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RESEARCH ON OXIDATIVE STABILITY OF MILK IN RELATION TO ITS CONTENT IN FUNCTIONAL FATTY ACIDS AND LIPOPHILIC ANTIOXIDANTS

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Abstract

*The aim of this research is to evaluate the influence of the preservation method of herbage (pastures) on the content of functional fatty acids (FA), non-enzymatic lipophilic antioxidants (β-carotene, retinol and α-tocopherol) and oxidative stability of sheep milk. A sample of 24 lactation sheep belonging to Turcana breed were distributed into three relatively homogeneous groups, after which they were randomly allocated one of three experimental diets as follows: **P** - maintained on pasture, **F** - fed with hay ration and **S** - fed with silage herbage ration. Food and milk samples were analyzed in terms of content in functional FA, lipophilic antioxidants and malondialdehyde (MDA- as lipid peroxidation byproduct). The nature of the food basic forage (i.e. pasture, hay, silage) resulted in profound changes in the profile of fatty acids, and the content of lipophilic antioxidants and MDA that was used to monitor the oxidative stability of milk. The pasture fresh grass provided the highest concentrations of functional FA in milk (C18:1 trans-11, C18:2 c9, t11; C18:3n-3, C20:5n-3 and C22:6n-3), the most amount of lipophilic antioxidants (β-carotene, retinol and α-tocopherol) and the best milk oxidative stability demonstrated by the low content of malondialdehyde (MDA). Of the two methods of preservation of pasture grass (drying or silage), the hay provided both a higher concentration of functional FA and lipophilic antioxidants in milk but also a better oxidative stability of milk compared to the silage. Oxidative stability of milk negatively correlated (inversely) with functional fatty acid composition and correlated positively with the concentration of α-tocopherol. Retinol concentration did not significantly contribute to providing oxidative stability of milk, but it correlated positively with the concentration of α-tocopherol in milk, most likely due to the antioxidant properties of tocopherol and retinol protection. One was unable to establish a correlation between the milk content in β-carotene content and the oxidative stability, and the milk content in MDA (malondialdehyde), respectively.*

Key words: milk, omega-3 FA, CLA, VA, antioxidant, MDA.

INTRODUCTION

Milk and dairy products' fat, due to its content in saturated fatty acids, cholesterol and *trans* fatty acids has been perceived by consumers as being harmful. Moreover, most nutritionists recommend eating skimmed fat dairy. However, scientific studies have shown that whole milk has been more effective in preventing cardiovascular disease than skimmed milk (Steinmetz et al., 1994). Manipulating the composition of milk fat, raising the concentration of Vaccenic acid (C18:1 *trans*-11, VA), conjugated linoleic acid (C18:2 *cis*-9, *trans*-11, RA) and Omega-3 (ALA C18:3n-3, EPA, C20:5n-3, DHA, C22:6n-3) and reducing the share of saturated fatty acids (SFA), represents a huge challenge for research in this area.

Improving fatty acid profile of milk has the disadvantage of increasing its sensitivity to autoxidation and the emergence of some toxic products (i.e. aldehydes, ketones), which may alter the psychosensorial quality of milk and dairy (alien flavours and tastes) (Reklewska et al., 2002). Therefore there is an interest to increase the content of non-enzymatic lipophilic antioxidants (β-carotene, retinol and α-tocopherol), in order to increase the oxidative stability of foods with a high content in poly-unsaturated fatty acids.

Natural pasture is an important source of PUFA and especially C18:3 n-3, and the lipophilic antioxidants in animal feed. Hay drying by exposure to sun leads to significant losses of unsaturated fatty acids in grass (Boufaied et al., 2003). Several studies have shown that increasing the share of PUFA in the structure of milk fat is corroborated with an increased susceptibility to autoxidation and development of alien flavours in milk (Kristensen et al., 2004) because PUFA favours the capture of a hydrogen atom and thus the oxidation of fats starts.

Few studies have been initiated to determine the oxidative stability of sheep milk in relation to its content in fatty acids and natural functional nonenzimatic lipophilic antioxidants. Furthermore, no studies have been undertaken on the influence of green fodder preservation method of meadows (hay or silage) on the fatty acid profile and the content of lipophilic antioxidants in milk and their combined effect on the oxidative stability of sheep raw milk.

The aim of this research is to evaluate the influence of the preservation method of grasslands (pastures) herbage on the content of functional fatty acids (FA) and lipophilic antioxidants (β-carotene, retinol and α-tocopherol) of sheep milk fat and the oxidative stability of the milk. Finally, one aims to maximize by naturally means the milk concentration in bioactive compounds (functional fatty acids and lipophilic antioxidants) which are beneficial to human health and preserving its quality for a longer period of time.

