Short communication: Cholesterol oxidation products in traditional buttermilk

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ABSTRACT

The aim of this study was to quantitatively and qualitatively assess the content of cholesterol oxidation products in traditional buttermilk after butter production. Cholesterol oxidation products (COP) exhibit a wide spectrum of biological activity, including cytotoxic, carcinogenic, and pro-oxidative properties. Buttermilk has about 2 mg of COP/kg of fat, including 7 β -hydroxycholesterol and 5,6 α -epoxycholesterol. Buttermilk immediately after production had a relatively high level of 7 β -hydroxycholesterol (1.47 mg/kg), which decreased to 0.61 mg/kg after storage for 10 h at 3°C. During storage, the content of 5,6 α -epoxycholesterol increased from 0.50 to 1.40 mg/kg. After 10 h of storage, the antioxidant potential of the buttermilk decreased (expressed as radical scavenging ability; change in 1,1-diphenyl-2-picrylhydrazyl = 32.2%). This study showed the presence of COP in fresh and stored buttermilk and the influence of time on changing the direction of cholesterol oxidation.

Key words: buttermilk, cholesterol, oxysterol, DPPH

Short Communication

Traditional buttermilk (BM) is a natural product resulting from the butter-making process during sweet cream or cream churning. It is intended for direct consumption or as an ingredient of cultured buttermilk. The components of BM depends on its acidity, the composition of the cream, and the conditions of churning. In traditional BM obtained in a butter churn machine, the fat content varies from 0.15 to 0.4%, by the continuous cream method, fat content can reach 0.65%, and from sweet cream up to 0.75%. Buttermilk usually has lower acidity than the plasma of churned cream. It resembles skim milk in its composition and appearance but contains most of the milk fat globule membrane (MFGM; Fu et al., 2014). During the manufacture of

butter, materials derived from the MFGM are recovered in the aqueous phase (BM) when milk fat globules are mechanically disrupted upon churning of cream. The MFGM fragments contain polar lipids (glycerophospholipids and sphingolipids), neutral lipids, proteins, glycoproteins, enzymes, and cholesterol (Fauquant et al., 2014). The major sterol present in milk—cholesterol—accounts for at least 95% of the sterols and is located mainly in the MFGM (Jensen and Newburg, 1995). Cholesterol content in milk ranges from 3.08 to 6.06 g/kg of fat (Mesilati-Stahy and Argov-Argaman, 2014); on average, it is 3.3 g/kg of fat, which equals 135 mg/kg of milk. In the MFGM, on the other hand, cholesterol content is 1.7 mg/m² MFGM (Et-Thakafy et al., 2017). For comparison, goat milk contains 1.5 mg/m² MFGM and sheep milk 1.8 mg/m². The content of cholesterol in MFGM is 40 mg/100 g of fat globules, which is 2 g/100 g of MFGM dry matter or 2% (Dewettinck et al., 2008). The amount of membrane fat globules is 1.9 m²/g of fat. In MFGM, there is 1.7 mg/m² of cholesterol (Et-Thakafy et al., 2017). The estimated mass of the MFGM is 2 to 6% of that of the total fat globules (Lopez, 2011).

Cholesterol [(3 β)-cholest-5-en-3-ol; **Ch**] is a 27-carbon animal sterol. Its structure contains a tetracyclic cyclopentanoperhydrophenanthrene, an 8-carbon saturated side chain, and a hydroxyl group in the 3 β position. Cholesterol levels in dairy products depend on, among other factors, the fat content, heat treatment, milk homogenization, and the type of lactic acid bacteria present. Milk contains approximately 12 mg of cholesterol per 100 g (Fletouris et al., 1998). According to Kovács et al. (2004), low-fat dairy products have a larger share of the Ch in fat high-fat products, because the small fatty beads have a relatively large surface area, which accumulates Ch.

