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Oxidative stability of buttermilk as influenced by the fatty acid composition of cows' milk manipulated by diet

Dorthe Kristensen[1], Rikke V Hedegaard[1], Jacob H Nielsen[2]* and Leif H Skibsted[1]

[1] Food Chemistry, Department of Dairy and Food Science, Royal Veterinary and Agricultural University (KVL), Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[2] Department of Food Science, Danish Institute of Agricultural Sciences, Research Centre Foulum, PO Box 50, DK-8830 Tjele, Denmark

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Milk from cows fed a low-fat diet high in cereals designed to stimulate fat synthesis *de novo* was lower in unsaturated fatty acids (21·3%) than milk from cows fed a diet high in fat, mainly from roasted soy beans (41·3% unsaturated fatty acids). Buttermilk from the more unsaturated milk was less oxidatively stable during storage (at 4 °C, followed for 11 d) than buttermilk from the more saturated milk, as monitored both by primary lipid oxidation products (lipid hydroperoxides) and by the secondary lipid oxidation product, hexanal. Fat-soluble antioxidants, β-carotene and α-tocopherol, analysed by HPLC, were not consumed during storage for either of the two types of buttermilk. In contrast, the antioxidative capacity of the serum phase decreased during storage as evaluated in a radical scavenging assay based on the semi-stable water-soluble radical nitrosodisulphonate (Fremy's salt). The time course for the decrease in water-soluble antioxidants was very similar for the two types of buttermilk suggesting that oxidation is initiated in the serum phase independently of fatty acid composition.

Keywords: Buttermilk, oxidation, free radicals, fatty acid composition.

Absence of lipid-derived off-flavours is central to consumer acceptance of milk and other dairy products (Christensen & Hølmer, 1996). A characteristic and unpleasant 'cardboard' flavour of milk is caused by oxidative cleavage of unsaturated lipids (Hall & Lingnert, 1984). Lipid oxidation may be initiated by enzymes present in the milk, by traces of metal ions from processing equipment or by exposure to light during storage (Kristensen et al. 2002). In some instances oxidation is already underway in the raw milk and is known as 'spontaneous oxidation', while more often lipid oxidation becomes evident after processing and storage (Granelli et al. 1998).

Lipids are protected against oxidation by natural antioxidants present in raw milk. Both α-tocopherol and β-carotene, hydrophobic antioxidants located in the lipid phase, and ascorbate and urate, located in the serum phase, are important as scavengers of free radicals and for the protection of unsaturated fatty acids (Fox & McSweeney, 1998) such as 18 : 1 and 18 : 2 in phospholipids and triglycerides. The balance between initiators of lipid oxidation and antioxidants is critical for the oxidative stability of milk. However, a detailed understanding of the interactions between pro-oxidants and antioxidants is not available and, especially, the influence of the degree of unsaturation of the milk fat is largely unknown. To further understanding of the importance of the degree of unsaturation of milk fat, we compared the oxidative stability of milk from cows from two feeding regimes. Buttermilk, a product especially sensitive to oxidation owing to its relatively high concentration of phospholipids and low pH, was selected as a product well suited for a chill-storage experiment to examine the effect of varying degrees of unsaturation.

Two different types of milk were obtained, one from cows fed a low-fat diet rich in cereal, resulting in a high synthesis of fatty acids *de novo*, and the other from cows fed a high-fat diet including roasted soya beans. Raw milk obtained from the first feeding regimen was characterized by a fatty acid profile dominated by saturated fatty acids, while milk from the other feeding regimen was more unsaturated. Buttermilk resulting from a traditional churning of cream from the two types of raw milk had a correspondingly similar difference in saturation.

During a chill-storage period of 11 d the concentration of lipid hydroperoxides was monitored to follow the initial phase in lipid oxidation together with determination of

*For correspondence; e-mail: jacobh.nielsen@agrsci.dk

hexanal as a secondary lipid oxidation product having a marked effect on flavour (Frankel et al. 1989; Park & Goins, 1992). The antioxidative capacity was also followed, for the lipophilic antioxidants, α-tocopherol and β-carotene, by monitoring their concentrations in the product during storage. Antioxidative capacity of the serum phase depends on a variety of low and high molecular weight compounds, and a radical scavenging method based on Electron Spin Resonance spectroscopy and a water-soluble semi-stable radical known as Fremy's salt was used to follow the overall antioxidative capacity of the serum phase of the buttermilk during storage.

