temperature and humidity). The final pH for both cheese types was 5.05 ± 0.03.

For each manufacturing process, cheese forms weighing approximately 800 g each were made from the two different types of milk obtaining cheese from the farm with hay supplementation (NOL) and cheese from the farm with olive leaf supplementation (OLI).

After reaching maturation times of 15, 30, and 60 days, the cheeses were transported to the laboratory to evaluate the physical and chemical composition, lipid oxidation, and fatty acid content. Nine shapes for each type of milk were sampled in three different ageing times for a total of 18 shapes (3 processes × 3 times of maturation × 2 types of milk).

All analyses were conducted in duplicate for each cheese shape after removing two centimetres of surface layer. Physical determination and proximate composition were performed on non-frozen samples, while the analysis of fatty acids was performed on samples frozen at −80 °C.

2.3. Physical and Chemical Analysis of Cheese

The shear force (SF) on cheese samples was measured using a Kramer apparatus as a dynamometer (Instron 1011) with a 1.5 cm^3 thick sample cube, crosshead speed was set at 2.5 cm/min [14]. Colours were estimated by L* (lightness), a* (redness), and b* (yellowness) with D illuminant, using the Konica Minolta CM-3006 in the CIELAB system (International Commission on Illumination CIE-L*a*b*) [15]. The mean of six measurements was used to obtain the colour data for each sample.

Dry matter, ash, fat, and protein content were determined according to AOAC (2006) [12]. Lipid oxidation was analysed using malondialdehyde (MDA) quantifications. MDA is a principal result of lipid oxidation process. The procedure was performed using a thiobarbituric acid reactive substance (TBARS) assay following the method described in Rinaldi et al. [16], using a Water Alliance HPLC instrument on Zorbax Plus C18 column

(250 mm × 4.6 mm) with isocratic phase, flow rate 1 mL/min and read in fluorescence with ex 515 nm and em 543 nm. The MDA sample peak was identified by confronting the peak of MDA standard peak and TBARS were expressed as mg MDA/kg.

The total fat was extracted according to Folch et al.'s [17] procedure, and the lipids were methylated by adding 2 N methanolic potassium hydroxide (IUPAC 1987) [18]. The fatty acid methyl esters (FAME) were quantified using a gas-chromatography (GC 6890N Agilent, Inc., Santa Clara, CA, USA) instrument equipped with a flame ionization detector (FID) and a CP-Sil88 fused silica capillary column (100 m, 0.25 mm internal diameter with 0.2 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Gas chromatograph conditions were set as reported by Rinaldi et al. [19]. Internal standards (19:0 and 23:0) were added to the samples before the fat extraction.

To analyse the different cis/trans isomers, SPE Cartridge Ag-ion was performed on 1 mg of FAME. The FAME sample was loaded into an Ag-ion SPE tube (Supelco, Merck KGaA, Darmstadt, Germany), and following the SPE sheet procedure the cis/trans isomers and conjugated linoleic acids (CLA) were separated; after that, each extraction was injected in GC with the same procedure for total FAME. The separation chromatograms of 18:1 isomers, C18:2 isomers, and CLA are reported in Figure S1 as Supplementary Data.

Fatty acid methyl esters were identified by comparing their peak retention times of each compound with standard peaks from Supelco mix 37, CLA mix (Sigma-Aldrich Merck, Darmstadt, Germany) and a mixture of branched chain fatty acids of BR2 and BR4 (Larodan, Solna, Sweden). Other peaks of the chromatogram obtained by Kramer et al. [20] with the same column were used to identify other peaks of isomers and CLA.

The different classes of fatty acids as the amount of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), and their subclasses as the sum of branch, odd, cis/trans isomers, and CLA isomers were expressed as percentages of total FAME. Furthermore, the atherogenic index (Ai) and thrombogenic index (Ti) were calculated with the equation proposed by Ulbricht and Southgate [21].

2.4. Statistical Analysis

The analyses of data were performed using PROC GLM procedure of SAS (SAS Inst. Inc., Cary, NC, USA, 2011) [22], with a bi-factorial model with interaction:

$$Yijk = \mu + \text{feeding} + \text{ripening} + (\text{feeding} \times \text{ripening})\ ij + \varepsilon ijk,$$

where µ = general mean; ε= residual error. Tukey's test with $p < 0.05$ set as a significant level among the factors.