Cholesterol, due to the presence of Δ^5 unsaturated bond, is subject to numerous chemical reactions, including oxidation. Ex vivo oxidation of Ch occurs primarily through free radicals under the influence of heat, UV, or ionizing radiation in the presence of oxygen. Oxidation of the carbon at the C-7 position is the easiest to accomplish. Through the intermedi-

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ate product, 7-hydroperoxide, isomeric hydroxyl derivatives are formed: 7 β -hydroxycholesterol (**7 β -OHC**) and 7 α -hydroxycholesterol (**7 α -OHC**). Another route is the oxidation of Ch on the carbon atoms forming the double bond (C-5 and C-6), where epoxidation occurs easily. As a result, isomeric epoxides are formed, including 5,6 α -epoxycholesterol (**α -epoxyC**) and 5,6 β -epoxycholesterol (**β -epoxyC**). Many researchers have reported that cholesterol oxidation products (**COP**) in foods can reach 1% of total cholesterol and occasionally 10% or more (Hur et al., 2007). In addition to cholesterol, the formation of COP requires reactive oxygen species and is favored by the presence of UFA or transition metals; in some rare cases, the oxidation is enzymatic (Rose-Sallin et al., 1996; Hur et al., 2007). The probability of COP forming in fresh dairy products is very low because the medium is liquid and the oxygen content is low (Sieber, 2005). Milk has a low level of PUFA and of pro-oxidant trace elements such as iron and copper. However, oxidation in fresh dairy products may be dominated by 7 β -OHC and α -epoxyC (Kumar and Singhal, 1992). The formation of COP in milk and dairy products can only occur under extreme conditions, such as high temperatures for a long period or prolonged storage at high temperatures (Sieber, 2005).

In very low concentrations, oxysterols are natural components of the human body and mediate many physiological functions (Kloudova et al., 2017). They participate in the regulation of Ch metabolism. However, oxysterol action is also connected with human pathologies (e.g., atherosclerosis, Alzheimer's disease, Parkinson's disease). Cholesterol oxidation products exhibit a wide spectrum of biological activity, such as cellular cytotoxicity, immunosuppressive effects, mutagenicity, carcinogenicity, and pro-oxidative properties (Jusakul et al., 2013; Kulig et al., 2016). The major nonenzymatically formed oxysterols, 7-ketocholesterol (**7-ketoC**), 7 β -OHC and β -epoxyC, have strong cytotoxic properties and are implicated in various pathological states (Lemaire-Ewing et al., 2005; Rimner et al., 2005; Massey, 2006). It has been suggested in the literature that COP produce different effects depending on whether they are applied separately or in mixtures. For example, a mixture of 7 β -OHC and 25-hydroxycholesterol (**25-OHC**) had weaker pro-apoptotic effects than 7 β -OHC alone (Aupeix et al., 1995; Kloudova et al., 2017). Because of the potential health risk of oxysterols, their formation and presence in foods have been the subject of many studies (Seckin and Metin, 2005). Bierzuńska et al. (2017) examined COP found in cheese from buttermilk and showed a correlation between the amount of oxysterols produced in cheese and the antioxidant potential. In the scientific and industrial literature, however, there is no information

about the composition and concentration of oxysterols in traditional buttermilk. In addition, observations on traditional BM production technology have prompted us to consider the effect of storage time on the amount and type of COP. The time that elapses between the end of churning and the further use of BM induces changes that may affect the direction of Ch oxidation, the number and type of COP, and the antioxidant potential. In these experiments, attention has been focused on the most common and abundant COP, including cholesterol 25-OHC, cholestanetriol (**triolC**), 7 α -OHC, 7 β -OHC, 7-ketoC, and α - and β -epoxyC.