MATERIAL AND METHOD

The experiment was conducted in a private farm located in submontane area (1248 m altitude). A pasture surfacing bout 8.6 ha was divided into three smaller parts of about equal size, which were randomly assigned to one of three means of use: grazing, harvesting in the early phase of grass blossoming and grass transformation into hay, respectively (drying by exposure to sun and baling) and in silage (prior wilting - 20 hours and then storage in concrete silage cells). In terms of flora, the meadow was comprised of *Festuca rubra*, *Phleum pratense*, *Poa pratensis*, *Dactylis glomerata*, *Trifolia repens* and different species of weed. A sample of 24 lactation sheep (lactation 3-4) belonging to Turcana breed were distributed into three relatively homogeneous groups, after which they were randomly allocated one of three experimental diets as follows: **P** - maintained on pasture, **F** - fed with hay ration and **S** - fed with silage herbage ration. Feed intake was completed in all three groups with 300 g/day of concentrate mixture. Basic fodder of ration was provided *ad libitum* and concentrate mixtures were administered twice daily in equal parts during milking.

On weekly basis (n = 3), samples consisted of milk (60:40, am:pm) were collected and analyzed in terms of content in functional FA, lipophilic antioxidants and malondialdehyde (MDA – as lipid oxidation byproduct).

Fat extraction and analysis of functional FA in milk was carried out as described by Mierliţă (2012), by gas chromatography.

Determination of Β-carotene was made by HPLC (High-Performance Liquid Chromatography), reverse phase, described by La Terra et al., (2010). The extraction was done by methanol in 20 µl aliquots and carotenoid extract separation was made by repeating the procedure until reaching the colourless filtrate phase. Saponification was done with by methanolic KOH solution (10%), after which the carotenoids were extracted by ethyl ether. Β-carotene analysis was done by a Parkin-Elmer LC-295 device, equipped with Alltech C18 column (particle size: 3µm, 150 x 4.6 mm internal diameter). The mobile phase was: 85% acetonitrile/15% methanol by the addition, after 8 min. of 2-propanol (30%).

Determination of α-tocopherol was made according to the method described by Terre et al., (2010) as follows: in one aliquot one added 2 ml of milk, BHT, KOH, distilled water and standard solution of α-tocopherol. The tubes were placed at 800ºC in a water bath with continual stirring. Α-tocopherol extraction was done by means of a mixture of hexane and water (2/1, v/v). After evaporation of the hexane phase, methanol was added, after which the separation of α-tocopherol was carried out by means of HPLC using the column with the features described above.

Malondialdehyde (MDA) is the main product resulted from the enzymatic peroxidation of PUFAs, and it is widely used as an index of lipid oxidation in food and biofluids (Kanner et al., 1991). Therefore, in our research, MDA was determined as a product of lipid peroxidation and used as an index of lipid oxidation in fodder and milk. Concentration of MDA (malondialdehyde) in feed and milk was established spectrophotometrically, as described by Mroczek (1967).

Data we obtained were subjected to analysis of variance using the GLM (General Linear Models) procedure, version 9.1.3. (SAS Institute, Cary, NC, USA). Multiple comparisons between mean values were carried out made by means of Duncan's test. The significance level was set at $p < 0.05$.

RESULTS AND DISSCUSIONS

The results clearly show that milk from the pasture corresponds best to principles of sanogeneous food nutrition of humans, with a high content of functional fatty acids (Omega-3, CLA and VA) and lipophilic antioxidants (β-carotene, retinol and α-tocopherol), and the best oxidative stability, demonstrated by the low concentration of lipid oxidation byproducts MDA (malondialdehyde).

Drying and storage in silage of pasture grass has led to a decrease by 3-4 times of the content of liposoluble provitamins (β-carotene and α-tocopherol), but also to the acceleration of fat oxidation (Table 1). MDA smaller amount stored in the silage compared with hay (67.3 vs. 93.7 μmol/kg), is caused by the fact that the lipoxygenase activity of plants is inhibited the low pH specific to pickled fodder (Lourenco et al., 2005; Zhong et al., 2006). In addition, oxidation of lipids involves the presence of oxygen, while the ensilage activity is based on anaerobiosis, and the lipoxygenase activity is thus much reduced (Charmley et al., 1994; Focant et al., 1998).