3. Results and Discussion

3.1. Proximate Composition of Cheese

The proximate composition and physical analyses of sheep's cheese at 15, 30, and 60 days of ripening are shown in Table 2. Both the diets and the ripening times influenced the chemical and physical attributes of cheeses.

Cheese from OLI group reported a significantly lower percentage of dry matter (DM) than NOL cheese ($p < 0.001$) and consequently also showed lower percentage of ash, fat and protein. The protein percentage was significantly lower in OLI than NOL cheese at 30 and 60 days ($p < 0.001$ for both times), by contrast, fat percentage was lower at 15 ($p < 0.01$) and 30 days ($p < 0.05$) compared to NOL, not showing significant differences at 60 days despite showing lower values.

The differences in moisture between the two types of cheese may be attributed to the different curds ability to retain whey during the cheesemaking process. However, several factors influence the curd's capacity to retain liquid and subsequently in the cheese. These factors also impact the transformation of chemical constituents over time through proteolysis and lipolysis, which are further modulated by the presence of compounds with antioxidant properties [23].

Table 2. Proximate composition and physical parameters of cheese obtained from ewes fed in two different ways.

Parameters	15 Days			30 Days			60 Days			RMSE	*p*-Value Time
	NOL	OLI	Sign	NOL	OLI	Sign	NOL	OLI	Sign		
DM (%)	57.9	55.9	***	67.4	64.5	***	68.9	65.9	***	0.62	<0.001
Ash (%)	3.7	3.4	*	4.3	4.2	ns	4.5	4.3	ns	0.19	<0.001
Fat (%)	28.6	27.4	**	32.2	31.5	*	33.4	32.9	ns	0.72	<0.001
Protein (%)	24.5	24.2	ns	30.2	27.8	***	30.5	28.3	***	0.74	<0.001
TBARS (mg/kg)	0.06	0.06	ns	0.07	0.06	ns	0.11	0.09	**	0.01	<0.008
SF (kg)	1.72	1.62	ns	2.32	2.09	ns	3.31	3.19	ns	0.43	<0.001
L*	82.0	88.2	***	71.7	70.5	ns	67.6	67.1	ns	2.19	<0.001
a*	−2.6	−2.5	ns	−3.1	−2.5	***	−3.1	−3.0	ns	0.17	<0.001
b*	10.9	10.3	ns	11.7	10.3	***	10.7	11.7	**	0.49	0.016

NOL = cheese from ewes fed without olive leaves; OLI = cheese from ewes fed with olive leaves. Time = ripening of cheese at different times; RMSE = root means square error. *, **, *** = significantly different for $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; ns = not significant. DM = Dry matter; SF = Shear force; L* = lightness; a* = redness; b* = yellowness; TBARS = substance of lipid oxidation that reacts with thiobarbituric acid expressed in mg MDA/kg (MDA = malondialdehyde).

Nevertheless, the impact of replacing ruminant feed with olive by-products on the proximate composition of cheese remains a topic of debate. Some authors have reported significant differences in the chemical characteristics of cheese when olive leaves were integrated into the ruminant diet [6,7], while other authors have found no differences in these parameters when olive by-products were included in the feed of sheep [24]. Our data disagree with Bolletta et al. [7], who reported an increase in DM and fat content in cheese produced from animals fed with a diet including olive leaves, while ash and protein levels remained unchanged. These results probably depend on the different diet composition, since in this study the olive leaves were added ad libitum in the diet as a replacement for hay, while in Bolletta et al. [7] 28% of olive leaves were replaced in the concentrate as a dry component.

The non-significant differences in fat percentage between NOL and OIL cheese at 60 days of ripening (despite a lower DM in OLI cheese) could probably depend on greater fat lipolysis and oxidation in NOL cheese during ripening. The lower fat oxidation at 60 days in OLI cheese also showed a significantly ($p < 0.01$) lower TBARS value compared to NOL cheese (0.09 vs. 0.11 mg MDA/kg of cheese, respectively), while no differences in TBARS were reported at 15 and 30 days of ripening (Table 2). Innosa et al. [6] also reported a lower MDA amount in cheese obtained from goats fed a dietary olive leaf supplementation. The presence of bioactive compounds such as phenols, carotenoids, and tocopherols in olive leaves increased the antioxidant capacity in milk with a subsequent transfer to the cheese [25]. Moreover, the level of MDA in our cheeses was very low (reaching a maximum of 0.11 mg MDA/kg cheese at 60 days of ripening) probably because even the cheese from sheep without olive leaf integration showed good oxidative stability due to bioactive compounds obtained from grazing. For this reason, no significant differences were found in oxidation levels at 15 and 30 days of ripening.