The research material was traditional buttermilk, which was left over after the industrial production of butter from sour cream. The butter production used traditional methods, sometimes on an industrial scale. To cream, comprising 35% fat [9° Soxhlet-Henkel (**SH**) of plasma] after pasteurization (92°C for 30 s), the starter culture Lyofast M030N (Sacco, Cadorago, Italy) was added at 10 units of activity per 1,000 L of cream. The sour cream, comprising 35% fat, pH 5.5 (13.1°SH, 21°SH of plasma), was churned at 10°C for 55 min to achieve a butter grain size of 2 to 4 mm. The BM was taken to the tank by a gravity drain directly after the churning process and immediately cooled to 3 $\pm$ 0.5°C (sample **BM-0**). Buttermilk was stored in a covered fermentation tank at 3 $\pm$ 0.5°C for 10 h (sample **BM-10**). The tank was manufactured from high-alloy austenitic steel (type 316L-AISI, X2CrNiMo 17 13 2-DIN), a stainless chromium-nickel steel, cold-rolled sheet, annealed, with surface roughness (roughness average, Ra) of 0.6 μ m. The current study examined the composition and properties of BM using the methods previously described by Cais-Sokolińska et al. (2016, 2018). The isolation of lactic acid bacteria and determination of the proteolytic and lipolytic activity of the microorganisms was carried out following the method of Bettache et al. (2012).

The main element of the study was to analyze the content of Ch and COP. Fat from BM was extracted according to the Röse-Gottlieb procedure following ISO method 14156 (ISO, 2001). The Ch content was determined using an AOCS Official Method (AOCS, 2009) by gas chromatography, after a prior sample saponification with 1 mol/L KOH in methanol for 18 h at room temperature and triple extraction of the unsaponifiables with hexane:*tert*-butyl methyl ether (**MTBE**) 1:1 (vol/vol). The solvent was evaporated under a stream of nitrogen. The dry residues were dissolved in 0.1 mL of pyridine and silylated with 0.4 mL of Sylon BTZ (Supelco, Bellefonte, PA). Derivatives of the sterols were separated on an HP 6890 gas chromatograph equipped with a DB-35MS capillary column (25 m $\times$ 0.20 mm; 0.33 μ m; J&W Scientific, Folsom, CA). A sample was

injected in splitless mode. The column temperature was held at 100°C for 5 min, and then programmed to increase to 250°C at 25°C/min, held for 1 min, increased to 290°C at 3°C/min, and finally held for 20 min. The detector temperature was set to 300°C. Hydrogen was used as a carrier gas at a flow rate of 1.5 mL/min. 5 α -Cholestane was used as the internal standard for sterol quantification.

The COP were determined according to the methodology described by Przygoński et al. (2000). Isolated fats were esterified with 10% sodium methylate in MTBE (4:6, vol/vol), and cholesterol and its oxidation products were extracted with chloroform, followed by fractionation on a Sep-Pak NH2 column (Waters, Milford, MA). Chromatographic analysis of silylated oxysterols was performed on a HP 6890 GC apparatus (Hewlett-Packard, Palo Alto, CA). Separation was carried out on a DB-5 capillary column (30 m $\times$ 0.25 mm, with a stationary phase film of 0.25 μ m; J&W Scientific), using a temperature program increasing at 25°C/min from 60°C to 270°C, followed by 2.5°C/min until 290°C was reached. Helium was applied as a carrier gas at a flow rate of 1 cm³/min. The injector and detector were held at 300°C, and a split ratio of 1:40 was used. 19-Hydroxycholesterol was applied as an internal standard. The COP were identified using retention data

for standards, previously verified by mass spectrometry utilizing laboratory library and published data.

The ferric reducing antioxidant power (**FRAP**) was measured; this assay evaluates the ability of analyzed substances to reduce the complex of Fe³⁺-2,4,6-tris(2-pyridyl)-s-triazine (**TPTZ**) to Fe²⁺-TPTZ. The ability of an antioxidant to scavenge stable 1,1-diphenyl-2-picrylhydrazyl (**DPPH**) radicals was evaluated spectrophotometrically, in relation to the radical scavenging ability of the reference substance 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Both methods are described by Bierzuńska et al. (2017).

To verify the statistical hypotheses, a level of significance at $\alpha = 0.05$ was adopted. The statistical calculations were carried out using Statistica data analysis software (version 10, StatSoft, Tulsa, OK).

We examined the effect of buttermilk storage time on its composition, acidity, water activity, the binding of water, the concentration of lactic acid bacteria, and its proteolytic and lipolytic activity (Table 1; $P > 0.05$).

The cholesterol content in BM was 10.86 (BM-0) and 11.04 (BM-10) g/kg of fat ($P > 0.05$, Table 1). Cholesterol content in 1 kg of buttermilk was 43.44 mg, which was much higher than the 2.281 g/kg indicated by Fletouris et al. (1998) in butter. Based on literature data, the average content of Ch in buttermilk is $7.6 \pm$

Table 1. Composition, physicochemical, and microbiological characteristics of buttermilk^{1,2}

Item	BM-0	BM-10
Moisture (g/kg)	914.5 $\pm$ 1.1 ^a	913.6 $\pm$ 1.6 ^a
Total N (g/kg)	5.58 $\pm$ 0.03 ^a	5.53 $\pm$ 0.04 ^a
NPN (g/kg)	0.37 $\pm$ 0.07 ^a	0.38 $\pm$ 0.05 ^a
Noncasein N (g/kg)	1.24 $\pm$ 0.07 ^a	1.24 $\pm$ 0.04 ^a
Total protein ³ (g/kg)	33.23 $\pm$ 0.05 ^a	33.23 $\pm$ 0.03 ^a
Casein ³ (g/kg)	25.32 $\pm$ 0.01 ^a	25.31 $\pm$ 0.04 ^a
Whey protein ³ (g/kg)	5.61 $\pm$ 0.04 ^a	5.60 $\pm$ 0.02 ^a
Fat (g/kg)	4.01 $\pm$ 0.05 ^a	4.00 $\pm$ 0.02 ^a
Cholesterol (g/kg of fat)	10.86 $\pm$ 0.10 ^a	11.04 $\pm$ 0.35 ^a
Salt (g/kg)	7.10 $\pm$ 0.04 ^a	7.10 $\pm$ 0.02 ^a
Titratable acidity (% lactic acid)	0.4 $\pm$ 0.1 ^a	0.4 $\pm$ 0.2 ^a
Water-holding capacity (%)	95.6 $\pm$ 0.2 ^a	95.4 $\pm$ 0.4 ^a
SWS ⁴ (%)	ND ⁵	ND
Water activity (–)	0.9879 $\pm$ 0.0007 ^a	0.9891 $\pm$ 0.0010 ^a
Lactic acid bacteria (10 ⁴ cfu/g)	2.14 $\pm$ 0.2 ^a	2.51 $\pm$ 0.6 ^a
Proteolytic activity ⁶	—	—
Lipolytic activity	—	—

^aValues within a row with different lowercase superscripts indicate statistically significant differences at $\alpha = 0.05$.

¹BM-0 = control sample buttermilk; BM-10 = buttermilk after 10 h of storage.

²Values represent mean $\pm$ SD ($n = 7$).

³Total protein = (total N – NPN) $\times$ 6.38; casein = (total N – noncasein N – NPN) $\times$ 6.38; whey protein = (noncasein N – NPN) $\times$ 6.38.

⁴Spontaneous whey syneresis.

⁵Not detected.

⁶Proteolytic activity using plate count agar with 1% and 2% (wt/vol) skim milk; a slight reaction was detected with 1% skim milk and no activity was observed with 2%.

3.4 g/kg of fat (Bourlieu et al., 2018). The Ch content in cheese from BM was 12.32 g/kg of fat (Bierzuńska et al., 2017). The higher content of Ch in BM than in butter is due to the presence of mainly small fat globules in BM. They have a relatively large surface area that allows Ch to accumulate between the MFGM and triacylglycerol (Martin, 1989). Bonczar et al. (2016) showed no effect of storage time (2 wk) on the Ch content in yogurt, kefir, and cultured milk.