Materials and Methods

Chemicals

Water was purified through a Millipore Q-plus purification train from Millipore Corporation (67120 Malshelm, France). β-Carotene was obtained from Roche Ltd (2650 Hvidovre, Denmark), ethanol from De Danske Spritfabrikker (9100 Aalborg, Denmark), 1,2,3-trihydroxybenzene (pyrogallol) from Aldrich (D-89555 Steinheim, Germany), heptan HPLC grade and sodium chloride from Merck (D-64271 Darmstadt, Germany), 2,6-di-t-butyl-p-cresol (butylated hydroxytoluene) from Sigma (St. Louis, MO 63178, USA), α-tocopherol from Calbiochem (San Diego, CA 92121, USA) and potassium nitrosodisulphonate (Fremy's salt) from Aldrich (D-89555 Steinheim, Germany). Chloroform, methanol, 2-propanol, hexane, methanol HPLC grade and trimethylamine synthetic grade were all from LAB-SCAN (Stillorgan, Dublin, Ireland). Nitrogen, class 2 and helium, were obtained from Hede Nielsen (8700 Horsens, Denmark).

Animal management and experimental design

Six multiparous Holstein cows, yielding >22 kg/d, were randomly distributed in two groups. Cows were in mid-lactation at the start of the experiment. Two isocaloric diets were given to the cows in order to produce two different types of milk, one containing a higher content of shorter-chain saturated fatty acid (type I) and the other a higher content of unsaturated fatty acids (type II):

Milk type I: Feed giving a low content of citrate and high rate of lipid synthesis *de novo,* resulting in a high content of short-chain fatty acids in the milk fat. The feed was supplemented with barley and soybean meal and a mineral blend and contained 15 g fatty acids/kg dry matter (DM).

Milk type II: Feed giving a high content of citrate, resulting in a relatively high content of unsaturated fatty acids. The diet was supplemented with oatmeal and heat-treated soybeans and a mineral blend and contained 53 g fatty acids/kg DM.

Table 1. Composition of diets fed to the cows

Values are daily intakes of dry matter (DM) and fatty acids

	Type I		Type II	
	DM, kg	Fatty acids, g	DM, kg	Fatty acids, g
Grass silage	7·2	120	7·2	120
Barley straw	0·3	—	0·7	—
Beet pills	5·0	—	4·0	—
Barley	3·4	100	—	—
Harrow	—		2·1	104
Crushed soya	2·2	54	—	—
Toasted soya beans	—	—	4·1	738
Total	18·1	274	18·1	962

Feeds were produced at the Danish Institute of Agricultural Science; their composition is given in Table 1. Before the experiment the cows had access *ad libitum* to grass pasture for at least 2 weeks, and the first week in the experiment was allowed for adjustment to the experimental diet. The experiment lasted for 12 weeks in September–November. Milk samples were collected from the morning milk, and were immediately cooled to 4 °C. Afterwards the samples were transported to the Royal Veterinary and Agricultural University where they were pasteurized and creamed in a pilot plant and subsequently transported to Arla Foods, Amba in Holstebro, where they were churned into butter and buttermilk on a UCR 060 churn from Paasch & Silkeborg (8600 Silkeborg, Denmark). Buttermilk was stored for 11 d at 4 °C and was analysed on days 0, 5 and 11.

Fatty acid composition

Before GC separation and quantification, lipid was extracted twice with chloroform : methanol (2 : 1 v/v) and the extracted lipid was transesterified to methyl esters in sodium methylate solution (2 g/l methanol). Analysis of fatty acid methyl esters was carried out by GC (Hewlett-Packard, Palo Alto, CA 94304, USA) using an FFAP-column (polyethylene glycol TPA 25 m × 200 μm × 0·30 μm, Hewlett-Packard) with helium as carrier gas and a flame ionization detector. Injection was splitless with an injector temperature of 250 °C. Detector temperature was 300 °C. Initial column temperature was 40 °C, which was held for 4 min. The temperature was then raised at 10 deg C/min to 240 °C, which was held for 1 min. Quantification was based on area and given as percentages of fatty acids.

α-Tocopherol

Concentrations of α-tocopherol were determined by HPLC analysis (Hewlett-Packard, HP 1100 Series) after saponification and extraction with hexane. Samples (500 mg) of milk were transferred to test tubes and 2·0 ml pyrogallol (10 g/l) was added to the samples, which were then saponified with an aqueous solution of 50% (w/v) KOH

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(0·3 ml) at 70 °C for 30 min, followed by cooling on ice. After addition of 2·0 ml water and 4·0 ml hexane containing butylated hydroxytoluene (0·01 mg/l) the resulting suspension was centrifuged at 7000 ***g*** for 10 min. The upper phase was transferred to a test tube and 3·0 ml hexane was added followed by centrifugation. Finally, the solvent was removed from the pooled hexane extracts by evaporation under nitrogen. Residues were resuspended in 500 µl of 96% ethanol. Quantification of α-tocopherol in the samples was performed with reversed-phase HPLC using a 125 mm × 4·0 mm, 5 µm C18 column Hypersil ODS from Agilent Technologies (2850 Nærum, Denmark) with methanol/water (94 : 6) at a flow rate of 0·7 ml/min as a mobile phase. Fluorescence detection (288/330 nm, ex/em) was employed, using an external standard for quantification.

β-Carotene

Concentrations of β-carotene were determined by HPLC after saponification and extraction with hexane. The saponification step for β-carotene determination was identical to the saponification step described for α-tocopherol. Residues were resuspended in the mobile phase consisting of heptan : 2-propanol : trimethylamine (95 : 5 : 1, v/v). Quantification of β-carotene was by normal phase HPLC (LC10, Shimadzu, Tokyo 101, Japan) using a 250 mm × 4·6 mm, 5 µm C18 Supercosil LC NH_2 column from Supelco (Bellefonte, PA 16823-0048, USA). Absorption detection (450 nm) was employed, using an external standard for quantification.

Lipid hydroperoxides

Primary oxidation products that accumulated in the buttermilk during storage were analysed by determination lipid hydroperoxides by a method based on the reaction FeII/thiocyanate as described by Østdal et al. (2000). The relative concentration of lipidhydroperoxides was expressed as absorbance at 500 nm.

Hexanal

Hexanal in the stored buttermilk was analysed by solid phase micro extraction (SPME) in combination with GC-FID (HP 6980; Hewlett-Packard). An aliquot (2·00 ml) of buttermilk was placed in a 4-ml headspace vial cleaned by a gas-flame with a glass coated stirring bar. NaCl (0·25 g) was added to get a salting-out effect. A SPME fibre (Carboxen-PDMS, 75 µm) from Supelco (Bellefonte, PA 16823-0048, USA) was inserted in the headspace of the vial, which was equilibrated for 30 min, while thermostatted at 45 °C. Analysis of the volatiles absorbed to the SPME fibre was carried out by GC using an HP-5 column (5% phenyl methyl siloxane, 30 m × 250 µm × 0·25 µm; Hewlett-Packard) and helium as carrier gas. Injection was in the splitless mode with an injector temperature of 250 °C, and the SPME fibre was desorbed for 5 min. Initial column temperature was 40 °C, which was held for 4 min, and the temperature was raised by 10 deg C/min to 150 °C and then to 250 °C at 30 deg C/min. Identification of hexanal was by retention time verified by GC-MS. Relative concentration was based on area of the hexanal peak.

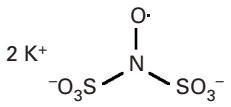

Fig. 1. Fremy's salt (potassium nitrosodisulphonate), a probe for water-soluble radical-scavengers.

Oxidative stability test by ESR spectroscopy

Radical-scavenging capacity of the serum phase of the milk was studied by ESR-spectroscopy using a standard solution made from 200 µl of 1·0 mM solution of Fremy's salt (potassium nitrosodisulphonate; Fig. 1) and 3 ml aqueous 12% ethanol to yield 63 µM radical concentration. The method was a modified version of that of Gardner et al. (1999) for alcoholic beverages.

After exactly 3 min, samples were transferred into an ESR flow cell (Wilmad, Buena, NJ 08310, USA) for measurement of aqueous solution, and the measurement started after 4 min and 30 seconds. Samples were prepared by adding 800 µl buttermilk to the standard solution which was measured in the ESR using the same procedure. A JES-FR30 ESR-spectrometer (JEOL Ltd. Tokyo 196, Japan) was used operating in X-band mode. The following parameters were used in all ESR experiments: microwave power, 4 mW; modulation frequency, 100 kHz; sweep width 5 mT; sweep time 4 min; modulation width 0·1 mT, time constant 0·2 s. The relative signal intensity was calculated using a manganese internal standard by measuring the peak-to-peak amplitude of the second line in the spectrum. Measurements were repeated and the amount of added buttermilk adjusted until a 50% reduction of the initial signal was obtained. The paramagnetic radical Fremy's salt can be measured by ESR-spectroscopy, while the reduction products are invisible in ESR. A solution with a high antioxidative potential reduces the Fremy's radical and the ESR signal. Solutions with no antioxidative potential thus result in an ESR signal at the same level as the blank. Antioxidative activity of the buttermilk was expressed as the volume of buttermilk required to reduce the initial signal to 50% and a low volume thus indicates high antioxidative capacity. ESR measurements were preformed during the storage experiment of the buttermilk, and samples were measured on days 0, 5 and 11.

Results and Discussion

Buttermilk produced from the two different types of milk had a very similar fat content and a very similar content of DM (Table 2). The noted difference in the oxidative

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Table 2. Dry matter and fat content (%) of the buttermilk together with fatty acids composition (%) of the two types of milk, buttermilk and butter

	Buttermilk		Butter		Milk	
	Type I	Type II	Type I	Type II	Type I	Type II
Fat %	0·62	0·65				
Dry matter %	8·90	8·98				
Fatty acids						
C4	2·97	3·47	3·54	3·80	2·78	2·78
C6	2·07	1·83	2·57	2·12	2·10	1·60
C8	1·63	1·13	1·81	1·26	1·50	0·90
C10	4·34	2·41	4·60	2·65	4·20	2·00
C12	5·41	2·69	5·53	2·89	5·30	2·00
C14	15·17	10·45	15·64	11·00	15·70	9·30
C16	36·58	25·19	38·95	25·65	41·50	24·10
C16 : 1	2·77	1·69	2·46	1·69	2·50	1·50
C18 : 0	6·30	11·42	6·23	11·01	6·00	13·40
C18 : 1	18·76	31·55	15·66	30·72	15·70	34·40
C18 : 2	3·29	5·93	2·38	5·09	2·60	5·80
C18 : 3	0·66	2·24	0·56	2·06	0·50	2·60
Sum	100·00	100·00	100·00	100·00	100·00	100·00

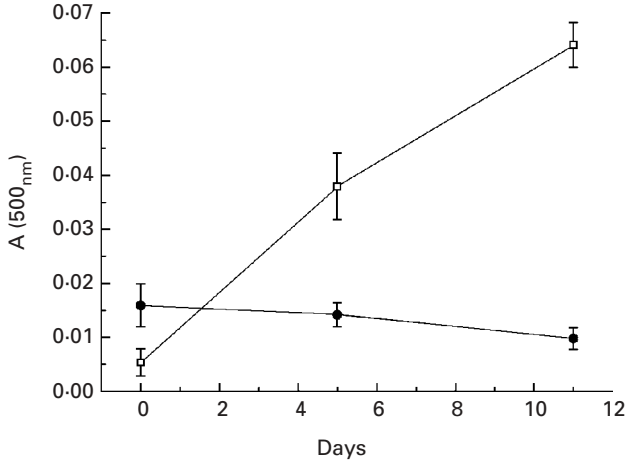

Fig. 2. Absorbance at 500 nm, as a relative measure of formation of peroxides in type I buttermilk (●), and in the more unsaturated type II (□) buttermilk during storage at 4 °C.

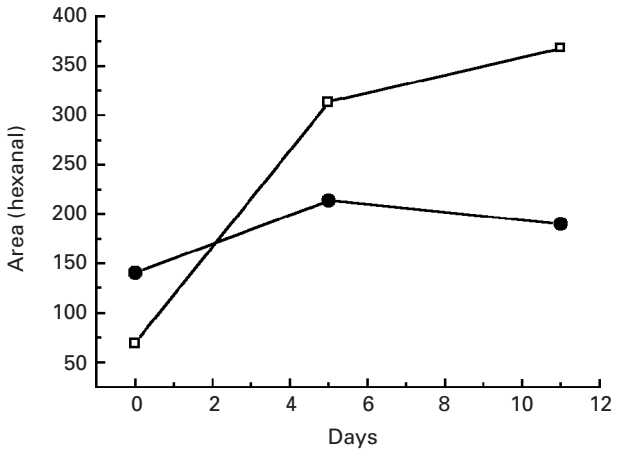

Fig. 3. Area of the integrated hexanal peak in the chromatogram (GC) as a relative measure of hexanal in type I (●) and in the more unsaturated type II (□) buttermilk during storage at 4 °C.

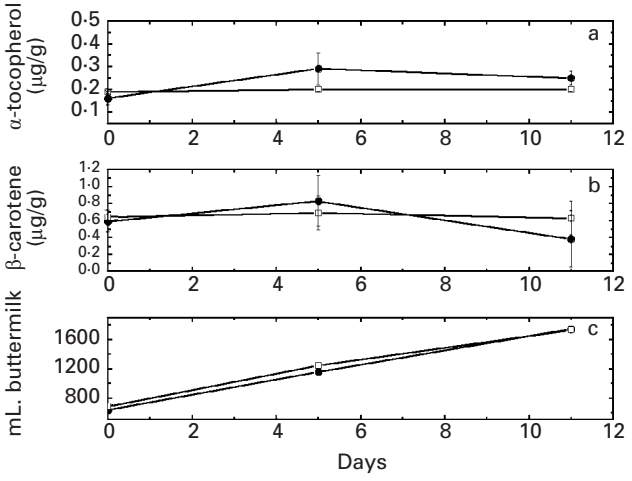

Fig. 4. Antioxidant capacity of type I buttermilk (●) and more unsaturated type II buttermilk (□) during storage at 4 °C; a: concentration of α-tocopherol; b: β-carotene; and c: volume of buttermilk required to scavenge 50% of the semi-stable, water-soluble radical, nitrosodisulphonate (63 μM).