3.2. Physical Parameters of Cheese

The texture of cheese is an important parameter because it is linked to the casein content and manufacturing [26]. The shear force (SF) did not show significant differences between the feed treatments, while the ageing increased the SF of the cheese ($p < 0.001$, Table 2), due to loss in moisture [26].

The colour indexes of the cheeses were affected by olive leaf supplementation. In particular, the cheese appeared lighter in OLI group than NOL (L* 88.18 vs. 82.00 respectively, $p < 0.001$) but only at 15 days, while the red index and yellow index did not show significant differences at this time because of the low maturation of cheese. After 15 days, the redness was greater in NOL group than OLI ($p < 0.001$), while at 60 days this significant

difference disappeared. The yellow index differed between the two groups both at 30 and 60 days, but in different ways. In fact, at 30 days NOL group had greater yellowness, while at 60 days OLI group showed a higher value than NOL. The yellow index, like the other colour parameters, depends on the maturation of the cheese, but also on some compounds present in milk (like carotenoids in particular xanthophylls), which become concentrated with dehydration [27]. Furthermore, over time these compounds can undergo oxidation processes, transforming into other compounds [6]. These processes were probably the cause of the fluctuating behaviour of the yellow index in the two cheeses. In fact, over time the NOL cheese reached peak yellowness at 30 days and then this parameter degraded, while the OLI cheese presumably reached the peak at 60 days, showing greater preservation of the colour due to the presence of antioxidant compounds.

The physical-chemical characteristics of cheeses are the result of the interaction of several factors, including the properties of the milk and the conditions during production and ageing [23]. Compared to cow's milk, the milk of ewes and goats contains mainly retinol and xanthophylls, and generally lower β-carotene due to a higher efficiency of conversion of carotenoids, such as β-carotene, into retinal [27]. Then, milk carotenoids are transferred into cheeses with minimal losses during their production [6].

3.3. Content of Principal Saturated Fatty Acids

The inclusion of olive leaves to the diet instead of clover hay influenced the content of fatty acids (FA) in cheese but these differences also arise due to ripening time (Table 3).

Table 3. Principal saturated fatty acids (% of total FAME) of cheese obtained from ewes fed in two different ways.

%	15 Days			30 Days			60 Days			RMSE	*p*-Value Time
	NOL	OLI	Sign	NOL	OLI	Sign	NOL	OLI	Sign		
C4:0	3.65	3.44	ns	3.61	3.87	ns	3.55	3.98	ns	0.67	0.686
C6:0	2.01	2.04	ns	2.01	2.05	ns	1.99	1.98	ns	0.23	0.867
C8:0	1.83	1.89	ns	1.83	1.91	ns	1.79	1.90	ns	0.21	0.970
C10:0	4.99	4.84	ns	4.86	4.71	ns	4.76	4.60	ns	0.32	0.211
∑SFA low chain	12.47	12.29	ns	12.10	12.21	ns	12.53	12.44	ns	0.94	0.936
C15:0	0.95	0.95	ns	0.96	1.07	**	0.92	1.05	***	0.08	0.146
C17:0	0.56	0.65	***	0.58	0.72	***	0.57	0.73	***	0.04	0.023
∑SFA Odd	2.06	2.13	ns	2.06	2.32	**	2.02	2.31	***	0.13	0.236
C15:0 *anteiso*	0.51	0.53	ns	0.52	0.55	ns	0.52	0.54	ns	0.04	0.642
C17:0 *iso*	0.64	0.68	ns	0.62	0.73	**	0.63	0.72	**	0.05	0.547
∑SFA Branch	2.36	2.47	ns	2.33	2.69	***	2.33	2.66	***	0.14	0.219
C12:0	2.87	2.89	ns	2.93	2.93	ns	2.94	2.97	ns	0.20	0.585
C14:0	8.90	8.69	ns	9.06	8.26	**	8.74	7.99	**	0.49	0.109
C16:0	25.29	21.96	***	24.84	21.04	***	24.71	21.56	***	1.36	0.455
C18:0	13.32	13.56	ns	13.47	12.99	ns	13.05	13.15	ns	0.86	0.630
∑SFA	67.69	64.39	***	67.47	63.25	***	66.39	63.57	***	1.30	0.144

FAME = Fatty acids methyl ester; Fatty acids more than 0.5% of total FAME were reported. NOL = cheese from ewes fed without olive leaves; OLI = cheese from ewes fed with olive leaves. Time = ripening of cheese at different times; RMSE = root means square error. *, **, *** = significantly different for $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; ns = not significant. ∑SFA low chain = sum of saturated fatty acids from C4:0 to C10:0; ∑SFA Odd = sum of odd saturated fatty acids; ∑SFA Branch = sum of branch saturated fatty acids; ∑SFA = total saturated fatty acids.

The total saturated fatty acids SFA were significantly lower ($p < 0.001$) in OLI than NOL group (63.74% vs. 67.18%, respectively). Principal low chain SFA (from 4:0 to 10:0) and their sum (∑SFA low chain) did not exhibit significant differences between the two groups. This disagrees with some authors [3,28], who reported a lower percentage of short chain fatty acids in dairy sheep or cows fed with olive by-products, probably due to an excessive concentration of MUFA in a diet supplemented with olive by-products.

We found different percentages for the sum of saturated odd (∑SFA Odd) and the sum of saturate branch fatty acids (∑SFA Branch), with the OLI group reporting higher values than NOL (2.25% vs. 2.05% and 2.61% vs. 2.34%, respectively). Additional data about branch fatty acids are reported in Table S2.

According to Vlaeminck et al. [29], changes in ruminal bacterial populations induced by dietary rich in fibre may affect branched and odd chain fatty acids, which are primarily of bacterial origin. Cheese is a good dietary source of odd and branch SFA for humans, despite its low incidence in the total amount of FA. Therefore, the content of these fatty acids is an important health parameter that has recently received great interest due to new evidence demonstrating their anti-inflammatory properties and their role in reducing the development of cardiovascular diseases [29].

Palmitic acid (16:0) was always lower in OLI compared to NOL for the three ripening times, while stearic acid (18:0) did not show differences among diets (Table 3). Furthermore, data showed a lower myristic acid (14:0) in OLI cheese compared to NOL cheese (8.90% vs. 8.31%, $p < 0.01$). The data partially agree with Castellani et al. [30], who reported only 16:0, a lower value in cheese produced by cows supplemented with dried olive pomace, probably because supplementation olive by-products, rich in long-chain FA, negatively affected the de novo synthesis of 14:0 and 16:0 in the mammary gland [30].

Reducing the percentages of SFA and medium-chain FA, especially palmitic acid, could enhance the nutritional and health quality of dairy products. Intake of medium-chain FA may be unhealthy and lead to an increase in the concentration of low-density lipoprotein cholesterol when it is not associated with the correct level of linoleic acid, while stearic acid is considered to have a neutral effect on health [7].

3.4. Content of Principal Monounsaturated Fatty Acids

The total monounsaturated fatty acids (ΣMUFA) were significantly increased in OLI cheese than in NOL cheese at each time of ripening (Table 4). Palmitoleic acid (16:1 cis-9) was higher in OLI cheese than NOL at 15 and 30 days of ripening (1.02% vs. 0.92% in mean, Table 4), while the oleic acid (18:1 cis-9) was significantly different between diets only at 30 and 60 days of ripening (21.16% vs. 18.91% in mean, OLI vs. NOL). For the principal trans isomers of C18:1, we report limited differences except for trans vaccenic acid (C18:1 trans-11) which showed a lower value in OLI cheese ($p < 0.001$) at 60 days, probably due to an oxidative effect during ripening. The sum of MUFA trans was significantly higher in OLI cheese than in NOL cheese at 15 days of ripening (4.90% vs. 4.52%). More data on individual cis/trans isomers of 16:1 and 18:1 is reported in Table S3.

Table 4. Principal monounsaturated fatty acids (% of total FAME) of cheese obtained from ewes fed in two different ways.

%	15 Days			30 Days			60 Days			RMSE	*p*-Value Time
	NOL	OLI	Sign	NOL	OLI	Sign	NOL	OLI	Sign		
C16:1 c9	0.92	1.03	**	0.92	1.00	**	0.97	0.98	ns	0.06	0.800
C18:1 c9	18.53	19.67	ns	18.72	21.13	***	19.09	21.18	**	1.11	0.070
C18:1 c11	0.49	0.50	ns	0.50	0.49	ns	0.48	0.48	ns	0.04	0.547
C18:1 t9	0.55	0.61	*	0.54	0.60	*	0.54	0.57	ns	0.05	0.599
C18:1 t10	0.72	0.63	*	0.64	0.61	ns	0.56	0.56	ns	0.07	0.003
C18:1 t11	2.23	2.27	ns	2.43	2.18	ns	2.80	2.22	***	0.23	0.024
∑MUFA trans	4.52	4.90	***	4.61	4.81	ns	4.95	4.70	ns	0.23	0.391
∑MUFA	26.52	28.72	**	26.83	29.99	***	27.72	29.94	***	1.13	0.041

FAME = Fatty acids methyl ester; Fatty acids more than 0.5% of total FAME were reported. NOL = cheese from ewes fed without olive leaves; OLI = cheese from ewes fed with olive leaves. Time = ripening of cheese at different times. RMSE = root means square error. *, **, *** = significantly different for $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; ns = not significant. c = cis; t = trans; ∑MUFA trans = sum of trans monounsaturated fatty acids; ∑MUFA = total monounsaturated fatty acids.

The higher percentage of oleic acid was probably due to direct transfer from the olive leaves as they have a high content of oleic acid (21.76%, Table 1). This is consistent with previous studies that used dietary olive pomace [31] and olive cake supplements in dairy sheep [24]. In addition, 18:1 cis-9 may result from 18:0 desaturation occurring in the mammary gland by Δ9 desaturase [31].

The positive effect of olive leaf feed on the oleic acid and its trans isomers level in cheese is particularly interesting since this FA shows potentially beneficial effects on human health such as anticancer properties by inhibiting the cycle of cancer cells [31].

According to the literature, an increase in MUFA trans may depend on the abundance of oleic fatty acids in diet and on better stearoyl-coenzyme A desaturase activity in the mammary gland [30], especially if this is associated with a high presence of phenolic compounds in the diet which induces a greater formation of 18:1 trans isomers, except for trans 9 [7] as also obtained in our data presented in Table S3.

3.5. Content of Principal Polyunsaturated Fatty Acids

The percentage of ΣPUFA in OLI cheese was significantly higher compared to NOL cheese (4.62% vs. 4.02%, respectively), corresponding to an increase in both total n-6 (ΣPUFA n-6) and n-3 PUFA (Table 5). The total sum of very long polyunsaturated fatty acids n-6 (ΣVLPUFA n-6) was not affected by the use of olive leaves in the diet, such as the percentage of C20:4 n-6, while C20:2 n-6 showed a lower value ($p < 0.001$) in OLI cheese. On the contrary, the total sum of very long polyunsaturated fatty acids n-3 (ΣVLPUFA n-3) showed significantly higher values in OLI cheese than NOL only at 15 days of ripening, while C20:3 n-3 was unchanged.

Table 5. Principal polyunsaturated fatty acids (% of total FAME) and nutritional indexes of cheese obtained from ewes fed in two different ways.

