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## **Seasonal variation of fatty acids composition of milk from grazing ewes in Thessaly, central Greece**

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## **Εποχιακή διακύμανση στη σύνθεση των λιπαρών οξέων γάλακτος από προβατίνες ελευθέρως βοσκής στη Θεσσαλία της κεντρικής Ελλάδος**

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**ABSTRACT.** The aim of this work was to evaluate the changes in fatty acids (FAs) profile and conjugated linoleic acid (CLA) concentration of milk from grazing ewes in winter (December and January) and spring (April and May) in Thessaly, central Greece. No significant changes ( $P>0.05$ ) in the physicochemical properties (pH and protein, lactose and total solids content) of winter and spring milk were observed. However, the fat content of spring milk was lower ( $P<0.05$ ) than the winter milk. The saturated FAs content of milk was not significantly changed ( $P>0.05$ ) during winter neither during spring, whereas the polyunsaturated FAs content was significantly changed ( $P<0.05$ ) in each of the four months examined. Nevertheless, in the ovine milk of spring, the saturated FAs content was significantly decreased ( $P<0.05$ ), but the monounsaturated and polyunsaturated FAs content was significantly increased ( $P<0.05$ ) as compared to that of winter milk. In contrast to the saturated FAs decrease in spring milk, the saturated stearic acid (C18:0) content showed a significant increase ( $P<0.05$ ) in the spring milk as compared to winter milk. In winter milk, the C18:2 *cis*-9, *trans*-11 CLA levels were  $0.89\pm 0.05$  and  $0.98\pm 0.03$ g/100 g Fatty Acid Methyl Esters (FAMES) in December and January, respectively, whereas, in spring milk, the CLA levels were significantly increased ( $P<0.05$ ) to  $1.36\pm 0.04$  and  $1.27\pm 0.03$ g/100 g FAMES in April and May, respectively. The atherogenicity index (AI) associated with proatherogenic and antiatherogenic FAs was found significantly ( $P<0.05$ ) lower in spring milk compared to winter milk.

**Keywords:** Fatty acids, conjugated linoleic acid, ovine milk, gas chromatographic analysis, grazed pasture.

**ΠΕΡΙΛΗΨΗ.** Σκοπός αυτής της εργασίας ήταν η αξιολόγηση των μεταβολών του προφίλ των λιπαρών οξέων και της συγκέντρωσης του συζευγμένου λινολεϊκού οξέος (CLA) του γάλακτος από προβατίνες ελευθέρως βοσκής του χειμώνα (Δεκέμβριος και Ιανουάριος) και την άνοιξη (Απρίλιος και Μάιος) στη Θεσσαλία της κεντρικής Ελλάδας. Δεν παρατηρήθηκαν σημαντικές μεταβολές ( $P>0,05$ ) στις φυσικοχημικές ιδιότητες (pH και περιεκτικότητα σε πρωτεΐνη, λακτόζη και συνολικά στερεά) του γάλακτος χειμώνα και άνοιξης. Ωστόσο, η περιεκτικότητα σε λιπαρά του ανοιξιάτικου γάλακτος ήταν χαμηλότερη ( $P<0,05$ ) εκείνης του χειμώνα. Η περιεκτικότητα σε κορεσμένα λιπαρά οξέα του γάλακτος δεν μεταβλήθηκε σημαντικά ( $P>0,05$ ) τόσο κατά τη διάρκεια του χειμώνα όσο και κατά τη διάρκεια της άνοιξης, ενώ η περιεκτικότητα σε πολυακόρεστα λιπαρά οξέα μεταβλήθηκε σημαντικά ( $P <0,05$ ) σε κάθε έναν από τους τέσσερις μήνες που εξετάστηκαν. Παρ' όλα αυτά, στο γάλα της άνοιξης, η περιεκτικότητα των κορεσμένων λιπαρών οξέων μειώθηκε σημαντικά ( $P<0,05$ ), αλλά η συγκέντρωση των μονοακόρεστων και πολυακόρεστων λιπαρών οξέων αυξήθηκε σημαντικά ( $P<0,05$ ) σε σύγκριση με το γάλα του χειμώνα. Σε αντίθεση με τη μείωση των κορεσμένων λιπαρών οξέων στο ανοιξιάτικο γάλα, η περιεκτικότητα του κορεσμένου στεατικού οξέος (C18:0) παρουσίασε σημαντική αύξηση ( $P<0,05$ ) στο γάλα της άνοιξης σε σύγκριση με εκείνη του χειμώνα. Στο γάλα του χειμώνα, τα επίπεδα του C18:2 *cis*-9, *trans*-11 CLA ήταν  $0.89\pm 0.05$  και  $0.98\pm 0.03$  g/100g FAMES τον Δεκέμβριο και τον Ιανουάριο, αντίστοιχα, ενώ στο γάλα της άνοιξης τα επίπεδα του CLA αυξήθηκαν σημαντικά ( $P<0,05$ ) σε  $1.36\pm 0.04$  και  $1.27\pm 0.03$  g/100g FAMES τον Απρίλιο και τον Μάιο, αντίστοιχα. Ο δείκτης αθηρογένεσης (AI), που σχετίζεται με λιπαρά οξέα που ευνοούν την πρόκληση αθηροσκλήρωσης αλλά και με λιπαρά οξέα που δεν ευνοούν την πρόκληση αθηροσκλήρωσης, βρέθηκε σημαντικά μικρότερος ( $P<0,05$ ) στο γάλα της άνοιξης σε σχέση με εκείνο του χειμώνα.

## INTRODUCTION

China is the largest producer of ovine milk in the world (12.2%) followed by Greece, which is the largest producer in Europe (8.7%) (Balthazar et al., 2017). According to the statistics of the Hellenic Agricultural Organization Elgo Dimitra, the production of the ovine milk amounted to 644,451,875 kg for the year 2017 (Elgo Dimitra, 2018). Also, according to the same data the regions with the highest production of ovine milk in 2017 were Thessaly (central Greece) followed by the Western Greece region. Karagouniko sheep breed is originated from Thessaly, but nowadays it is reared in several other regions in Greece. Among other local sheep breeds, Karagouniko sheep breed is characterized by a high milk production and lambing properties.

The nutritional value of ovine milk is considered quite high due to its protein, fat, minerals and vitamins

content. Although the fat of the ovine milk is high and rich in saturated fatty acids (SFAs), which are incriminated for an increased risk of cardiovascular diseases, several (FAs) possess beneficial health properties, such as the monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (Palmquist et al., 2005). Among MUFAs, the vaccenic acid C18:1 *trans*-11 VA exhibits a protective role against heart diseases and the metabolic syndrome (Wenjie et al., 2015). Among PUFAs, conjugated linoleic acid (CLA) was initially recognized for its anticancer activity (Pariza et al., 1979). CLA consists of several geometric and positional isomers of linoleic acid (C18:2) with conjugated double bonds, naturally found in ruminant dairy or meat products. In ovine milk, the predominant CLA isomer is C18:2 *cis*-9, *trans*-11 CLA (80 to 90% of total CLA) followed by C18:2 *trans*-10, *cis*-12 CLA but in lower quantities (Meluchova et al., 2008). The C18:2

*cis*-9, *trans*-11 CLA shows also, apart from its anticancer activity, anti-atherogenic, anti-diabetic, anti-inflammatory, and increased immune response properties, while C18:2 *trans*-10, *cis*-12 CLA exhibits anti-obesity properties and improves osteosynthesis (Fuke and Nornberg, 2017).

The FAs content of ovine milk may vary due to various reasons such as sheep breed, feeding conditions or season (De La Fuente et al., 2009). Grazing pasture is an effective feeding ways of improving the levels of MUFAs, PUFAs including CLA content in milk of lactating ewes (Soják et al., 2013). The FAs profiles of pasture varies in different seasons and thus, it affects also the profile of FAs of milk produced by lactated ewes under grazed pasture feeding conditions (Cabiddu et al., 2005; De Renobales et al., 2012). The FAs profile of milk produced by lactating Karagouniko sheep were mostly examined under conventional feeding conditions (Sinanoglou et al., 2015).

The aim of this study was to evaluate the changes in fatty acids (FAs) profile and conjugated linoleic acid (CLA) concentration of milk from grazing ewes in winter and spring in Thessaly, central Greece.

## MATERIALS AND METHODS

### Milk samples

Raw milk samples were taken from a Karagouniko sheep breed farm located in eastern Thessaly, central Greece. The lactating ewes were exclusively fed on grazing at the plain pastures of eastern Thessaly. Milk samples were collected in December 2016 and January 2017 as well as April 2017 and May 2017 from a bulk milk tank (a mixture of night and morning milk) for the winter and spring sampling seasons, respectively. The milk was obtained from two groups of 40 lactating ewes in each sampling season, approximately after 30-45 lambing days, according to the International Dairy Federation guidelines (IDF, 1995).

### Physicochemical analysis of milk.

The fat, protein, lactose and total solids content of milk were measured using a Milko Scan 4000 (FOSS Electric, Integrated Milk Testing™, Hillerød, Denmark). The pH of the milk samples was estimated using a pH meter (WTW, type 525, Wissenschaftlich-Technische Werkstätten, GmbH, Weilheim, Germany)

### Extraction of Fat

Milk fat was extracted using the method of Folch et al., (1975) with certain modifications as described

by Castro-Gomez et al., (2014) and Fletouris et al., (2015). In brief, milk samples (1 ml) were mixed with 15 ml of chloroform – methanol solution (2:1, v/v). The resulting mixtures were initially homogenized for 20 min and subsequently centrifuged at 6500 rpm for 10 min at 4 °C. After 1 min pause, the mixtures were further shaken for 2 min and centrifuged at 6500 rpm for 5 min at 4 °C. The mixture was filtered through filter paper and the resulting filtrate was made to 15ml with chloroform – methanol solution. Then, 3ml of 0.74% KCl was mixed with the filtrate. After centrifugation at 6500 rpm for 5 min at 4 °C, the upper layer was removed and the bottom chloroform layer was mixed with 3g of anhydrous sodium sulfate and filtrated by using a Whatman 1-phase separator filter (Whatman, Maidstone, UK). Finally, the extract was concentrated by evaporating the chloroform in a rotary evaporator (Rotavapor RE111, BÜCHI, Switzerland) and subsequently dried over a gentle stream of nitrogen. The fat extract was kept in amber vials at -18 °C pending for (FAs) analysis.

### Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAMES) were produced by using base-catalyzed methanolysis of the acylglycerides with potassium methoxide following a standard IDF method (IDF, 2002). This IDF procedure was selected in order to restrict isomerization of FAs and CLA during methylation.

### Gas chromatographic analysis of FAMES

FAMES analysis was accomplished using a GC-17A Shimadzu gas chromatograph (Shimadzu Scientific Instruments Inc., Kyoto Japan). The GC was equipped with an automatic injector, flame ionization detector (FID) and a split injection port. A fused capillary column of 60 m with internal diameter of 0.25 mm and film thickness 0.25 µm (model 122-2362 Agilent J&W, Agilent Technologies, Santa Clara, California, USA) was used for the FAMES separation. After injection, the column temperature was held at 70 °C for 1min. Then, the column temperature was raised as follows, to 130 °C at a rate of 5 °C/min and kept for 14 min, to 170 °C at a rate of 5 °C/min and kept for 15 min, to 215 °C at a rate of 2 °C/min and kept for 2 min, and finally to 230 °C at a rate of 5 °C/min and kept for 6 min. The FID temperature was set at 270 °C, while the injector temperature was set at 250 °C. Helium was used as a carrier gas at a flow rate of 0.7 ml/min. The injection volume was 1 µl, while the split ratio was 1:50.

The FAMES standards of a) Supelco FAME Mix 37 components (Sigma-Aldrich, Steinheim, Germany) and b) a mixture of *cis*-9 *trans*-11 CLA methyl esters and *trans*-10, *cis*-12 CLA methyl esters (Sigma-Aldrich, Steinheim, Germany) were used for the identification of the individual FAME by comparing their retention times. FAs percentages were measured by direct normalization of peak areas and using the Shimadzu GC solution data system program. Milk FAs were expressed as g/100 g FAMES estimated by multiplying peak areas with correction factors according to AOAC 963.22 method (AOAC 2000). FAs analyses were carried out in triplicate.

### Atherogenicity and $\Delta^9$ desaturase activity indexes

The atherogenicity index (AI) is associated with antiatherogenic and proatherogenic properties of FAs. The atherogenicity index (AI) was estimated as follows (Tsiplakou et al 2010):

$$AI = (C12:0 + 4 \times C14:0 + C16:0) / (PUFA + MUFA).$$

The  $\Delta^9$ -desaturase enzyme catalyzes the introduction of a *cis* double bond between carbons 9 and 10 of the saturated FA chain forming unsaturated FAs. The  $\Delta^9$ desaturase activity indexes were estimated using two pairs of FAs that represent products and substrates for  $\Delta^9$ desaturase action of the mammary gland. The fatty acids pairs were *cis*-9 C14:1/C14:0 and *cis*-9 *trans*-11 CLA/*trans*-11 C18:1. Thus, the desaturase activity index was calculated as: (product of  $\Delta^9$ desaturase)/(product of  $\Delta^9$ desaturase + substrate of  $\Delta^9$ desaturase). The two  $\Delta^9$ desaturase activity indexes C14DI and CLADI were estimated as follows (Lock and Garnsworthy, 2003):

1. C14DI: C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0)
2. CLADI: *cis*-9 *trans*-11 CLA / (*cis*-9 *trans*-11 CLA + C18:1 *trans*-11).

### Statistical analysis

All experimental data were subjected to statistical analysis of variance using the one-way ANOVA procedure of SPSS 10.05 statistical package (SPSS Ltd., Woking, Surrey, UK). The Tukey's test was applied in order to find the statistical differences between least-squares means. A probability level of  $P < 0.05$  was used in testing the statistical significance.

## RESULTS AND DISCUSSION

The physicochemical properties of the ovine milk

from lactating ewes of the Karagouniko sheep breed fed on grazing pasture at the Thessaly region, central Greece, in winter and spring, are shown in Table 1. No significant differences ( $P > 0.05$ ) as regards the pH and the protein, lactose and total solids content of ovine milk produced in winter (December or January) as well as that in spring (April or May) were observed. However, the fat content of the milk produced in spring was lower ( $P < 0.05$ ) than that produced in winter. The lower fat content in spring may be due to the diet inducing milk fat depression (MFD) syndrome (Bauman and Griinari 2001, Bauman et al., 2003). The MFD syndrome in lactating ruminants is caused by the high amounts of MUFA and PUFA found in the consumed feeds or pasture. It was previously reported that high amounts of MUFA and especially PUFA in ruminants' diet affects the biohydrogenation fat procedures in the rumen, forming certain fat components with antilipogenic properties which cause the fat content decrease of the milk (Bauman and Griinari, 2001). The pasture in spring was found to contain higher amounts of MUFA and PUFA than the one in winter in various Mediterranean countries (Cabiddu et al., 2005). Seasonal variations of the fat content have been reported in previous studies for ovine milk (Prandiniet al., 2004; Carloni et al., 2010), bovine (Lu et al., 2018) or caprine milk (Malissiova et al., 2015). The supplementation of feeds with fish oil, which is rich in MUFA and PUFA, affected the lipogenic genes of mammary gland and decreased the fat content of the ovine milk (Carreño et al., 2016). Previous studies on conventional feeding (hay and concentrates) of lactating Karagouniko ewes on the milk composition revealed that the fat content of the milk was dependent on the type of concentrates (Papadopoulos et al., 2002; Sinanoglou et al., 2015 ; Skoufos et al., 2017).

The changes of FAs profile of the milk produced by the Karagouniko sheep breed grazing in the pasture in winter and spring months are shown in Table 2. In all months, the predominant FA in the milk was the saturated palmitic (C16:0). Similarly, other major FAs were the myristic (C14:0) and stearic (C18:0) among SFA, *cis*-9 oleic (C18:1 *cis*-9) among MUFA, and *cis* linoleic (C18:2 *cis*) among PUFA. In line with the present study, previous studies on FA profile of ovine milk produced by sheep under grazing pasture (Atti et al., 2006; Carloni et al., 2010; Papaloukas et al., 2016) or conventional feeding conditions (Castro et al., 2009; De La Fuente et al., 2009), showed the same predominant FAs.

**Table 1.** Physicochemical properties of ovine milk produced by the Karagouniko sheep breed fed on grazing pasture in winter and spring

Month	Physicochemical properties of ovine milk*				
	pH	Fat (g/kg)	Protein (g/kg)	Lactose (g/kg)	Total Solids (g/kg)
December	6.69 ± 0.10	66.15 ± 1.15 <sup>a</sup>	54.24 ± 1.24	46.07 ± 1.39	176.36 ± 2.52
January	6.70 ± 0.09	68.22 ± 1.27 <sup>a</sup>	55.12 ± 1.32	45.09 ± 1.35	178.23 ± 2.23
April	6.70 ± 0.08	63.52 ± 1.65 <sup>b</sup>	53.62 ± 1.45	± 1.27	172.14 ± 2.59
May	6.69 ± 0.09	62.75 ± 1.41 <sup>b</sup>	52.93 ± 1.29	45.93 ± 1.35	171.51 ± 2.56

\*Mean values ± standard deviation (n=9).

<sup>ab</sup>Mean values followed by different letters in the same column are significantly different (P<0.05).

Comparing the sum of FA in the milk of all examined months, SFA were higher than MUFA, while PUFA were lower than MUFA (SFA>MUFA>PUFA). The same decreasing content order of the sum of the saturated and unsaturated FAs was also reported in previous studies in ovine (Ostrovsky et al., 2009; Tsiplakou et al., 2010), bovine (De Noni and Battelli, 2008) or caprine (Sampelayo et al., 2007) milk.

The FAs analysis of ovine milk produced in spring months showed that the SFA content was significantly decreased (P<0.05), but in contrast the MUFA and PUFA content was significantly increased (P<0.05) as compared to that of winter months. The FAs of ruminants' milk fat are derived from two sources: *de novo* synthesis in the mammary gland and the plasma lipids originating from the feed (Palmquist et al., 2005). MUFA and PUFA in blood plasma are also originated from the feed, by biohydrogenation of feed FA by rumen bacteria, and are excreted by the mammary gland in the milk (Bauman and Griinari 2001). The pasture plants are richer in unsaturated FAs in spring than in winter in countries with a moderate climate (Cabiddu et al., 2005). Feeding lactating ewes with pasture or lipid supplements rich in PUFA results in milk rich in MUFA and PUFA (Mierlita et al., 2011). In accordance with the present results, similar seasonal effect with a decrease in SFA, and an increase in MUFA and PUFA of the milk produced by lactating ewes (Atti et al., 2006; Meluchova et al., 2008; Abilleira et al., 2009; Carloni et al., 2010), cows (Sasanti et al., 2015) or goats (Sampelayo et al., 2007), under grazing pasture feeding conditions, were observed in previous studies.

The saturated stearic acid (C18:0) content showed a significant increase (P<0.05) in the spring milk as compared to that of winter, although total SFA showed a different behavior with a significant decrease

(P<0.05) between the spring and winter months. This might be attributed to changes of the MUFA of the pasture by the rumen bacteria (Meluchova et al., 2008). It is known that stearic acid (C18:0) found in ruminants' milk is synthesized by biohydrogenation of linoleic or  $\alpha$ -linolenic acids of the pasture to the end-product by the rumen bacteria (Palmquist et al., 2005). The levels of  $\alpha$ -linolenic acid and linoleic acid of pasture flora are substantially increased by a percentage ranging between 20-55% in spring months as compared to those in winter months in the Mediterranean countries (Cabiddu et al., 2005). In line with our findings, an increase of stearic acid (C18:0) and a parallel decrease in SFA were also reported in previous studies for the milk produced in spring months as compared to that of winter months of lactating ewes (Abilleira et al., 2009), or cows (Chion et al., 2010) under grazing pastures feeding conditions.

