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REVIEW

Natural antioxidants in milk and dairy products

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Milk antioxidants, both lipophilic (conjugated linoleic acid, α-tocopherol, β-carotene, vitamins A and D_3, coenzyme Q_{10}, phospholipids) and hydrophilic antioxidants (proteins, peptides, vitamins, minerals and trace elements) play a key role in maintaining pro-oxidant and antioxidant homeostasis in the human body. Lipophilic antioxidants are characterised by high thermal stability and they are active in all dairy products. Lipophilic and hydrophilic antioxidants interact in the process of deactivating reactive oxygen species and the final products of lipid peroxidation. A negative correlation between milk consumption and the incidence of diet-dependent diseases confirms that the consumption of milk and dairy products delivers health benefits.

Keywords Bovine milk, Antioxidants, Milk proteins, Lipids, