Effect of forage preservation method on fatty acid composition and oxidative stability of organic sheep milk

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Abstract

The aim of this study was to evaluate the effect of different forage preservation method in mountainous areas on the relationship between the fatty acid (FA) profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk. Twenty-four multiparous Turcana ewes were randomly assigned to 3 treatments: grazed grass (G), hay grass (H), and grass silage (GS). Indoor ewes were offered *ad libitum* grass hay or grass silage. All animals received 300 g DM grain mix (triticale and barley,1:1) per day. Conservation of grass by drying compared with ensiling resulted in lower forage 18:2n-6, 18:3n-3 and fat-soluble vitamin concentrations. Milk from ewes grazed, has a considerably higher concentration of n-3 FA and n-6 FA but and a higher content of nutritionally beneficial *trans*-fatty acids (e.g. CLA; conjugated linoleic acid, VA; vaccenic acid) than milk from ewes fed hay or grass silage. In spite of lower content, milk fat 18:2n-6 and 18:3n-3 content was higher ($P < 0.05$) for hay than for silage diets (2.34 *vs*. 1.64 and 2.11 *vs*. 1.70 % of total FA, for hay, and silages, respectively). Forage conservation method had no clear effects on milk VA or CLA content. Compared with silage, hay diets resulted in milk containing lower ($P < 0.001$) α-tocopherol and retinol concentrations, but had no effect on γ-tocopherol. There was no clear association between increased levels of the α-tocopherol and retinol in milk and a lower risk of lipid oxidation (lower concentration of malondialdehyde). There was a marked effect of composition of the lipids on the oxidative stability of milk, where high concentrations of PUFA (polyunsaturated FA), especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation.

Keywords: *roughage, fodder preservation, functional fatty acids, CLA, fat-soluble antioxidants, organic sheep milk*

Zusammenfassung

Die Wirkung von Raufutter-Konservierungsmethoden auf die Fettsäure-Zusammensetzung und Oxidationsstabilität der Milch von Schafen im Ökolandbau

Ziel der Untersuchung war die Bewertung der Einflüsse unterschiedlich konservierter Raufuttermittel, die ökologisch produziert wurden, auf das Fettsäurenmuster, den Gehalt an fettlöslichen Antioxidantien und der oxidativen Stabilitat in Schafmilch. 24 multipare Milchschafe der Rasse Turcana wurden in drei Interventionsgruppen randomisiert mit Weidegras (G), Heu (H) oder Grassilage (GS) gleicher Herkunft (Bergwiesen) *ad lib.* gefüttert. Alle Schafe erhielten pro Tag zusätzlich 300 g TM geschrotetes Getreide. Im Vergleich zu dem Silagefutter enthielt das Heu niedrigere Konzentrationen an Linolsäure (18:2n-6), Linolensäure (18:3n-3) und fettlöslichen Vitaminen. Die Schafe, die mit frischem Weidegras gefüttert wurden, hatten signifikant höhere Konzentrationen an wertgebenden n-3 bzw. n-6 Fettsäuren als auch trans-Fettsäuren (z. B. CLAs: konjugierte Linolsäure; VA: Vaccensäure) im Vergleich zu den Konzentrationen in der Milch von Schafen, die mit Heu oder Silage gefüttert wurden. Der Gehalt an 18:2n-6 und 18:3n-3 Fettsäuren ($p \leq 0,05$) war in der Milch von Schafen, die mit Heu gefüttert wurden, höher als in der Milch von Schafen, die mit Silage gefüttert wurden (2,34 vs. 1,64 und 2,11 vs. 1,70 % totale Fettsäuren für Heu bzw. Silage). Die Futterkonservierungsmethode hatte keinen signifikanten Effekt auf den Milchgehalt an CLA bzw. VA. Es konnte keine signifikante Korrelation zwischen den höheren Konzentrationen an α-Tocopherol und Retinol und einem niedrigeren Risiko an Fett-Oxidation (z. B. niedrigere Malondialdehydekonzentrationen) festgestellt werden.

Schlüsselwörter: *Raufutter, Futterkonservierung, funktionale Fettsäuren, CLA, Anti-Oxidanten, Ökologische Schafmilch*

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1 Introduction

Organic sheep milk production systems, compared with conventional farms, produce milk with markedly low levels of milk fat SFA (saturated fatty acids) and high levels of FA (fatty acids) having healthy properties (also named functional fatty acids), especially vaccenic acid (VA; *t*11-C18:1), conjugated linoleic acid (CLA; in particular isomer *c*9,*t*11-C18:2), and omega-3 FA (ALA; α-linolenic acid, EPA; eicosapentanoic acid, DHA; docosahexaenoic acid). These effects are essentially due to grass feeding because of the specific environmental conditions of the mountains as well as of the particular high botanical diversity of grass species which could specifically affect the FA composition of milk fat (Collomb et al., 2008).

The organic sheep sector is dominated by three Member States: the United Kingdom, Italy and Spain, representing together 62.7 % of the entire EU organic herd (3.9 million heads). Sheep breeding into organic farming system has a strong growing tendency in Romania in the future. The sheep livestock farmed organically in 2010 was 18883 heads and in 2012 the reached to 51722 heads (Eurostat, 2015).

Forages, even though containing a relatively low level of lipids, are the cheapest and often the major source of beneficial unsaturated fatty acids in ruminant diets.

Sheep farmed in mountainous areas are fed mainly on pastures, with little or none concentrates supplementation of concentrate during summer for the remainder of the year on hay from mountain grass and/or grass silage and low amounts of concentrate. Fresh forages are an important natural source of fatty acids and vitamins in ewes diets. Hay or silage from specific plant species of mountain areas could also affect the FA composition of milk fat (Shingfield et al., 2005; Collomb et al., 2008). Addis et al. (2005) showed that the percentages of CLA, VA and n-3 FA in milk fat seem to be strongly linked to the linolenic acid content of forages.

The disadvantage of milk enriched with functional fatty acids is the possibility to suffer oxidation due to its high content of double-bonded molecules, which are prone to oxidation onset (Puppel et al., 2012). The delicate balance between anti- and oxidative processes in milk is influenced by factors such as ruminant nutrition, degree of fatty acid unsaturation, content of transition metal ions, and content of antioxidants such as tocopherols and carotenoids (Havemose et al., 2006).

Lipophilic antioxidants (vitamin A and E) are naturally occurring compounds that can prevent some of the processes involved in the development of cancer (protecting DNA from oxidative damage) and cardiovascular disease (inhibiting oxidative damage to LDL-low density lipoprotein) (Schönfeldt and Holden, 2009). One of the most important indicators of oxidation process in milk is malondialdehyde (MDA), which is formed during peroxidation of PUFA by the action of reactive oxygen species. Mutagenic and carcinogenic properties of reactive oxygen species have been confirmed by Klaunig et al. (2010). Storage at low temperatures efficiently preserves saturated fats but is not sufficient to protect polyunsaturated fats against oxidation (Dervishi et al., 2012).

Vitamin A can be found in ewe's milk, in contrast to that of cows, only as retinol because dietary β-carotene is converted entirely into this form (Revilla et al., 2016), thus explaining differences in colour between bovine and sheep dairy products. Vitamin E is found in milk in three forms, α-, β-, and γ-tocopherols but α-tocopherol is the most abundant form (Revilla et al., 2014).

While some studies have failed to show an antioxidative capacity of α-tocopherol in milk (Havemose et al., 2006), others conclude that α-tocopherol is one of the most important antioxidants in cow's milk. Puppel et al. (2012) have demonstrated the protective role of vitamin E in relation to the introduction of PUFA supplementation, and thus demonstrated that tocopherol protects against growth of MDA in milk.

The aim of this study was to evaluate the effect of different forage preservation methods in mountainous areas on the relationship between the fatty acid profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk.

2 Materials and methods

2.1 Experimental design and treatments

The study was conducted at the University of Oradea (Romania) over a 8-week period (May to July, 2015). The first three weeks were used as a covariate period (week 1) and for adaptation to dietary treatments (weeks 2 and 3).

This study was undertaken in organic dairy sheep farm (a family farm) that has met the criterions stipulated by the Council Regulations of the EEC, 1991 (No. 2092/1991) and EU, 1990 (No. 1804/1999); Rahmann, 2013) on standards of organic animal husbandry. The organic farm status was checked with the existence of a valid certificate. The farm was located in the mountain area of western Carpathian Mountains (north-western Romania, 46^{0}40'N, 22^{0}29'W, 1240 m above sea level, total annual rainfall 778 mm; mean annual temperature 9.3 °C).

Sheep breeding into organic farming system in Romania is based on native breeds, which are well adapted to mountain and submontain regions with large areas of pastures, and prevalent breed is Turcana (over 50 % of the sheep in Romania belong to this breed). Milk production of the breed is generally low, with ewes producing yields of 40 to 60 kg of milked milk, with the average fat content ranging between 7 to 8 % and 6 % proteins.

Twenty-four multiparous (lactation number 3 to 4) Turcana ewes, were balanced for average body weight (45.7 ± 1.71 kg) and milk yield and were randomly assigned to three treatments: grazed grass (G), hay grass (HG), and grass silage (GS).

An area of pasture of 3.6 ha, situated on soil type argiluviosol, were subdivided into 3 equal parts who were randomly assigned to one of three ways to use: grazing, getting the

grass hay and getting grass silages. The most common species in the pasture in terms of occurrence were ca. 37.4 % *Festuca rubra*, 14.8 % *Dactylis glomerata*, 9.1 % *Phleum pratense*, 8.4 % *Poa pratensis*, 5.2 % *Agrostis capillaris*, 18.3 % legumes (mainly *Trifolium repens*) and other grasses. Botanical composition was determined on forage samples taken randomly by quadratic frame (0.25 × 0.25 m^2) by manual separation of plant species. Botanical composition was calculated by dividing individual species weight by the total weight collected (wet basis).

The pasture was harvested at the early flowering stage. Hay and silages were prepared simultaneously in May: grass used to prepare silages was cut and field-wilted for 6 h, and baled in bags while grass used to prepare hay was left to dry for 4 day, before baling. All the experimental animals were grazing sward before the feeding experiment.

The ewes were managed in experimental conditions exclusively. Outdoor grazing ewes had free access on pasture (12 to 14 h/day) and they were housed overnight. Rotational fenced grazing was practiced, pasture having three identical in size areas. Indoor ewes were offered ad libitum grass hay or grass silage and they had free access in outdoor paddock. All animals received 300 g DM grain mix (triticale and barley, into equal proportions) per day, divided into equal halves and given during the morning and evening milkings. All animals had non-restricted access to water and salt blocks. All forages (pastures, hay, silage and concentrates) were organically produced on farm. Ewes were milked twice per day, by hand, to a sheltered area.

The animals were managed according to the Animal Welfare and Good Clinical Practice (European Commission, 2010) and to those laid down by the local Bioethics Committee (Rahmann et al., 2016).

2.2 Sampling and chemical analyses

Forage samples were collected twice weekly and combined into one sample per week. All the forage samples collected were freeze-dried and ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA). Samples were analysed for dry matter (DM) (ISO 6496, 1999), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest et al., 1991) on a Fibersac analyser (Ankom Technology, Fairport, NY) and nitrogen (N) (Kjeldahl technique: Vapodest Distillation Systems – Gerhardt; AOAC International, 1995).

Samples of forage (n = 5) collected for determine the FA profile were stored immediately at -20 °C, and later lyophilized and kept until analysis.

The milk samples were collected once a week for a period of five weeks. Thus, five samples for analysis were obtained from each ewe (40 milk samples for each group and 120 total samples, respectively). Each sample (200 mL milk) was divided into two parts, one for fatty acid determination and the other one for analysis fat-soluble antioxidant content and MDA (malondialdehyde). Milk samples were kept in 100 mL containers and were protected from light, refrigerated immediately and then brought directly to the laboratory and was stored at -20 °C.

To determine the concentration of FAs in the diets, fatty acid methyl esters (FAME) were prepared by the one-step extraction-methylation method of Sukhija and Palmquist (1988). In order to determine the composition of FAs in milk, the fat was extracted according to the international standard, ISO 14156 / IDF 172, 2001. FAME were prepared according to the method proposed by Christie (1982) and Chouinard et al. (1999) and were determined by gas chromatography using a Varian GC 3600 equipped with FID and a fused silica capillary column (SP 2560 Supelco), 100 m × 0.25 mm i.d., film thickness 0.20 µm. Helium was used as the carrier gas at a flow rate of 1 mL/min. The split ratio was 1 : 100. The oven temperature was programmed at 90 °C and held for 1.50 min, then increased to 210 °C at a rate of 9 °C/min, held at this temperature for 25 min, then increased to 230 °C at 15 °C/min, and held for 7 min. The temperatures of the injector and the detector were set at 270 °C. The FA identification was based on external standards, and calculation of the distribution (in weight percentage) was based on the area of each FA ester corrected for the response factors for the individual FAs. Internal standards were used to determine the percentage of recovery. The CLA isomer reported is *c*9,*t*11-CLA and *t*10,*c*12-CLA. The percentage of each fatty acid was calculated by dividing the area under the FA peak by the sum of the areas under total reported FA peaks.

Determination of vitamins, was made by HPLC (High-Performance Liquid-Chromatography), the extracts obtained from milk samples. Extraction of fat soluble vitamins has been made with a mixture of diethyl ether and petroleum ether (1:1), in aliquots of 20 ml. The ether phase, after separation, was saponified with methanolic KOH solution (10 %), after which vitamins have been extracted in hexane, washed with water in a separator funnel and evaporated to dryness. For the determination of retinol was conducted in a first stage, curve standard using total-trans-retinol solutions (10 to 80 mg/ml). Standard solutions (Sigma) and samples were injected into a system Parkin-Elmer LC-295, equipped with Alltech C18 column (length 15 cm, 4.6 mm internal diameter and particle size 3µm). The mobile phase consisted of acetonitrile/methanol (85:15), with the addition of 2-propanol (30 %) after 8 min.

The analysis of carotenoids in the forages, ethanol: methanol:tetrahydrofuran (75:20:5) was used as a HPLC buffer. The flow rate was at 1 ml/min and the injection volume was 100 µl. The wave length for detection was 450 nm. External standards of β-carotene were used in order to quantify the carotenoids.

Dosage tocopherol was made using the same analysis conducted extract retinol. After evaporation of the hexane phase was added to methanol, after which separation of the tocopherol was by HPLC using the column with characteristics described above. The mobile phase for the different tocopherols analysed was acetonitrile:methanol (85:5)/isopropanol 90/10. Tocopherol analysis in feed samples was performed in the same way as in the milk samples. Instead of 2 ml of milk, 0.4 g of feed was taken to start the analysis. Detection wavelengths for α- and γ-tocopherol were set at 290 nm and 296 nm respectively. By injecting known

amounts of α-tocopherol (Sigma-Aldrich) and γ-tocopherol (Sigma-Aldrich) standard containing internal standard Tocol, realized a standard curve. Quantitative analysis of α- and γ-tocopherol in the samples was performed by HPLC Millennium software using peak area measurement and standard curve.

Malondialdehyde (MDA) is the main product of oxidation of PUFAs, it is widely used as a marker of lipid peroxidation in food and biofluid (Andrei et al., 2008). Thiobarbituric acid (TBA) reacts with MDA to form a pink compound in an acid medium, which was measured photometrically at 532 nm using a spectrophotometer UV-VIS Jenway 6315. The standard curve to calculate the required concentration of MDA was obtained by acid hydrolysis of 1,1,3,3-tetrametoxipropan (TMP) (Andrei et al., 2008).

2.3 Statistical analysis

Statistical analyses were carried out with SAS (SAS Institute Inc., 2005). Data for fatty acids profile and fat-antioxidants in milk were analysed using ANOVA model with factorial term for diet composition. Comparison among means was carried out using Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Pearson correlations between the parameters content was done with the XLSTAT Pearson Edition (Addinsoft SARL, 75018 Paris). Multivariate analysis was carried out with PAST (Paleolontological Statistical Software) (Hammer and Harper, 2005). There was used a multivariate sequence (PCA – Principal component analysis, MANOVA – Multivariate analysis of variance and HCA – hierarchical cluster analysis) in order to get the proper number of clusters with 95 % level of significance.

3 Results

The chemical composition, FA levels and fat-soluble antioxidants of the forages from diets is presented in Table 1. As expected, the fresh grass had a higher PUFA concentration, especially α-linolenic acid (C18:3n-3, ALA) (51.8 % of total FAME) and fat-soluble antioxidants, compared with the hay grass, which had a higher SFA content. The sums of total PUFA were 41.3 % and 17.1 % greater in the fresh pasture compared to hay and grass silage. Conservation of grass by drying compared with ensiling resulted in lower forage linoleic acid (C18:2n-6, LA), α-linolenic acid and fat-soluble vitamin (α-tocopherol and β-carotene) concentrations. The grain mix was good source of LA and MUFA.

Table 2 shows the results of fatty acids and fat-soluble antioxidants contents in milk in the three groups. The results clearly show a higher concentration of beneficial *trans*-fatty acids (e.g. CLA, VA), sum of n-3 FAs and fat-soluble

Table 1
Fatty acid composition and fat-soluble antioxidants content of experimental forages and concentrate supplement

	Forages			SEM	p-value	Concentrate
	Grass fresh	Grass hay	Grass silage			supplement
Dry matter (DM), g/kg	207	846	267	-	-	877
CP, g/kg DM	184	139	107	-	-	134
NDF, g/kg DM	461	526	486	-	-	178
ADF, g/kg DM	241	320	305	-	-	94
Fatty acids, (% of FAME)						
C12:0	0.46^{a}	0.61^{a}	0.11^{b}	0.06	< 0.05	0.04
C14:0	0.50^{b}	0.70^{b}	1.53^{a}	0.04	< 0.01	0.21
C16:0	16.71^{c}	26.18^{a}	22.43^{b}	0.63	< 0.01	17.43
C16:1	0.19	0.32	0.40	0.05	NS	0.64
C18:0	1.90^{c}	5.24^{a}	3.17^{b}	0.10	< 0.01	3.29
C18:1	3.71^{b}	6.97^{a}	3.32^{b}	0.14	< 0.001	24.69
C18:2 n-6 (LA)	19.40^{a}	16.18^{b}	18.43^{a}	0.21	< 0.01	41.20
C18:3 n-3 (ALA)	51.80^{a}	34.20^{c}	42.35^{b}	0.92	< 0.01	3.52
SFA	19.57^{c}	32.73^{a}	27.24^{b}	0.74	< 0.01	20.97
MUFA	3.89^{b}	7.69^{a}	3.74^{b}	0.09	< 0.01	25.33
PUFA	71.20^{a}	50.38^{c}	60.78^{b}	0.86	< 0.01	44.72
Fat-soluble antioxidants, mg/kg DM						
β-carotene	63.70^{a}	19.30^{c}	46.90^{b}	0.98	< 0.01	1.80
α-tocopherol	71.70^{a}	8.40^{c}	54.80^{b}	1.17	< 0.001	9.10
γ-tocopherol	15.80^{a}	5.20^{b}	8.90^{b}	0.52	< 0.01	12.30

CP – Crude Protein; NDF – Neutral Detergent Fibre; ADF – Acid Detergent Fibre.
FAME – fatty acid methyl esters; SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA – polyunsaturated acid.
^{a,b,c} – Values within rows with different superscript are significantly different ($P < 0.05$).

Table 2
Effects of feeding either grazing fresh, hay or grass silage on fatty acid composition, fat-soluble antioxidants and oxidative stability of milk

	Grass fresh (G)		Grass hay (H)		Grass silage (GS)	
	Mean	SEM	Mean	SEM	Mean	SEM
Fatty acid composition (% of FAME)						
C4:0 – C10:0	17.26^{b}	0.93	21.52^{a}	1.24	19.23^{a}	1.18
C12:0	2.07^{c}	0.41	2.81^{b}	0.50	3.54^{a}	0.55
C14:0	7.83^{b}	0.70	9.37^{a}	0.65	8.99^{a}	0.83
C16:0	18.12^{b}	1.34	22.68^{a}	1.52	22.44^{a}	1.39
C18:0	9.76	0.81	10.11	0.80	10.28	0.92
C18:1 n9t	0.59	0.04	0.47	0.04	0.52	0.04
C18:1 *trans*-11 (VA)	5.69^{a}	0.40	3.28^{b}	0.28	3.05^{b}	0.32
C18:1 n9c	24.51^{a}	1.72	20.07^{b}	1.59	23.46^{a}	1.64
C18:1 *cis*-11	0.64^{a}	0.18	0.41^{b}	0.21	0.40^{b}	0.20
C18:2 n6t	0.39	0.12	0.36	0.11	0.38	0.14
C18:2 n6c (LA)	2.87^{a}	0.39	1.76^{b}	0.27	1.02^{c}	0.20
CLA *cis*-9, *trans*-11 (RA)	3.06^{a}	0.35	1.81^{b}	0.32	1.82^{b}	0.31
CLA *trans*-10, *cis*-12	0.17^{a}	0.03	0.11^{b}	0.02	0.09^{b}	0.02
CLA-total	3.23^{a}	0.38	1.90^{b}	0.35	1.93^{b}	0.29
C18:3 n-3 (ALA)	2.17^{a}	0.51	1.63^{b}	0.47	1.15^{c}	0.44
C20:4 n-6	0.28	0.02	0.22	0.02	0.24	0.02
C20:5 n-3 (EPA)	0.41^{a}	0.06	0.20^{b}	0.04	0.21^{b}	0.04
C22:6 n-3 (DHA)	0.47^{a}	0.07	0.28^{b}	0.05	0.34^{b}	0.05
Unidentified FA	3.71	0.62	2.93	0.78	2.82	0.59
SFA	55.04^{c}	2.34	66.49^{a}	2.73	64.48^{b}	2.47
UFA	41.25^{a}	1.64	30.58^{c}	1.32	32.70^{b}	1.26
MUFA	31.43^{a}	2.86	24.23^{c}	2.59	27.43^{b}	2.71
PUFA	9.82^{a}	0.88	6.35^{b}	0.61	5.27^{c}	0.64
PUFA n-6	3.54^{a}	0.36	2.34^{b}	0.29	1.64^{c}	0.32
PUFA n-3	3.05^{a}	0.41	2.11^{b}	0.30	1.70^{c}	0.24
HFA[1]	28.02^{b}	1.73	34.86^{a}	2.11	34.97^{a}	1.97
PI[2]	7.60^{a}	0.82	5.38^{b}	0.67	3.70^{c}	0.41
Fat-soluble antioxidants (µg/100 g of milk)						
Retinol	93.46^{a}	3.16	48.81^{c}	2.04	71.16^{b}	2.27
α-tocopherol	187.39^{a}	14.11	89.34^{c}	9.72	122.60^{b}	10.34
γ-tocopherol	16.23	0.70	13.36	0.54	14.11	0.63
Oxidative stability of milk						
MDA – g/L of milk	3.31^{a}	0.18	3.04^{a}	0.13	2.67^{b}	0.13

1 HFA (hypercholesterolaemic fatty acids) [C12:0 + C14:0 + C16:0].
[2] PI (polyunsaturated index) = C18:2 n-6 + (C18:3 n-3 x 2).
FAME – fatty acid methyl esters; CLA – conjugated linoleic acids. SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA – polyunsaturated acid; FA – fatty acid; MDA – Malondialdehyde.
[a,b,c] – Means within the same row with different letters differ significantly according to Duncan's tests ($p < 0.05$).

antioxidants in milk produced from grazing animals, than ewes fed hay or silage grass. G ewes presented a significantly greater proportion of PUFA in milk than the H and GS group, which agrees with their higher proportion in grazed pasture compared to hay and grass silage (Table 1). Total PUFA n-6 milk fatty acids were greater in the G group than in the H and GS group due to the greater amount of linoleic acid in milk from G ewes than in milk from H and GS ones (Table 2).

In spite of lower intakes of LA and ALA, their contents were higher in the milk fat of ewes fed hay than in that of ewes fed grass silages. The forage conservation method had no clear effect on milk *trans*-18:1 acids or CLA contents.

Table 3
Pearson's correlation matrix between the bioactive compounds (fatty acids and fat-soluble antioxidants) and oxidative stability (MDA concentration) of milk.

	MDA	PUFA	n-3 FA	n-6 FA	CLA	Retinol	γ-tocopherol	α-tocopherol
MDA	**1**	**-0.5971**	**-0.6351**	**-0.5792**	**-0.5374**	0.1076	0.1389	0.1988
PUFA	**-0.5971**	**1**	**0.9462**	**0.9864**	**0.9585**	0.2036	0.3510	**0.5196**
n-3 FA	**-0.6351**	**0.9462**	**1**	**0.9165**	**0.8584**	0.1127	0.3790	0.4747
n-6 FA	**-0.5792**	**0.9864**	**0.9165**	**1**	**0.9155**	0.1425	0.3210	0.5068
CLA	**-0.5374**	**0.9585**	**0.8584**	**0.9155**	**1**	0.3429	0.3423	**0.5191**
Retinol	0.1076	0.2036	0.1127	0.1425	0.3429	**1**	0.3730	0.4970
γ-tocopherol	0.1389	0.3510	0.3790	0.3210	0.3423	0.3730	**1**	-0.0217
α-tocopherol	0.1988	**0.5196**	0.4747	0.5068	**0.5191**	**0.5970**	0.0217	**1**

Bold values significantly different, $p < 0.05$
MDA – malondialdehyde, PUFA – polyunsaturated acid; FA – fatty acid, CLA – conjugated linoleic acids.

Milk fat of ewes fed hay or grass silages than in that of ewes fed grass fresh has a higher HFA (hypercholesterolaemic FA: C12:0+C14:0+C16:0) proportion, whereas the polyunsaturated index is lower.

Concentrations of α-tocopherol and retinol were higher ($P < 0.05$) in milk from grazing fresh. Relative to silages, milk from hay diet contained lower ($P < 0.05$) amounts of α-tocopherol and retinol, but the concentrations of γ-tocopherol were independent of forage conservation method (Table 2).

The TBA test (thiobarbituric acid) provides information about the level of MDA, a secondary compound formed in lipid oxidation. The highest level of MDA was recorded in group G (3.31 g/l of milk). On the other hand, the lowest level was recorded in GS group, 2.67 g/l of milk. In the milk we found a negative association between the MDA and PUFA for all PUFA categories analysed (PUFA total, n-3 FA, n-6 FA and CLA) (Table 3). There was no clear association between increased levels of the α-tocopherol and retinol in milk and a lower risk of lipid oxidation (lower concentration of MDA, respectively). There was a marked effect of composition of the lipids on the oxidative stability of milk, where high concentrations of PUFA, especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation.

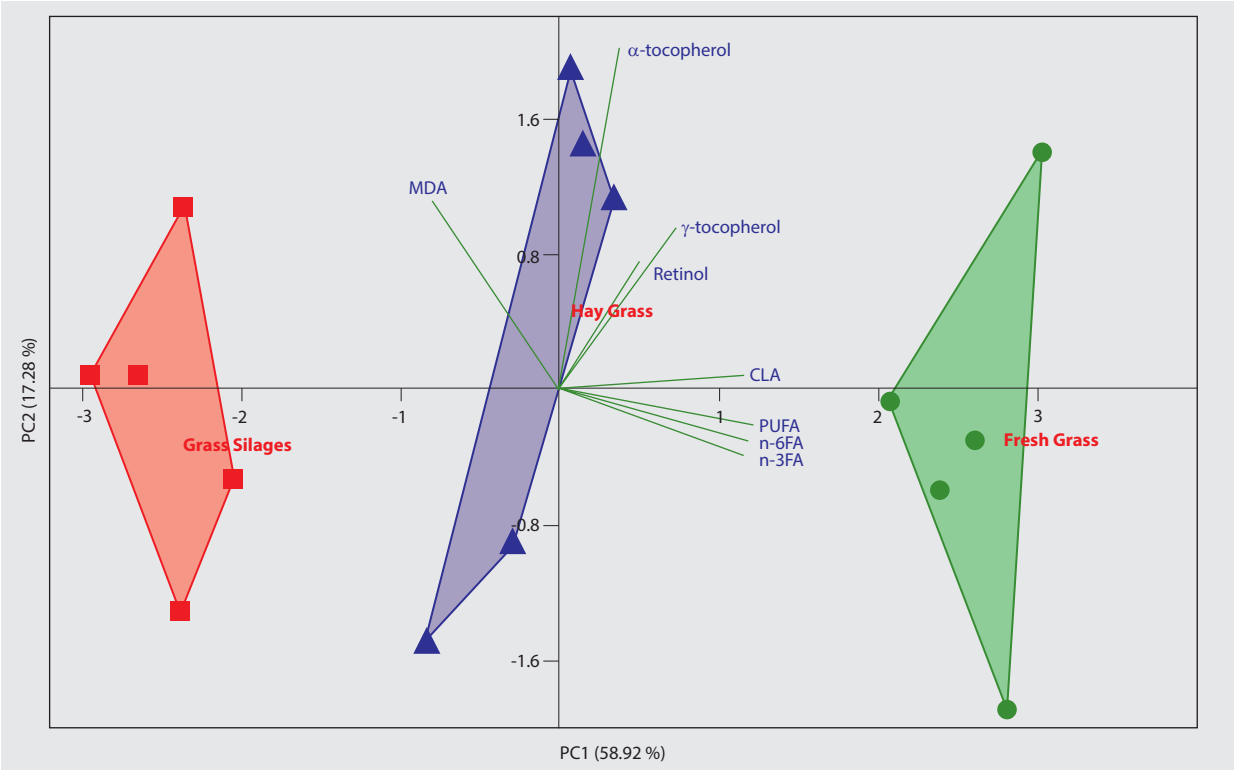

Figure 1
Relationship between healthy fatty acid, fat-soluble antioxidants concentration and oxidative stability of milk (component analysis biplot).

In order to present a complete evaluation of the effect of different forage preservation method in mountainous areas using the fatty acid profile, fat-soluble antioxidants contents and oxidative stability of organic sheep milk, a multivariate sequence was performed.

The multivariate sequence was intended to assess the sample clusters, and consists of: principal component analysis (PCA), multivariate analysis of variance (MANOVA) and hierarchical cluster analysis (HCA). Figure 1 shows the PCA biplot that contains both the scores (i.e. samples principal coordinates) and loadings (i.e. variables/parameters principal coordinates). Loadings are represented as vectors with origin as starting points. The vectors are correlated each other and with the principal axes (PC1 and PC2). From the PCA biplot (see Figure 1), it can be considered four variable groups. First group gathers: CLA, PUFA, n-6 FA and n-3 FA; the second group: retinol and γ-tocopherol; third group has only one variable α-tocopherol and the same the fourth group MDA. These groups are "shell" distributed over the three quadrants: first, second and fourth, and geometrically generates non-overlapping sample groups distributed along the first principal axis. The fresh grass sample group has highest abundance of first group of variables. The hay grass sample group is divided in two parts; one has highest content of α-tocopherol and second variable group, and the other part has average abundance of the first variable group. The grass silages sample group has the highest content of MDA and the lowest content of the first variable group.

4 Discussion

Forages are often the main source of fatty acids in the diet but the effects of conservation method on milk fatty acid composition and oxidative stability of milk are not well defined (Chilliard et al., 2001; Shingfield et al., 2005). Exposure to solar radiation and the duration of wilting affecting oxidative losses of unsaturated fatty acids and fat-soluble antioxidants (e.g. α-tocopherol and β-carotene) from cut grass. In the present experiment, hay contained lower amounts of all measured fatty acids, α-tocopherol and β-carotene than silages.

Fresh grass is a rich source of ALA and compared to hay or grass silage diets, results in increased milk fat concentrations of *c*9-C18:1, *t*11-C18:1, total n-3 FA and *c*9,*t*11-CLA, and decreased C4:0-C16:0 concentrations. Fresh grass resulted in higher concentration of *c*9-C18:1 in milk fat than hay and silage that may arise from higher uptake of *c*9-C18:1 from arterial plasma across the mammary glands (Halmemies et al., 2013). Feeding fresh grass decreased milk fat C4:0-C16:0 content relative to dried hay and grass silage primarily due lower de novo synthesis in the mammary glands, consistent with increases in the supply of preformed fatty acids inhibiting synthesis of C4:0-C16:0 de novo (La Terra et al., 2010; Shingfield et al., 2013).

The effects of fresh grass on increased the milk fat content of ALA, VA and CLA are related to the high content of ALA in green pasture, which is partly biohydrogenated into VA in the rumen and then secreted into milk and partially converted into *c*9,*t*11-CLA in the mammary tissue by the action of stearoyl-CoA desaturase (Nudda et al., 2014). Grazing pasture may enhance the growth of specific bacteria in the rumen, stimulating the production of CLA and/or blocking the final reduction of VA to stearic acid (C18:0), as well as increasing n-3 FA (Dervishi et al., 2012). Cabiddu et al. (2005) obtained a linear relationship between the total daily intake of ALA and the estimated daily amount of VA and *c*9,*t*11-CLA in sheep milk. In the present study, fresh pasture are richer in ALA (51.8 and 34.2 *vs*. 42.35 for fresh pasture and hay *vs*. grass silage, respectively), which can stimulate the CLA production.

Grass conservation through hay making or ensiling leads to decreases in ALA concentrations, with hay having lower LA and ALA concentrations than grass silage (16.18 *vs*. 18.43 and 34.2 *vs*. 42.35 % of FAME, respectively) (Table 1). Higher oxidative losses of LA and ALA in hay as compared with silage of Italian ryegrass (*Lolium multiflorum*) were reported by Dewhurst et al. (2006). Nevertheless, milk from hay diets they are richer in LA and ALA than milk from silage diets (Table 2), due to higher transfer efficiency from diet to milk with hay than with grass silage (Shingfield et al., 2005). Incubations in vitro show that the rate and extent of LA and ALA biohydrogenation are higher for ensiled than for dried grass (Boufaied et al., 2003). Earlier studies show the rate of C18:0 formation during incubations with mixed rumen bacteria in vitro to be higher for silage than for hay prepared from the same grass (Boufaied et al., 2003), which suggests that both lipolysis and biohydrogenation of forage lipids are lower when grass is dried rather than ensiled.

The forage conservation method had no clear effect on milk *t*11-C18:1 acids or CLA contents. This result was not expected as Collomb et al. (2008) reported a positive association between proportion of grass silage in the cows diet and content of *c*9,*t*11-CLA and *t*11-C18:1 in milk. Previously, forage conservation by drying compared with ensiling has been reported to decrease (Onetti et al., 2004) or have no effect (Nozière et al., 2006) on milk fat *c*9,*t*11-CLA content. The inconsistencies in milk fat responses between studies may be explained by the large differences in treatment effects on LA and ALA intake.

Compared to the hay diet the decrease in the concentration of SFA in milk fat from ewes fed grass silage may be due in part to the more important amounts of dietary PUFA provided by silage than by hay. These PUFA and/or their biohydrogenation products are potent inhibitors of mammary SFA synthesis by directly inhibiting acetyl-CoA carboxylase activity (Kalač and Samkova, 2010). Milk fat of ewes fed hay or grass silages than in that of ewes fed grass fresh has a higher HFA proportion, whereas the proportion of FA having healthy properties (e.g. VA, *c*9,*t*11-CLA and n-3 FA) is lower. Extensive lipolysis during forage preservation can be among the causes of these differences. The higher concentration of oleic acid (23.46 and 20.07 % of FAME) in milk fat from ewes fed grass silage compared to hay may be due to the fact that silage contains more PUFA than hay.

The effect of forage preservation method on α-tocopherol and β-carotene concentrations reflected more extensive

losses of these vitamins during the drying than during the ensiling of grass. Havemose et al. (2006) also report higher concentrations of α-tocopherol in silage than in hay (35 *vs*. 15 mg/kg DM) prepared from the same mixed timothy-alfalfa swards. There can be losses of up to 80 to 90 % of α-tocopherol and β-carotene in hay, but in well-fermented silages the losses are often less than 20 % (Havemose et al., 2006; Marino et al., 2012).

Fresh grass is rich in β-carotene and α-tocopherol and higher intake of α-tocopherol has been shown to result in a higher output of α-tocopherol in milk (Focant et al., 1998). In the present study, α-tocopherol and retinol in milk increased when ewes were feeding to pasture, which could be considered as a natural consequence of what was stated above. This increase, not found to be sufficient to prevent milk oxidation. Focant et al. (1998) showed improved oxidative stability of the milk when cows were fed supplements of α-tocopherol to the diet, and the concentration of α-tocopherol in the milk reached 2,670 µg/L. This indicates that concentrations of α-tocopherol in the milk (893 to 1873 µg/L) in this study needed to be higher to improve the oxidative stability.

In this studies we found a negative association between the MDA and PUFA for all PUFA categories analysed (PUFA total, n-3 FA, n-6 FA and CLA) (Table 3). These results are in agreement with previous studies in which lipid oxidation have been shown to be highly dependent on the content and composition of the PUFA in milk (Havemose et al., 2006; Juhlin et al., 2010). A higher degree of lipid oxidation was found in milk of ewes fed the grass fresh compared of hay or grass silages diet. The higher content of natural antioxidants (retinol and α-tocopherol) did not prevent oxidation and higher contents of n-3 FAs (3.05 %, 2.11 % and 1.7 % of total FAs in fresh grass, hay and grass silages, respectively) were thought to be the cause. The role of α-tocopherol and retinol on the oxidative stability of the milk appears to be less important than the variation in the fatty acid profile. It was observed that oilseed (rapeseed and linseed) included in grass silage-based cow diets could increase the concentration of n-6 FA and n-3 FA in milk fat and the content of α-tocopherol but the resistance to oxidation was reduced (Focant et al., 1998). The relationship between levels of α-tocopherol, retinol and lipid oxidation is not clear and there are also other antioxidants present in milk which in combination with various prooxidants may add to the complexity of PUFA oxidation (Juhlin et al., 2010).

5 Conclusions

The nature of forages consumed by ewes has a large effect on fatty acid composition and oxidative stability of milk. High intakes of PUFAs and natural antioxidants (lipid-soluble vitamins) from fresh pasture, result in higher concentrations of FA n-3, CLA and antioxidants in milk fat. Ensiling is superior to haymaking in preserving PUFA, α-tocopherol and β-carotene in forages. Milk from hay diet, had concentration higher FA n-3 and CLA but lower levels of antioxidants, compared with the silage diet. The high concentrations of PUFA, especially the concentration of n-3 FA were associated with an increasing risk of lipid oxidation from milk.

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