In winter, the C18:2 *cis*-9, *trans*-11 CLA levels of the milk were 0.89±0.05 and 0.98±0.03 g/100 g FAMES in December and January, respectively (Table 2). In spring, the C18:2 *cis*-9, *trans*-11 CLA levels of the milk were significantly increased (P<0.05) to 1.36±0.04 and 1.27±0.03 g/100 g FAMES in April and May, respectively. The C18:1 *trans*-11 VA showed a similar increasing trend (Table 2). In winter, the C18:1 *trans*-11 VA levels were 2.16±0.07g/100 g FAMES and 2.25±0.05 g/100 g FAMES in December and January, respectively. In spring, the C18:1 *trans*-11 VA levels were significantly increased (P<0.05) to 2.52±0.09g/100 g FAMES and 2.38±0.06 g/100 g FAMES in April and May, respectively. The C18:2 *cis*-9, *trans*-11 CLA and C18:1 *trans*-11 VA are intermediate products of biohydrogenation of linoleic acid (C18:2) to stearic acid (C18:0) in the rumen. The C18:2 *cis*-9, *trans*-11 CLA is the initial intermediate product, while C18:1 *trans*-11 VA is rapidly formed by further biohydrogenation of the C18:2 *cis*-

9, *trans*-11 CLA. Before the complete transformation of C18:1 *trans*-11 VA to stearic acid (C18:0), parts of these two intermediate products of either C18:2 *cis*-9, *trans*-11 CLA or C18:1 *trans*-11 VA are absorbed in the small intestine of the ruminants and transferred by the blood plasma to milk. This biohydrogenation process of linoleic acid (C18:2) is accomplished by the action of *cis*, *trans* linoleate isomerase enzymes found in certain ruminant bacteria such as *Butyrivibrio fibrisolvens*, *Ruminococcus*, *Eubacterium* or, *Fusocillus* spp. (Palmquist et al., 2005). Other FAs such as  $\alpha$ -linolenic acid and  $\gamma$ -linolenic acid, found in high amounts in the pasture, are also biohydrogenated to stearic acid in the rumen, with intermediate products of C18:2 *cis*-9, *trans*-11 CLA or C18:1 *trans*-11 VA (Bauman et al., 2003). In spring, the higher amounts of C18:1 *trans*-11 VA and C18:2 *cis*-9, *trans*-11 CLA found in ovine milk are derived from the higher PUFA amounts in grazing pasture (Abilleira et al., 2009).

In line with the present findings, seasonal variations of C18:2 *cis*-9, *trans*-11 CLA in ovine milk were observed in previous studies. Meluchova et al., (2008) reported that the bulk ovine milk produced by various Slovak sheep breeds under grazing pasture feeding conditions presented *cis*-9, *trans*-11 CLA levels of 0.71, 1.5 and 2.76 g/100 g FAME in April, June and September, respectively. De La Fuente et al., (2009) reported seasonal variations of *cis*-9, *trans*-11 CLA levels in ovine bulk milk produced by Churra sheep breeds under combined conventional and grazing pasture feeding conditions in Spain, which were 0.68, 1.02, 0.94 and 0.96 g/100 g FAMEs in winter, spring, summer and autumn, respectively. Carloni et al., (2010) examined ovine milk produced by various sheep breeds (Suffolk, Fabrianese, Sopravvissana, Sarda) in the plains and mountainous areas in Marche region (central Italy) under combined conventional or grazed pasture feeding conditions and reported seasonal variations of *cis*-9, *trans*-11 CLA values ranging from 0.01 to 0.07%, 0.08 to 1.96% and 0.50 to 2.22% FAME in December, May and July, respectively. Papaloukas et al., (2016) reported that bulk ovine milk produced by various sheep breeds fed on grazing pasture in mountainous areas in Northern Greece showed *cis*-9, *trans*-11 CLA values of 0.58, 0.98 and 1.12% FAME in winter, spring and summer, respectively. Seasonal variations of C18:2 *cis*-9, *trans*-11 CLA in milk from cows (Chion et al., 2010; Sasanti et al., 2015) or goats (Milewski et al., 2018) produced under grazing pasture feeding conditions were also reported.

Signorelli et al., (2008) examined the FAs composition of ovine milk produced by lactated ewes under the same combined pasture and concentrate feeding conditions and found *cis*-9, *trans*-11 CLA levels of 1.905%, 1.958% and 1.617% FAME for the Italian breeds, Altamura, Gentile di Puglia and Sarda, respectively. Mierlita et al., (2011) examined the ovine milk produced by lactated ewes under the same conventional feeding conditions (hay, maize silage and concentrates) and reported high differences for the *cis*-9, *trans*-11 CLA reaching values of 1.01 and 2.67 g/100 g FAME for the Spanca and Turcana sheep breeds, respectively. However, Soják et al., (2013) did not observe any significant difference between the *cis*-9, *trans*-11 CLA levels (ca 1.54 g/100 g FAME) in the ovine milk produced under the same feeding conditions for the Tsigai, Valachian, Lacaune sheep breeds, respectively. Sinanoglou et al., (2015) examined the ovine milk produced by the Karagouniko sheep breed under conventional feeding conditions and found *cis*-9, *trans*-11 CLA levels of 0.52% and 0.66% FAME in December and June, respectively. Tsiplakou et al., (2010) reported *cis*-9, *trans*-11 CLA levels of 1.1% or 1.3% FAME for the ovine milk produced by the Karagouniko sheep breed under conventional and organic feeding conditions, respectively.

Previous studies also showed seasonal variations of C18:1 *trans*-11 VA in the ovine milk produced by lactating ewes under pasture grazing feeding conditions. The C18:1 *trans*-11 VA content in ovine milk was increased from ca 4 g/100 g FAME in March to 4.5 g/100 g FAME in April (Nudda et al., 2005). Ovine milk produced by lactating ewes fed on grazing pasture showed C18:1 *trans*-11 VA levels of 1.86%, 3.08% and 2.94% FAME in winter, spring and summer, respectively (Papaloukas et al., 2015). Various levels of C18:1 *trans*-11 VA were also reported for ovine milk produced from lactating ewes under grazing pasture feeding conditions such as 1.91-2.14 g/100 g FAME (Atti et al., 2006) or 1.05-3.95 g/100 g FAME (Meluchova et al., 2008). Sinanoglou et al., (2015) reported C18:1 *trans*-11 VA levels of 0.35 – 0.48% FAME in milk produced by the Karagouniko sheep breed under conventional feeding conditions. Seasonal variations of C18:1 *trans*-11 VA levels were also reported for milk from cows (Rego et al., 2008) or goats (Salari et al., 2016) fed grazed pasture.

**Table 2.** Fatty acid composition (g/100 g FAMES) of ovine milk from lactating grazed pasture Karagouniko sheep breed in winter and spring months

Fatty acids	December		January		April		May	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>C4:0</b> Butyric	5.51	0.12 <sup>a</sup>	5.36	0.12 <sup>a</sup>	5.19	0.11 <sup>b</sup>	5.31	0.13 <sup>b</sup>
<b>C6:0</b> Caproic	3.62	0.05	3.57	0.07	3.50	0.06	3.61	0.09
<b>C8:0</b> Caprylic	2.77	0.07 <sup>a</sup>	2.65	0.08 <sup>a</sup>	2.49	0.06 <sup>b</sup>	2.52	0.07 <sup>ab</sup>
<b>C10:0</b> Decanoiccapric	9.24	0.23	9.43	0.24	9.16	0.21	9.28	0.26
<b>C11:0</b> Undecanoic	0.09	0.02	0.10	0.03	0.09	0.02	0.08	0.02
<b>C12:0</b> Lauric	5.48	0.14 <sup>a</sup>	5.39	0.13 <sup>a</sup>	5.08	0.12 <sup>b</sup>	5.14	0.14 <sup>b</sup>
<b>C13:0</b> Tridecanoic	0.73	0.08	0.75	0.09	0.79	0.07	0.77	0.08
<b>C14:0</b> Myristic	11.51	0.19 <sup>a</sup>	11.32	0.21 <sup>a</sup>	10.67	0.24 <sup>b</sup>	10.85	0.13 <sup>b</sup>
<b>C15:0</b> Pentadecanoic	0.91	0.08	0.90	0.09	0.83	0.07	0.76	0.09
<b>C16:0</b> Palmitic	23.23	0.31 <sup>a</sup>	22.97	0.34 <sup>a</sup>	21.69	0.33 <sup>b</sup>	21.94	0.27 <sup>b</sup>
<b>C17:0</b> Heptadecanoic	0.54	0.05	0.59	0.08	0.59	0.06	0.62	0.09
<b>C18:0</b> Stearic	8.67	0.17 <sup>a</sup>	8.58	0.19 <sup>a</sup>	9.31	0.13 <sup>b</sup>	9.36	0.14 <sup>b</sup>
<b>C20:0</b> Arachidic	0.27	0.05	0.28	0.04	0.25	0.05	0.24	0.06
<b>C21:0</b> Heneicosanoic	0.06	0.01	0.06	0.02	0.08	0.02	0.07	0.02
<b>C22:0</b> Behenicacid	0.06	0.02	0.06	0.02	0.08	0.01	0.08	0.02
<b>C23:0</b> Tricosanoic	0.14	0.03	0.16	0.02	0.10	0.04	0.12	0.03
<b>C14:1</b> Myristoleic	0.21	0.07	0.22	0.05	0.31	0.06	0.29	0.05
<b>C15:1</b> <i>cis</i> -10- Pentadecanoic	0.25	0.04	0.24	0.03	0.28	0.06	0.29	0.04
<b>C16:1</b> Palmitoleic	0.87	0.05 <sup>a</sup>	0.85	0.06 <sup>a</sup>	1.05	0.04 <sup>b</sup>	1.01	0.05 <sup>b</sup>
<b>C17:1</b> <i>cis</i> -10- Heptadecanoic	0.25	0.02	0.26	0.04	0.23	0.02	0.24	0.02
<b>C18:1 trans -n9</b> <i>trans</i> -9-Elaidic	0.41	0.02 <sup>a</sup>	0.40	0.03 <sup>a</sup>	0.50	0.04 <sup>b</sup>	0.51	0.05 <sup>b</sup>
<b>C18:1 trans 11-n7</b> Vaccenic	2.16	0.07 <sup>a</sup>	2.25	0.05 <sup>a</sup>	2.52	0.09 <sup>b</sup>	2.38	0.06 <sup>b</sup>
<b>C18:1 cis-9 n9</b> <i>cis</i> -9-Oleic	17.81	0.29 <sup>a</sup>	18.13	0.17 <sup>a</sup>	18.89	0.26 <sup>b</sup>	18.52	0.18 <sup>b</sup>
<b>C22:1</b> Eruric	0.30	0.06	0.31	0.05	0.42	0.07	0.40	0.04
<b>C24:1</b> Nervonic	0.24	0.02	0.26	0.02	0.26	0.03	0.27	0.02
<b>C18:2</b> <i>cis</i> -9. <i>trans</i> -11 CLA	0.89	0.05 <sup>a</sup>	0.98	0.03 <sup>b</sup>	1.36	0.04 <sup>c</sup>	1.27	0.03 <sup>b</sup>
<b>C18:2</b> <i>trans</i> -10. <i>cis</i> -12 CLA	0.08	0.01 <sup>a</sup>	0.09	0.01 <sup>a</sup>	0.14	0.02 <sup>b</sup>	0.13	0.02 <sup>b</sup>
<b>C18:2 trans</b> <i>trans</i> Linolelaidic	0.37	0.05	0.39	0.06	0.44	0.04	0.41	0.05
<b>C18:2 cis</b> <i>cis</i> Linoleic	2.35	0.08 <sup>a</sup>	2.42	0.05 <sup>a</sup>	2.59	0.10 <sup>b</sup>	2.47	0.06 <sup>ab</sup>
<b>C20:4 n6</b> Arachidonic	0.17	0.03	0.18	0.02	0.19	0.02	0.17	0.04
<b>C18:3 n6</b> $\gamma$ -Linolenic	0.05	0.01	0.04	0.02	0.07	0.02	0.06	0.03
<b>C18:3 n3</b> $\alpha$ -Linolenic	0.06	0.02	0.06	0.01	0.07	0.02	0.07	0.01
<b>C20:2</b> <i>cis</i> -11-14- Eicosadienoic	0.08	0.02	0.09	0.02	0.08	0.02	0.09	0.02
<b>C20:4 n3</b> <i>cis</i> 5.8.11.14 Eicostetranoic	0.29	0.05	0.31	0.07	0.31	0.04	0.29	0.05
<b>C22:2</b> <i>cis</i> 13.16 Docosadienoic	0.10	0.02	0.11	0.02	0.13	0.03	0.12	0.02
<b>C20:5</b> <i>cis</i> -5.8.11.14.17 Eicosapentanoic	0.12	0.03	0.12	0.02	0.14	0.02	0.13	0.03
<b>C22:6</b> <i>cis</i> -4.7.10.13.16.19 Docosahehexaenoic	0.11	0.02	0.12	0.03	0.12	0.02	0.13	0.02
SFA	72.83	0.68 <sup>a</sup>	72.17	0.58 <sup>a</sup>	69.9	0.62 <sup>b</sup>	70.75	0.54 <sup>b</sup>
MUFA	22.5	0.29 <sup>a</sup>	22.92	0.23 <sup>a</sup>	24.46	0.22 <sup>b</sup>	23.91	0.24 <sup>c</sup>
PUFA	4.67	0.10 <sup>a</sup>	4.91	0.11 <sup>b</sup>	5.64	0.15 <sup>c</sup>	5.34	0.12 <sup>d</sup>
$\Delta^9$ Desaturase index C14DI*	0.017	0.001 <sup>a</sup>	0.019	0.002 <sup>a</sup>	0.028	0.002 <sup>b</sup>	0.026	0.001 <sup>b</sup>
$\Delta^9$ Desaturase index CLADI**	0.291	0.02 <sup>a</sup>	0.303	0.02 <sup>a</sup>	0.350	0.02 <sup>b</sup>	0.347	0.01 <sup>b</sup>
AI***	2.868	0.07 <sup>a</sup>	2.760	0.05 <sup>a</sup>	2.307	0.07 <sup>b</sup>	2.409	0.06 <sup>b</sup>

<sup>a,b,c,d</sup>Mean values followed by different letters in the same row are significantly different ( $P < 0.05$ ).

Abbreviations are: SFA=saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA=polyunsaturated fatty acids

\* $\Delta^9$ desaturase index C14DI = C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0)

\*\* $\Delta^9$ desaturase index CLADI= *cis*-9 *trans*-11 CLA/ (*cis*-9 *trans*-11 CLA+ C18:1 *trans*-11)

\*\*\*AI = (C12:0 + 4x C14:0 + C16:0)/ (PUFA + MUFA).



**Table 3.** CLA concentration (g/100 g lipids and g/100 g sample) in winter and spring months

Month	<i>cis-9.trans-11</i> CLA		<i>trans-10. cis-12</i> CLA	
	g/100 g lipids	g/100 g sample	g/100 g lipids	g/100 g sample
<b>December</b>	0.81 ± 0.08 <sup>a</sup>	0.05 ± 0.01 <sup>a</sup>	0.07 ± 0.01 <sup>a</sup>	0.004 ± 0.001 <sup>a</sup>
<b>January</b>	0.89 ± 0.07 <sup>a</sup>	0.06 ± 0.01 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>	0.005 ± 0.001 <sup>a</sup>
<b>April</b>	1.27 ± 0.09 <sup>b</sup>	0.08 ± 0.01 <sup>b</sup>	0.11 ± 0.01 <sup>b</sup>	0.007 ± 0.001 <sup>b</sup>
<b>May</b>	1.18 ± 0.07 <sup>b</sup>	0.07 ± 0.01 <sup>ab</sup>	0.10 ± 0.01 <sup>b</sup>	0.006 ± 0.001 <sup>ab</sup>

CLA analyses were carried out in triplicate.

<sup>a,b</sup>Mean values followed by different letters in the same column are significantly different ( $P < 0.05$ ).

Since CLA possess several beneficial properties in human health and the large number of factors affecting its presence in dairy products, its concentration in milk as g/100 g lipids and g/100 g sample, were also estimated (Table 3). The recorded levels of *cis-9, trans-11* CLA and C18:2 *trans-10, cis-12* CLA in either winter or in spring milk, indicate that the ovine milk is an important source for the daily intake of CLA in the human diet. It is worthwhile to note that the recommended dietary daily intake of CLA is 0.8-3g per person (Benjamin et al., 2015).

The C18:2 *trans-10, cis-12* CLA levels found in milk in winter (ca 0.08 g/100 g FAMES) were also significantly increased ( $P < 0.05$ ) in spring (ca 0.13 g/100 g FAMES). Compared to C18:2 *cis-9, trans-11* CLA, the milk in both seasons presented lower levels of C18:2 *trans-10, cis-12* CLA. Previous studies have also showed that the major CLA isomer is C18:2 *cis-9, trans-11* CLA in ruminants' milk including ovine milk (Sampelayo et al., 2007). Under conventional feeding conditions of lactating ewes, Toral et al., (2010) and Buccioni et al., (2010) reported C18:2 *trans-10, cis-12* CLA levels of 0.01-0.02 g/100 g FAME and 0.017 g/100 g FAME in the ovine milk, respectively. Under seasonal grazing feeding conditions of lactating ewes, the C18:2 *trans-10, cis-12* CLA levels showed a significant increase ( $P < 0.05$ ) in spring (0.03-0.10% FAME) as compared to winter milk (0.01-0.02% FAMES) (Carloni et al., 2010).

In the present study, the ovine milk produced in spring presented  $\Delta^9$ -desaturase activity indexes C14DI and CLADI higher ( $P < 0.05$ ) than those in winter. The use of  $\Delta^9$ -desaturase activity index C14DI represents better the  $\Delta^9$ -desaturase activity,

since C14:0 of the milk is totally formed via *de novo* synthesis in the mammary gland while *cis-9 trans-11* CLA used in CLADI index is either formed in the rumen as an intermediate product of biohydrogenation of linoleic acid or endogenously in the mammary gland by the action of the  $\Delta^9$ -desaturase enzyme. In agreement to our work, similar seasonal variations of  $\Delta^9$ -desaturase activity indexes were reported for milk produced by lactating ewes (Soják et al., 2013) or cows (Lock and Garnsworthy, 2003) under grazed pasture feeding conditions.

The atherogenicity index (AI) is associated with proatherogenic and antiatherogenic FAs and may reflect also the atherogenicity status of FAs in produced milk. The AI index of the milk was found significantly ( $P < 0.05$ ) lower in spring than in winter. In agreement to the present study, seasonal variations of the atherogenicity index (AI) were reported for milk produced by lactated ewes (Gómez-Cortés et al., 2009) or cows (Nantapo et al., 2014) under grazed pasture feeding conditions.

In conclusion, the SFA were significantly higher ( $P < 0.05$ ) in the ovine milk of winter than that of spring, but the saturated stearic acid (C18:0) showed a different behavior with significantly lower ( $P < 0.05$ ) values in winter than in spring. The MUFA including C18:1 *trans-11* VA, PUFA including C18:2 *cis-9, trans-11* CLA and C18:2 *trans-10, cis-12* CLA of the ovine milk were significantly higher ( $P < 0.05$ ) in spring than in winter months.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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