Two oxosteroids were identified in the tested BM samples: 7 β -OHC and α -epoxyC (Table 2). The amount of the former compound was 2.4 times more in BM-0 than in BM-10, decreasing significantly to 0.61 mg/kg of fat ($y = -0.09x + 1.47$; $P < 0.05$) after 10 h. The reverse was shown for α -epoxyC content. During storage, the amount of α -epoxyC increased from 0.50 mg/kg of fat in BM-0 to 1.40 mg/kg of fat in BM-10 ($y = 0.09x + 0.5$; $P < 0.05$). In both samples, the sum of COP was about 2 mg/kg, which accounted for 0.02% of the cholesterol content. Cholesterol can be oxidized by free radical reactions or by enzymatic reactions. 7-Hydroxy derivatives of cholesterol are not stable and can react with oxygen formed by other products. Their content changes during storage and it depends on many factors, including temperature, access of light, enzymes, and water content in product. The probability of COP presence in fresh dairy products is very low. Because of the high content of PUFA, BM is susceptible to oxidative deterioration. In the case of the studied BM, the occurrence of COP was caused by exposure to oxygen and water as well as the decreased pH level. A number of research data indicate that fresh cream and butter do not contain COP (Pie et al., 1990; Sieber, 2005). Bierzuńska et al. (2017) noted a total COP concentration of 1.17 mg/kg of fat in fresh white cheese made from buttermilk. Only technological activities, such as heating, can cause their presence or increase their amount. In fresh white cheese made from buttermilk, 7 β -OHC, β -epoxyC, and 25-OHC dominated (Bierzuńska et al., 2017). However, during refrigerated storage after 30 d, total COP concentration reached 4.88 mg/kg. Such a large increase in COP was halted by the introduction of whey protein concentrate. After 30 d of storage with whey protein concentrate, only 7 β -OHC and β -epoxyC were found in the cheese from buttermilk, in a total amount of 1.02 mg/kg of fat (Bierzuńska et al., 2017). The content of oxysterols in foods is highly variable and depends on processing. For example, total COP contents in raw and boiled shrimp are 38.9 and 43.6 mg/kg of fat, respectively (Hernández Becerra et al., 2014). Often total COP are expressed not per kilogram of fat, but per kilogram of sample. Iberian pork contains COP at 1.7 mg/kg of muscle (raw), 3.3 mg/kg

Table 2. Levels (mg/kg of fat) of oxysterols in buttermilk^{1,2}

Oxysterol ³	BM-0	BM-10
7 α -OHC	ND ⁴	ND
7 β -OHC	1.47 $\pm$ 0.05 ^b	0.61 $\pm$ 0.06 ^a
β -EpoxyC	ND	ND
α -EpoxyC	0.50 $\pm$ 0.06 ^a	1.40 $\pm$ 0.07 ^b
TriolC	ND	ND
25-OHC	ND	ND
7-KetoC	ND	ND
Σ COP	1.97 $\pm$ 0.07 ^a	2.01 $\pm$ 0.10 ^a

^{a,b}Values within a row with different lowercase superscripts indicate statistically significant differences at $\alpha = 0.05$.

¹BM-0 = control sample buttermilk; BM-10 = buttermilk after 10 h of storage.

²Values represent mean $\pm$ SD ($n = 7$).

³7 α -OHC = 7 α -hydroxycholesterol; 7 β -OHC = 7 β -hydroxycholesterol; β -epoxyC = 5,6 β -epoxycholesterol; α -epoxyC = 5,6 α -epoxycholesterol; triolC = cholestanetriol; 25-OHC = 25-hydroxycholesterol; 7-ketoC = 7-ketocholesterol; Σ COP = sum of cholesterol oxidation products.

⁴Not detected.

of muscle (fried), 3.4 mg/kg of muscle (grilled), and 3.6 mg/kg of muscle (roasted; Broncano et al., 2009). Mixed vegetables in canned baby foods contain total COP of 0.8 mg/kg, protein athletic supplement powder contains 8.6 mg/kg, and vanilla yogurt contains 1.3 mg/kg (Sander et al., 1989).