%	15 Days			30 Days			60 Days			RMSE	*p*-Value Time
	NOL	OLI	Sign	NOL	OLI	Sign	NOL	OLI	Sign		
C18:2 n-6	1.47	1.96	***	1.50	1.95	***	1.56	1.87	***	0.12	0.973
C20:2 n-6	0.19	0.12	***	0.18	0.11	***	0.22	0.12	***	0.03	0.157
C20:4 n-6	0.17	0.18	ns	0.19	0.19	ns	0.17	0.18	ns	0.02	0.076
∑VLPUFA n-6	0.38	0.40	ns	0.37	0.39	ns	0.42	0.39	ns	0.04	0.270
∑PUFA n-6	2.34	2.78	***	2.36	2.78	***	2.50	2.68	*	0.15	0.878
C18:3 n-3	1.01	1.26	***	0.93	1.18	***	0.94	1.11	***	0.09	0.013
C20:3 n-3	0.25	0.28	ns	0.30	0.27	ns	0.29	0.27	ns	0.03	0.370
∑VLPUFA n-3	0.23	0.28	**	0.26	0.29	ns	0.27	0.28	ns	0.03	0.208
∑PUFA n-3	1.62	1.95	***	1.62	1.87	***	1.63	1.79	**	0.12	0.311
CLA *c*9, *t*11	0.88	1.02	*	0.82	0.99	*	0.83	0.93	ns	0.11	0.373
∑CLA	1.75	2.06	*	1.66	2.03	**	1.68	1.93	ns	0.24	0.604
∑PUFA *trans*	0.075	0.086	***	0.078	0.083	ns	0.082	0.085	ns	0.005	0.315
∑PUFA	3.96	4.74	***	3.98	4.65	***	4.13	4.47	**	0.21	0.844
n-6/n-3	1.46	1.43	ns	1.47	1.49	ns	1.53	1.50	ns	0.11	0.299
Ti index	2.43	2.02	***	2.41	1.91	***	2.30	1.95	***	0.12	0.137
Ai index	2.10	1.78	***	2.08	1.65	***	1.97	1.64	***	0.13	0.040

FAME = Fatty acids methyl ester; Fatty acids more than 0.2% of total FAME were reported. NOL = cheese from ewes fed without olive leaves; OLI = cheese from ewes fed with olive leaves; Time = ripening of cheese at different times; RMSE = root means square error. *, **, *** = significantly different for $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; ns = not significant. *c* = *cis*; *t* = *trans*; ∑VLPUFA = total very long polyunsaturated fatty acids; ∑PUFA n-6 = sum of C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:4 n-6, and other long-chain PUFA n-6 in trace; ∑PUFA n-3 = sum of C18:3 n-3, C20:3 n-3, and other long-chain PUFA n-3 in trace; CLA = conjugated linoleic acid; ∑CLA = sum of total conjugated linoleic acids; ∑PUFA *t* = *trans* isomers of C18:2 n-6 and C18:3 n-3; ∑PUFA = sum of PUFA n-6 and n-3. n-6/n-3 = ∑PUFAn6/∑PUFAn3; Ai, atherogenic index = ((C12:0 + (4 * C14:0) + C16:0)/(∑MUFA + ∑PUFA n-6 + ∑PUFA n-3); Ti, thrombogenic index = (C14:0 + C16:0 + C18:0)/((0.5 × C18:1) + (0.5 × other MUFA) + (0.5 × ∑PUFA n-6) + (3 × ∑PUFA n-3) + (n-3/n-6)).

Furthermore, we observed higher percentages of 18:2 n-6 and 18:3 n-3 fatty acids in OLI group than NOL ($p < 0.001$ for both). The finding is consistent with Innosa et al. [6]

who also showed an increase in α-linolenic acid (18:3 n-3), which essentially depends on its presence in the diet since olive leaves are richer in 18:3 n3 than the replaced hay.

The increase in α-linolenic acid, due to its higher presence in olive leaves, likely plays a decisive role in increasing the VLPUFA n-3 in milk and consequently in cheese [24], even if VLPUFA n-3 was significantly different only at the first time of ripening, because lipid oxidation of long chain fatty acids occurred during ripening.

The principal PUFA were not affected by the time of ripening (p-value time > 0.05), except for 18:3 n-3 which decreased during ripening (p-value time = 0.013). These data also highlight the good antioxidant potential of both diets that protected the long chain n-3 PUFA from the oxidation process; in fact, the n-3 PUFA did not oxidize more than n-6 PUFA during ripening time and their rate remained unchanged [6]. Although the tannins in olive leaves can inhibit the activity of rumen microorganisms, the presence of fibre and oleic and linoleic fatty acids stimulates biohydrogenation and the production of trans/cis isomers and conjugated isomers (CLA) of 18:2 n-6. This effect was confirmed by the increase in these isomers in OLI cheese as reported in Tables S4 and S5.

The total conjugated linoleic acids (ΣCLA) and the main CLA of the fatty acid biosynthetic pathway CLA cis-9, trans-11 (rumenic acid) was significantly higher in OLI cheese compared to NOL cheese at 15 and 30 days of ripening, while there were no significant differences at 60 days of ripening (Table 5). The other CLA isomers are reported in Table S5. Fernández et al. [32] reported a positive correlation between the increase in CLA cis-9, trans-11 and the highest MUFA and PUFA, particularly α-linolenic acid contents in cheese. In addition, Tsiplakou and Zervas [33] reported an increase in the content of both CLA cis-9, trans-11 and C18:1 trans-11 in the milk of dairy sheep fed with dried olive tree leaves, as shown in Bolletta et al.'s study [7] on sheep cheese.

