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Original article

STUDY ON THE OXIDATIVE CHANGES IN MILK LIPIDS, INDUCED BY SUBCLINICAL MASTITIS AND DIFFERENT MILKING REGIMES IN COWS

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Summary

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The fatty acid composition, the amount of free fatty acids and primary and secondary products of oxidation were investigated in relation to quality of raw cows milk produced and stored at a farm with a central milk duct. Raw milk samples were collected from the evening milking, from bulk milk (evening and morning milkings) and from animals with signs of subclinical mastitis. It was established that bulk milk and the milk from cows with subclinical mastitis contained more short- and medium-chain saturated fatty acids (C4:0 to C16:0) and less long-chain unsaturated fatty acids (C18:1 and C18:2), mainly because of the altered amount of oleic and linoleic acids. In mastitic milk, apart the increased somatic cell counts, increased values of free fatty acids (FFA), peroxide value and malondialdehyde (MDA) concentrations were found out, while total phospholipids were decreased. Similar tendencies in FFA, peroxide value and MDA were established in bulk milk samples. The main reason for these changes were oxidative changes in bulk milk in the milk collection tank.

Key words: cow milk, fatty acids, free fatty acids, lipid peroxidation, mastitis

INTRODUCTION

The appearance and progression of oxidised flavour is an important defect affecting cow milk and dairy products trading. This defect could be described as milk with flavour of cardboard, paint, oil or metal (Alvarez, 2009) and could be the main reason for refusal from dairy products consumption. In a large-scale survey performed in 1995 in New Zealand, one-third of female respondents did not consume milk as they found its taste unpleasant (Howard *et al.*, 1995). Another survey reported that the off-flavours of milk were the major reason for dislike among people who did not consume milk (Porubcan & Vickers, 2005). The same study outlined that samples submitted to light-induced oxidation were defined as having acid and oily off-flavour which persisted for the longest time among all tested samples. Also, these samples received the lowest

scores for taste and were ranked second among most undesirable drinks.

The development of the oxidised flavour has been associated with the impaired balance between prooxidative and antioxidative factors in milk (Nielsen *et al.*, 2002). Lipid oxidation is the main reason for this defect and that is why the effects of general prooxidants as light and metals have been throughly studied (O'Connor & O'Brien, 2006). The presence of oxidised flavour was attibuted to using semi-transparent packages and light-permeable bottles (Mestdagh *et al.*, 2005). In another research on milk left in a box for at least 8 hours, non-trained consumers were able to detect differences between light-exposed (2000 lx) and non-exposed samples after 54 min to 2 hours after exposure (Chapman *et al.*, 2002). Gutierrez (2014) provided evidence that the addition of prooxidants as copper sulfate (0.05 and 1.0 mg/kg) and/or light exposure (2300 lx) of raw milk stored for 11 days (3.3 ºC) resulted in substantial increase in oxidation products and reduction of total antioxidant capacity, with light being the most powerful factor. The oxidation product hexanal was the main compound produced in all oxidised samples, and light was important for the formation of malondialdehyde – a marker of lipid peroxidation.

On the other part, mastitis and mastitis-induced immune response have a negative impact on cow milk composition and quality (Kitchen, 1981). Unwated processes as proteolysis (milk proteins degradation) and lipolysis (hydrolysis of triacylglycerols) occur in mastitic milk making the taste of dairy products sour or rancid. Some previous studies demonstrated that milk lipase activity increased in animals with udder infections (Azzara & Dimick, 1985), but others found out that it was reduced or unchanged (Salih & Anderson, 1979). Murphy *et al.* (1988) observed that in cows with mastitis, the lipase activity in milk was not higher, and high rates of lipolysis were due to infection-induced increased susceptibility of milk fat substrates. The concentrations of free fatty acids (FFA) in raw milk were high in mastitic milk at milking and after its cold storage (Gudding, 1982). Increased FFA amount in cold-stored milk from animals with mastitis is associated with „spontaneous lipolysis". The latter is defined as substantial elevation of FFA and occurs only after milk is cooled. After resolving of the infection, the milk could not be evaluated as free, as it preserves the features of milk with „spontaneous lipolysis" (Downey, 1980).

Numerous studies, especially on cow milk reported correlation between somatic cell counts and changes in milk composition. These changes could result from injury of udder cells and reduced rate of milk synthesis. Another possible explanation is the change in permeability of membranes and interstitial spaces leading to migration of blood componentns in milk. It is acknowledged that mastitic milk lipids are susceptible to lipases produced by white blood cells in the udder in response to infection. The action of these enzymes provoked reduction of triglycerides with simultaneous enhancement of fatty acid peroxidation. Milk with high somatic cell counts was far more susceptible to spontaneous lipolysis (Petrovski & Stefanov, 2006). Kisza & Botura (1969) affirmed that mastitic milk contained less phospholipids, more short- and medium-chain fatty acids ($C_{4:0}$-$C_{14:0}$) and reduced amount of unsaturated fatty acids than milk from healthy animals. Andrei *et al.* (2010) confirmed higher content of fatty acids ($C_{10:0}$-$C_{14:0}$) – 66.85% in mastitic

milk vs 60.69% in normal milk, higher content of stearic acid and malondialdehyde (MDA) up to 26.7 nmol/mL (vs average content of 14.62 nmol/mL) and low levels of monounsaturated (24.24%) and polyunsaturated (8.9%) fatty acids as compared to 28.9% and 10.39% respectively in normal milk. In their study, the authors found out also low cholesterol in mastitic milk (7.28 mg/100 mL vs 11.04 mg/100 mL in normal milk).

The aim of the present study was to determine the 24-hour fatty acid profile, free fatty acids and total phospholipids in milk from cows with subclinical mastitis and from healthy cows with respect to different milking regimes. Parallelly, the oxidation of milk fat was assayed by the classical methods of malondialdehyde content and peroxide value determination.

MATERIALS AND METHODS

The raw cow milk samples from evening milking, from bulk milk (evening and morning milkings) and from animals with signs of subclinical mastitis were collected at a farm with 90 cows in Central South Bulgaria.

A total 54 samples (n=18 of each type) were obtained. Raw milk (500 mL) was sampled directly from the tank in sterile glass flasks and cooled on ice up to 4.5 ºC. Samples from animals showing signs of subclinical mastitis were obtained using the same method. These cows were detected with Mastitis Test NK (Bioveta AD).

Somatic cell counts in milk were determined on an automated apparatus (Ecomilk Scan).

The fatty acid composition was analysed by gas chromatography (BSS EN ISO 15304:2004). The gas chromatograph (Agilent Technologies 6890 N) was equipped with flame ionisation detector, capillary column Supelco SP^{TM} 2560 with dimensions 100 m × 0.2 μm × 0.25 mm and nitrogen as a carrier gas. The temperature of the oven ranged from 60 °C to 240 °C. The isolation of raw milk fat was performed according to BSS EN 1528–2:2001 (point 6.1.4.1.2). Fatty acids in the residue were methylated via preesterification with trimethylsulfonium hydroxide (BSS EN ISO 5509:2004). For identification and quantification of fatty acids of milk fat, Supelco 37 Component FAME Mix (CRM 47885, Sigma-Aldrich) analytical standard containing 37 fatty acids was used.

The amount of free fatty acids was assayed titrimetrically as described by Mehieu (1984) on the principle of free fatty acids extraction with organic solvents and quantitation through titrimetry.

Lipid peroxidation was evaluated by the spectrophotometric method of King (1962). As a result of peroxidation, thiobarbituric acid reacted with malondialdehyde in acid medium forming a red adduct whose absobtion was quantitated at wavelength of 535 nm (Spekol 11). MDA concentrations were calculated from a standard curve obtained from the acid hydrolysis of 1,1,3,3,-tetrametoxypropane (Andrei *et al.*, 2008).

Peroxide value was determined as per BSS EN ISO 3960:2007 – Animal and vegetable fats and oils – Determination of peroxide value. The principle of the method consisted in processing of the sample in acetic acid-isooctane with potassium iodide and titration of the liberated iodine with standard sodium thiosulfate solution. The results are expressed as milliequivalents of active oxygen per kg.

Statistical analysis of data (descriptive statistics including mean standard error of

the mean and t-test) was performed using the GraphPad Software.

RESULTS

The analysis of milk samples from 90 dairy cows with the quick mastitis test detected 18 samples positive for subclinical mastitis. After analysis of the 18 positive samples on Ecomilk Scan, they exhibited total somatic cell counts between 500,000 and 1,200 000 cells/mL.

Saturated and unsaturated fatty acid proportions from the total content of fatty acids in milk are presented in Table 1. The amount of saturated fatty acids after the evening milking was 64.2%, in bulk milk (morning and evening milking) – 67.6% and in milk from cows with subclinical mastitis: 68.8%. The respective values for unsaturated fatty acids were 29.6%, 27.2% and 25.7%.

Table 2 presents the results for the content of 10 main fatty acids, whose amount in milk fat was over 1% (butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic and linoleic) which are relevant for the fatty acid profile of milk. Bulk and mastitic milk contained by 7% more butyric (C4:0) and caprylic (C8:0) acids as compared to evening milk. Caproic acid content (C6:0) was by 15% higher and that of capric acid (C10:0) – by 23%. Most obvious changes have occurred in lauric acid proportion (C12:0). In evening milk, it was 2.8%, in bulk milk: 3.3%, and in mastitic milk: 3.5% or

Table 1. Quantity of saturated (SFA) and unsaturated (UFA) fatty acids in raw cow's milk as percentage from the total fatty acids amount. Data are given as mean ±SEM (n=18)

Evening milking		Bulk milk (evening+ morning milking)		Milk from cows with subclinical mastitis	
SFA	UFA	SFA	UFA	SFA	UFA
64.2 ± 1.3	29.6 ± 1.1	67.6 ± 1.1	27.2 ± 1.3	68.7 ± 1.4	25.7 ± 1.1

Table 2. Quantity of ten fatty acids in raw cow's milk as percentage from the total fatty acids amount. Data are given as mean ±SEM (n=18)

Fatty acids	Evening milking	Bulk milk (evening+ morning milking)	Milk from cows with subclinical mastitis
Butyric (C 4:0)	2.8 ± 0.2	3.0 ± 0.2	3.0 ± 0.3
Caproic (C 6:0)	1.9 ± 0.1	2.1 ± 0.1	2.2 ± 0.1
Caprylic (C 8:0)	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Capric (C 10:0)	2.6 ± 0.2	3.0 ± 0.2	3.2 ± 0.5
Lauric (C 12:0)	2.8 ± 0.2	3.3 ± 0.3	3.5 ± 0.6
Myristic (C 14:0)	9.5 ± 0.5	10.6 ± 0.5	10.9 ± 0.7
Palmitic (C 16:0)	29.0 ± 0.8	31.3 ± 1.1	31.4 ± 0.7
Stearic (C 18:0)	11.3 ± 0.7	10.0 ± 0.8	9.7 ± 0.9
Oleic (C 18:1)	23.2 ± 0.9	20.9 ± 1.1	19.3 ± 0.8
Linoleic (C 18:2)	2.7 ± 0.4	2.7 ± 0.4	2.3 ± 0.6

Table 3. Quantity of free fatty acids (FFA), peroxide value, malondialdehyde (MDA) and total phospholipids in raw cow's milk. Data are given as mean±SEM (n=18)

Milk samples	FFA, meq/L	Peroxide value meq/kg	MDA, µmol/L	Total phospholipids, mg%
Evening milking	0.23±0.02	4.54±0.60	10.29±0.37	113.40±7.68
Bulk milk (evening+ morning milking)	0.39±0.06	6.06±0.50	11.58±0.31	88.21±9.66
Milk from cows with subclinical mastitis	0.63±0.09*	7.61±0.90*	18.54±0.55*$	82.69±10.94

* – P<0.05 vs evening milking values; $ – P<0.05 vs bulk milk values.

by 25% more. Myristic (C14:0) and palmitic (C16:0) acids increased by 14% and 8% respectively. In bulk and mastitic milk, the levels of unsaturated fatty acids were lower than in evening milk. Oleic acid content (C18:1) decreased by 17%, and linoleic acid (C18:2) – by 15%.

The FFA levels, malondialdehyde (MDA) concentrations, peroxide values and total phospholipids for the three types of milk samples are shown in Table 3. The results indicated statistically significantly increased FFA levels, peroxide values and MDA concentrations (P<0.05) and lower content of phospholipids in the milk of cows with subclinical mastitis compared to milk from healthy animals. A similar tendency was observed between morning+evening (bulk) milk vs evening milk (P<0.05).

DISCUSSION

The analysis of raw cow milk from evening milking showed variations of saturated fatty acids from 61.7% to 66.5%, and of unsaturated – from 28.1% to 31.7%. O'Donnell-Megaro *et al.* (2011) reported average unsaturated fatty acids of 63.7% in cow milk. Almost the same (63.8%) was the percentage established by Palmquist *et al.* (1993). The respective levels of unsaturated fatty acids found out by both research teams were 31.2% and 31.0%. Peichevski *et al.* (1988) affirmed that the content of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic and linoleic acids are appropriate for evaluation of the fatty acid profile of milk and factors influencing it. The increased content of saturated fatty acids in our results for bulk milk and milk from cows with subclinical mastitis deserves attention. This is due to the higher amount of short- and medium-chain fatty acids (from C4 to C16). Only stearic acid (C18:0) decreased by 1.3% to 1.6% from evening milk amounts. Lower content of unsaturated fatty acids is mainly a result from reduced oleic and linoleic acids. The comparision of the content of 10 analysed fatty acids in bulk milk and mastitic milk showed that there were no substantial differences. It could be concluded that the overnight storage of raw milk in the cooling tank and its mixing with the milk from the morning milking triggered oxidation processes which changed its fatty acid composition. At the same time, FFA,

MDA and peroxide values were higher. A similar tendency was reported by Andrei *et al.* (2010) and Randolph & Erwin (1974).

Malondialdehyde is one of products from peroxidation of unsaturated fatty acids – aldehyde produced from linoleic acid-derived hydroperoxyepidioxides and bicycloendoperoxides used commonly as a marker of lipid peroxidation (Gullien-Sans & Guzman-Honzas, 1998). According to DelRio *et al.* (2005) it is used as peroxidation marker of mastitic milk. Our studies demonstrated that its concentrations increased statistically significantly in the milk from cows with subclinical mastitis to 18.54 ± 0.55 μmol/L vs 11.58 ± 0.31 μmol/L in healthy cows ($P<0.05$). The mastitic milk fat is easily hydrolysed by lipases produced by leukocytes in the inflammed udder. The action of these enzymes on triglycerides results in higher amount of peroxidation products and unpleasant flavour and taste. Milk with increased somatic cell counts is rather more susceptible to lipolysis and peroxidation (Petrovski & Stefanov, 2006). With this respect, the milk from animals with subclinical mastitis with increased SCC had higher MDA concentrations (Table 3). A similar relationship was reported by Andrei *et al.* (2008): milk MDA 26.7±12.77 nmol/mL in mastitic milk vs 14.62±4.91 nmol/mL in healthy cows' milk, At the same time, FFA and peroxide value of milk increased along with reduciton of total phospholipids. Similar changes but of higher magnitude were reported by Randolph & Erwin (1974).

The results for bulk (morning+evening) milk showed considerably higher MDA concentrations (11.58 μmol/L vs 10.29 μmol/L in evening milk; $P<0.05$). An experiment performed by Gutierrez (2014) demonstrated increased MDA levels as early as the first day of 11-day cold storage of raw milk, with peak on the 4th day when MDA concentrations were high enough to detect organoleptically the oxidised flavour; light was reported as the primary factor for this elevation. Jenq *et al.* (1998) affirmed that the content of MDA in milk should be higher than 0.055 mg/kg in order to have a detectable oxidised flavour. The degradation of milk fat and occurrence of lipid peroxidation during the initial production and storage of raw milk are due to spontaneous, induced and bacterial lipolysis.

From toxicological point of view, the consumption of foods containing lipid peroxidation products should be limited as they have a variety of adverse biological effects. These products are reported as potential cytotoxins, mutagens and carcinogens and possibly involved in the pathogenesis of cancer, atherosclerosis and diabetes (Wasovicz *et al.*, 2004).

CONCLUSIONS

The milk produced by healthy cows after the evening milking which has not undergone oxidative changes had a normal fatty acid profile.

The milk from cows with subclinical mastitis as well as bulk milk from evening and morning milkings was characterised with increased amount of short- and medium-chain fatty acids (C4:0–C16:0) and lower content of unsaturated long-chain fatty acids (C18:1; C18:2).

The overnight storage of raw milk in the cooling tank provoked oxidation which altered its fatty acid profile and made it similar to that of milk from cows with subclinical mastitis. This is attributed to endogenous (spontaneouss lipolysis) and exogenous (induced and bacterial lipolysis) factors.

The milk of animals with increased somatic cell counts had higher values of free fatty acids, peroxide value and malondialdehyde concentrations were found out, while total phospholipids were decreased. A similar tendency was established in bulk milk samples.

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