stability of the two types of buttermilk may therefore be assigned to other factors, such as the content of anti-oxidants and the degree of unsaturation of the lipids in the buttermilk. Contents of the fat-soluble antioxidants, α-tocopherol and β-carotene, were very similar in the two types of buttermilk, as was the radical-scavenging efficiency of the serum phase of the products (Fig. 4). In contrast, fatty acid profiles of the raw milk, the butter and the buttermilk made from the milk were very different (Table 2). The two different feeding regimens thus affected the composition of the milk in ways that might influence lipid oxidation in dairy products made from them.

Fatty acid composition of type I buttermilk (Table 2) was characterized by a higher content of short-chain saturated fatty acids (C6–C16) than that of the more unsaturated type II buttermilk, which, on the other hand, contained more long-chain unsaturated fatty acids (e.g., 18 : 0, 18 : 1, 18 : 2 and 18 : 3). Fatty acid composition of the butter made from either of the two types of milk was similar to the corresponding buttermilk (Table 2). Some difference was expected owing to a larger quantity of phospholipids in buttermilk than in butter, which would result in a larger fraction of unsaturated fatty acids in the buttermilk (Christie et al. 1987). A difference between the butter and the buttermilk was seen for fatty acids between C16 and 18 : 1 (Table 2) although the difference was not as pronounced as might have been expected.

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Oxidative stability of the two types of buttermilk was followed by analysis of lipid hydroperoxides. Hydroperoxides increased during the storage period for the type II buttermilk (Fig. 2) in contrast to the type I buttermilk, indicating that an increase in the unsaturated fatty acid content enhances the oxidation in buttermilk.

Hexanal, a known product of peroxidation of n-6 polyunsaturated fatty acids, is a good indicator of lipid peroxidation (Frankel et al. 1989) and is a characteristic volatile secondary lipid oxidation product formed during oxidation of milk products (Kristensen et al. 2002). Hexanal content increased during storage in the type II buttermilk (Fig. 3) and reached a higher level at days 5 and 11 than for the more saturated type I buttermilk. Increased proportions of unsaturated fatty acids in the buttermilk thus promoted formation of secondary oxidation products, as also observed for primary oxidation products, i.e., the hydroperoxides. Hexanal content in type I buttermilk increased until day 5 but decreased again at day 11.

Milk contained more α-tocopherol (type I 0·69 μg/g, and type II 0·70 μg/g) than the buttermilk produced from it (Fig. 4), since α-tocopherol from the milk followed the lipid into the butter. Content of β-carotene was twice as great in type I raw milk (3·83 μg/g) as in type II raw milk (1·48 μg/g) because of the higher content of cereals in type I feed. This difference, however, did not influence the content of β-carotene in the buttermilk. Fat contents of the two types of buttermilk were approximately the same (Table 2) as were the initial content of β-carotene in both types of buttermilk. Hence β-carotene seemed to follow the phospholipids into the buttermilk.

During chill storage, amounts of fat-soluble antioxidants, α-tocopherol and β-carotene, did not change significantly (Figs 4a and 4b). In contrast, the ability of the buttermilk to scavenge the water-soluble semi-stable free radical nitrosodisulphonate decreased during storage (Fig. 4c). The ability was comparable for the two types of buttermilk, as was the time-course for the decrease.

The oxidative stability of buttermilk, as an example of a sensitive dairy product, clearly depended on the degree of unsaturation of the lipids. The degree of lipid oxidation was low, however, and was not reflected in a decrease in the lipid-soluble antioxidants. In contrast, the radical-quenching capacity of the serum phase decreased significantly during chill storage. Formation of the radicals initiating oxidative deterioration seemed to be related to the serum phase, where enzymes and metal ions may catalyse oxygen activation. Apparently this oxygen activation depended not on the degree of unsaturation of the lipids, but on the concentration of water-soluble antioxidants. The effect of radical formation was seen in the lipid phase and the degree of unsaturation was important for the formation of both primary and secondary lipid oxidation products.

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