The 10-h storage time for BM did not alter FRAP (Δ FRAP potential = 0.002 mmol/L; $P > 0.05$, Table 3). In contrast, the DPPH radical scavenging ability (Δ DPPH = 32.2%, $P < 0.05$, Table 3) was decreased. The DPPH value in BM00 was 0.152 mmol/kg, which is comparable to the antiradical power (against the DPPH radical) noted by Najgebauer-Lejko and Sady (2015) in BM that consisted of defatted milk powder and milk proteins [0.131 mmol of Trolox equivalents (TE)/kg]. The same BM had FRAP = 0.235 mmol of Fe²⁺/L. On the other hand, in a sample of defatted milk powder and natural buttermilk, the antiradical power amounted to 0.140 mmol of TE/kg. Flavored BM with strawberry and rhubarb exhibited 0.88 mmol of TE/kg and FRAP = 1.64 mmol of Fe²⁺/L (Najgebauer-Lejko and Sady, 2015). Antioxidant properties are caused by antioxidant compounds present in fermented milk: casein, whey proteins, peptides and amino acids, coenzyme Q10, enzymatic systems (superoxide dismutase, catalase, glutathione peroxidase), as well as the antioxidant activity of lactic acid bacteria (Najgebauer-Lejko et al., 2011). The efficiency of the lipophilic antioxidants in BM is the result of a greater variety, high thermal stability and, especially, the synergistic interaction between individual lipophilic and hydrophilic antioxidants. They play an important role in the processing of BM, protecting against the formation of undesirable compounds.

Table 3. Antioxidant activity characteristics of buttermilk^{1,2}

Characteristic ³	BM-0	BM-10
FRAP (mmol/L)	0.242 ± 0.015 ^a	0.240 ± 0.010 ^a
DPPH (mmol/kg)	0.152 ± 0.056 ^b	0.103 ± 0.021 ^a

^{a,b}Values within a row with different lowercase superscripts indicate statistically significant differences at $\alpha = 0.05$.

¹BM-0 = control sample buttermilk; BM-10 = buttermilk after 10 h of storage.

²Values represent mean ± SD (n = 7).

³FRAP = ferric reducing antioxidant power in mmol of Fe²⁺; DPPH = antiradical power (1,1-diphenyl-2-picrylhydrazyl) in mmol of Trolox equivalents.

Only 2 COP were identified directly after butter production: 7 β -OHC and α -epoxyC. The toxicity of individual COP is not the same. Triol, which is formed during oxidation of epoxy-derivatives, is the most toxic oxysterol. When the content of epoxides decreases, that of triol increases, so it is important to know how and when this process occurs. Model studies on the formation of volatile compounds generated by a thermal treatment of stigmaterol were performed and hypothetical pathways leading to formation of 2 compounds were presented (Derewiaka et al., 2017). Identification of volatiles formed during cholesterol oxidation permits the use of a rapid and sensitive method for determination of the content of COP in food products. If determination of 1 or 2 volatile compounds indicates that COP are formed, it could be a good tool for producing safer food products because individual COP have different biological activities and different roles in the pathogenesis of diseases.

Total COP quantity amounted to 1.97 g/kg. Although the sum of COP after 10 h of storage did not change, the proportion of oxidation products did change. The content of 7 β -OHC decreased by 59%, and that of α -epoxyC increased almost 3-fold. Other COP were not identified after storage. During storage of BM, its antiradical power decreased by 32% (expressed as mmol of TE). If the total content of COP in BM does not change during storage but DPPH decreases significantly, this implies that other factors, such as lipid oxidation, were more significant than cholesterol oxidation. Fatty acids are more easily oxidized than cholesterol (Ch is more stable). The current study has shown that even a short storage time (10 h) affects the synthesis of COP and the antioxidant potential of BM. Most likely, factors such as exposure to oxygen and light during and after storage were involved. The results of this preliminary study point to new attributes that may be used to increase the commercial value and health-beneficial properties of buttermilk.

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