In an in vitro study using olive cake, the increase in rumenic acid was related to a decrease in stearic acid [34]. This was due to the inhibition of rumen biohydrogenation of C18 unsaturated FA, which was associated with differences in microbial populations. However, this effect is not observed under real breeding conditions, because various dietary components modulate ruminal microorganism activity and the absorption of fatty acids from the diet. The biosynthesis of rumenic acid and the total CLA occurs through two pathways: the isomerization of linoleic acid by incomplete rumen biohydrogenation and the synthesis by desaturation of C18:1 trans-11 in the mammary gland [30].

The significance of CLA content in animal products is well-established, the CLA are essential fatty acids with a high influence on human health and wellness [32]. In 1996, the National Academy of Science National Research Council (NRC) defined CLA as "fatty acids that unequivocally show inhibition of tumor development".

3.6. Health Indexes

Diets and ripening time did not influence the n-6/n-3 ratio in cheese (Table 5), and the values obtained were very low (<2), representing a typical ratio found in dairy products from grazing ewes. The optimal n-6/n-3 ratio in foods should be lower than 4 to have a positive effect on anti-inflammatory processes. Therefore, a ratio of less than 2 indicates the high nutritional value of cheese produced by animals raised on pasture due to the reduction in n-6 PUFA content in favour of n-3 PUFA regardless of the addition of leaves in the diet.

Health indices, such as atherogenic (Ai) and thrombogenic (Ti) index, were lower in OLI than NOL cheese (1.99 vs. 2.43 and 2.17 vs. 2.68, respectively). Although both groups were grazing, the cheese obtained from sheep fed with the addition of olive leaves reported a better quantity of MUFA and n-3 PUFA fatty acids, essential compounds for improving these health indexes [7].

The ripening time had no significant effect on the proposed nutritional indices, except for the Ai (p = 0.040), which improved over time, demonstrating the good oxidative stability of the polyunsaturated fatty acids due to the presence of polyphenols which are abundant in the olive leaves and easily absorbed and accumulated in the cheese [35].

The richness of PUFA in a diet with olive leaves did not inhibit the formation of CLA cis-9, trans-11, odd and branch SFA. These combined effects resulted in an improvement in the Ai and Ti index in sheep cheese with a clear increase in positive effects on human health [29].

4. Conclusions

In conclusion, the milk obtained from sheep fed with fresh olive leaves produced a cheese characterised by a higher content of C18:1 cis-9, C18:3 n-3, and CLA and a lower presence of 16:0 compared to clover hay. Despite the random factors inherent in real farming conditions, the use of non-dried olive leaves enhanced the quality of the resulting cheese, which exhibited improved health indices with clear benefits for consumers. The cheese was enriched with nutraceutical compounds, which possess anti-inflammatory properties essential for improving human health and preventing metabolic diseases.

Furthermore, since this study was conducted under normal farm conditions rather than experimental models, it provides valuable insights into the applicability of a sustainable system that uses olive by-products in animal diets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dairy5040054/s1, Figure S1: GC-chromatograms (a) of C18:1 isomers extracted with SPE Ag+ cartridge (Red peaks were trans isomer whilst blue peaks were cis isomers), (b) of C18:2 isomers and CLA extracted with SPE Ag+ cartridge; Table S1: Proximate composition and main classes of fatty acids (% of total FAME) in cheese 60 days ripened from the two farms before the change of diet. Table S2: Branch fatty acids expressed as mg/g of cheese obtained from ewes fed in two different ways; Table S3: cis/trans isomers of C16:1 and C18:1 monounsaturated fatty acids expressed as mg/g of cheese obtained from ewes fed in two different ways; Table S4: Isomers of C18:2 n-6 and C18:3 n-3 expressed as mg/g of cheese, obtained from ewes fed in two different ways. Table S5: Conjugated linoleic acid (CLA) isomers, expressed as mg/g of cheese obtained from ewes fed in two different ways.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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