Table 1

Influence of method of preserving herbs on the content in lipophilic antioxidants and oxidative stability of fodder used in sheep feeding

	Treatments			
	Meadow (Pasture)	Hay	Silage	Concentrat e
Content in lipophilic antioxidants (μg/g DM)				
β-carotene	63.7	27.2	31.7	3.6
α-tocopherol	46.8	21.9	15.1	24.1
Oxidative stability				
MDA (μmol/kg fodder)	-	93.74	67.32	37.2

Omega-3 fatty acids were the most affected by the nature of the basic fodder of food were; their share has gradually decreased from 3.05% (% of FAME) in the case of sheep maintained on pasture (P) down to 2.11% in the case of those fed with hay and 1.70% in the case of those fed with fodder from storage (S). When compared with the hay and silage, the meadow (pasture) ensured in the fat composition of milk a significantly higher share for both α-linolenic acid (1.63 - 1.15% vs. 2.17%) and for its metabolites i.e. Eicosapentaenoic acid (C20:5n-3, EPA) and Docosahexaenoic acid (C22:6 n-3, DHA) (Table 2).

Table 2

Influence of forages preservation method of the content in functional fatty acids and antioxidants lipophilic of milk

		Treatments		
		Meadow (Pasture)	Hay	Silage
Functional fatty acids (% of FAME)	PUFA n-3	3.05[a]	2.11[b]	1.70[c]
	C18:3 n-3 (ALA)	2.17[a]	1.63[b]	1.15[c]
	C20:5 n-3 (EPA)	0.41[a]	0.20[b]	0.21[b]
	C22:6 n-3 (DHA)	0.47[a]	0.28[b]	0.34[b]
	CLA cis-9, trans-11 (RA)	3.06[a]	1.81[b]	1.32[c]
	C18:1 trans-11 (VA)	5.69[a]	3.28[b]	3.05[b]
Lipophilic antioxidants (µg/100 g fat)	β-carotene	40.3[a]	28.2[b]	35.8[ab]
	Retinol	4.8	4.0	4.0
	α-tocopherol	34.2[a]	22.1[b]	18.2[c]

The concentration of CLA and VA in milk was about 2-3 times higher in the milk obtained from sheep maintained on pasture, compared to those fed with rations based on conserved forage (hay or silage) (P > F > S). However, the increase of the concentration of CLA c9,t11 in sheep milk was lower compared to data on dairy cows reported by Dhiman et al., (2005), who mentions a 5.7 time increase in the concentration of CLA c9,t11 in milk from sheep maintained on pastures compared to that obtained from sheep fed based on hay and concentrate feed.

Nutrition of sheep maintained on pasture ensured a concentration of lipophilic antioxidants (β-carotene and α-tocopherol) in milk 2-3 times higher when compared to rations based on hay or silage (P > F > S), confirming that β-carotene and α-tocopherol content in milk is correlated with their concentration in food, because these biomolecules can not be synthesized by animals (Marino et al., 2012). Higher levels of lipophilic antioxidants in milk in response to sheep maintenance on pasture are confirmed by Calderon et al., (2006), Butter et al., (2008) and La Terra et al. (2010), who emphasized the importance of green fodder on the milk content in pro-vitamins. According to studies conducted by Key et al., (2005) and Noziere et al., (2006), green forage lose 30-80% of the β-carotene and α-tocopherol content during harvesting and preservation processes.

Although the β-carotene concentrations from food and milk were remarkably higher in sheep maintained on pasture, concentrations of retinol in milk were statistically not different from those recorded in the case of sheep fed by hay or silage based rations, suggesting that the body capacity to turn β-carotene in retinol is limited. In the case of cows one noted that carotenoids in the feed can pass intact into the milk, which at its turn can influence the colour of the cheese, while in the case of sheep and goats the carotenoids are degraded into compounds of low molecular weight, influencing the oxidative stability but also the colour and flavour of cheese as a result of oxidation of PUFAs (Yang et al., 2002). Thus one can explain the lower concentration of β-carotene compared to that in milk or that mentioned in numerous studies on cow milk obtained in similar feeding conditions.

The content of α-tocopherol in milk ranges between 34.2-18.2 μg/100g fat and is higher than that reported by Jensen and Nielsen (1996) in dairy cattle. The concentration of α-tocopherol in milk was directly proportional to that of food, the following hierarchy being set: P > F > S. Previous studies conducted by La Terra et al. (2010) led to results consistent with those obtained by us, showing an increase of the α-tocopherol concentration in milk by 37% and 68%, respectively when the pasture secured 30% and 70%, respectively of forage rations in cattle, compared with a mixed ration consisting of preserved forage.

Oxidative stability of milk was monitored on the basis of lipid oxidation byproducts. TBA (thiobarbituric acid) test provides information on the level of MDA (malondialdehyde) which is a compound formed in the oxidation of lipids in milk, along with other compounds. However there is a correlation between MDA values and the degree of oxidation of fat in milk (Guillen et al., 1998). The highest concentration of MDA was recorded in milk of sheep fed with rations based on silage (0.746 mg/l), while by opposite the lowest concentration was obtained in the case of sheep on maintained on pasture (0.43 mg/l) (S > F > P). During the storage of milk at 2ºC (for 2-24 hours) there was a considerable MDA increase in milk to all three sheep lots; 24 hours after milking, MDA concentration in milk was about 2 times higher than the one recorded 2 hours after milking. Fatty acids oxidation processes in milk were more intense in the first 6 hours, when the concentration of MDA basically doubled in all three experimental variants (fig. 1). On the other hand, MDA concentration in milk within the 12-24 hours interval (hours of milking), recorded relatively small increases: 8.5% in sheep maintained on pastures; 9.5% in the lot fed with silage based rations and 35.8% for the lot fed with hay. Increasing the concentration of MDA in milk is because the lipase initiates the process of lipolysis of fat in raw milk, thus the free fatty acids appear in milk and they are oxidized and converted initially into lipid peroxides and then into aldehydes (including MDA), and thus influencing the nutritional and sanogenous quality of milk but also its technological features (Deeth et al., 2006).

Havemose et al., (2006) found a higher degree of oxidation of fat in the milk of cattle fed with silage compared to those fed with hay, despite the higher level of PUFA in the milk of cattle fed with hay which yet showed a higher concentration of natural antioxidants (α-tocopherol especially). The higher concentration of C18:3n-3 in the milk of cows fed favoured the absorption and transfer of α-tocopherol in milk, thereby reducing the fatty acid oxidation (Havemose et al., 2006).

Fig. 1. Influence of fodder preservation method on the oxidative stability of milk fat

Therefore, in our study, the higher concentrations of C18:3n-3 in pasture favoured the absorption and transfer of α-tocopherol in milk where its concentration was almost double as against the one in the lot fed with hay or silage. Consequently the MDA level in the milk of sheep maintained on pasture was considerably lower.

MDA concentration in milk correlated inversely with lipophilic antioxidant content in milk and α-tocopherol especially. Thus, the milk of sheep maintained on pasture even though it had the highest content of functional fatty acids and consequently prone to a stronger oxidation it still had the best oxidative stability, i.e. the lowest concentration of MDA, because it contains natural lipophilic antioxidants and α-tocopherol especially. Opposite results were obtained in the case of milk obtained from sheep fed with silage, while in the case of those fed with hay intermediate results were recorded. The presence of lipophilic antioxidants in milk, even in high concentrations, could not stop lipid oxidation processes and the MDA increase in milk, but had an important role in limiting them.

Similar results were obtained by Puppel et al. (2013) and Gladine et al., (2007) who stated that in the case of dietary supplement of cows with PUFA rich fat, the addition of tocopherols significantly reduces the lipid oxidation in milk, and the concentration of MDA, respectively. Sawsz (2000) noticed that when pig feed was supplemented with fat rich in PUFA, the E vitamin concentration in muscle tissue decreased thereby proving its role in protecting the fatty acids against oxidation.

Among the strategies to improve the nutritional quality of milk, pasture is the only forage ensuring simultaneously the increasing of milk content both in functional fatty acids and non-enzymatic lipophilic antioxidants. Therefore further studies are required to establish whether these bioactive compounds in milk can be used as biomarkers for the identification of "milk and dairy products of declared origin".

CONCLUSIONS

Improving the milk content in functional FA in the sheep maintained on pasture was accompanied by an increase in the concentration of non-enzymatic lipophilic antioxidants (i.e. β-carotene, α-tocopherol), which proves that one milk can be added in a natural manner some extra nutritional value and an increased oxidative stability that would qualify it as a "functional food". Fodder preservation through drying (hay) provided a higher concentration of functional fatty acids and a better oxidative stability of milk as against the fodder preservation through silage in which case the milk' share of functional FA and the oxidative stability increased.

Oxidative stability of milk negatively correlated (inversely) with functional fatty acid composition and positively with the concentration of α-tocopherol. Retinol concentration did not significantly contribute to providing oxidative stability of milk, but positively correlated with the concentration of α-tocopherol in milk, likely due to the antioxidant properties of tocopherol and retinol protection. One was unable to establish a correlation between the milk content in β-carotene content and the oxidative stability on the one hand, and the milk content in MDA (malondialdehyde), on the other.

REFERENCES

1. Deeth HC., Fitz-Gerald CH. 2006. Lipolytic Enzymes and Hydrolytic Rancidity. Adv Dairy Chem Vol 2 Lipids, 481-556.
2. Dhiman T.R., Seung-Hee N., and AMY L.U. 2005. Factors Affecting Conjugated Linoleic Acid Content in Milk and Meat. Critical Reviews in Food Sci. and Nutr., 45:463–482.
3. Dhiman, T.R., Anand G.R., Satter L., and Pariza M.W. 1999. Conjugated linoleic acid content of milk from cows fed different diets. J. Dairy Sci., 82:2146-2156.
4. Gladine C., Morand C., Rock E., Bauchart D., Durand D. 2007. Plant extracts rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids from animals fed n-3 PUFA supplemented diets. Anim Feed Sci Technol 136: 281-296.
5. Griinari, J.M., Corl, B.A., Lacy, S.H., Chouinard, P.Y., Nurmela, K.V. & Bauman, D.E., 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. J. Nutrition 130, 2285-2291.
6. Havemose M.S., Weisbjerg M.R., Bredie W.L.P., Poulsen H.D., Nielsen J.H. 2006. Oxidative stability of milk influenced by fatty acids, antioxidants, and copper derived from feed. J. Dairy Sci. 89: 1970–1980.
7. Jensen SK., Nielsen KN. 1996. Tocopherols, retinol, beta-carotene and fatty acids in fat globule membrane and fat globule core in cows' milk. J. Dairy Res. 63: 565-574.
8. Kalac, P. & Samkova, E., 2010. The effects of feeding various forages on fatty acid composition of bovine milk fat: A review. Czech J. Anim. Sci. 55(12), 521-537.
9. Kay J.K., Roche J.R., Kolver E.S., Thomson N.A., Baumgard L.H. 2005. A comparison between feeding systems (pasture and TMR) and the effect of vitamin E supplementation on plasma and milk fatty acid profiles in dairy cows. J. Dairy Res., 72:322–332.
10. La Terra S., Marino V.M., Manenti M., Licitra G., Carpino S. 2010. Increasing Pasture Intakes Enhances Polyunsaturated Fatty Acids And Lipophilic Antioxidants In Plasma And Milk Of Dairy Cows Fed Total Mix Ration. Dairy Sci. Technol., 90:687-698.
11. Marino V., Schadt T., Caccamo M., Licitra G. 2012. Influence of season and pasture feeding on the content of α-tocopherol and β-carotene in milk from Holstein, Brown Swiss and Modicana cows in Sicily. Dairy Sci. Technol., 92 (5):501-513.
12. Mierlita, D., 2012. Effect Of Feeding Type (Pasture *Vs*. Total Mixed Rations) Of Turcana Ewes On Animal Performance And Milk Fatty Acid Profile. J. Food Agric. Environ. 10, 815- 818.
13. Noziere P., Graulet B., Lucas A., Martin B., Grolier P., Doreau M. 2006a. Carotenoids for ruminants: From forages to dairy products, Anim. Feed Sci. Technol., 131:418-450.
14. Pilarczyk, R., Wójcik, J., Sablik, P. & Czerniak, P., 2015. Fatty Acid Profile And Health Lipid Indices In The Raw Milk Of Simmental And Holstein-Friesian Cows From An Organic Farm. S. Afr. J. Anim. Sci. 45(1), 30-38.
15. Puppel K., Nałęcz-Tarwacka T., Kuczyńska B., Gołębiewski M., Kordyasz M. 2013. Effect of different fat supplements on the antioxidant capacity of cow's milk. Archiv Tierzucht 56 (17): 178-190.

16. Reklewska B., Oprządek A., Reklewski Z., Panicke L., Kuczyńska B., Oprządek J. 2002. Alternative for modifying the fatty acid composition and decreasing the cholesterol level in milk of cows. Livest Prod. Sci., 76: 235-243.
17. Shingfield K.J., Salo-Vaananen P., Pahkala E., Toivonen V., Jaakkola S., Piironen V., Huhtanen P. 2005. Effect of forage conservation method, concentrate level and propylene glycol on the fatty acid composition and vitamin content in cows´ milk. J. Dairy Resch., 72: 349–361.
18. Simopoulos A.P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med., 233:674–688.
19. Steinmetz K.A., Childs M.T., Stimson C., Kushi L.H., McGovern P.G., Potter J.D. Yamanaka W.K. 1994. Effect of consumption of whole milk and skim milk on blood lipid profiles in healthy men. American J Clin Nutr., 59: 612-618.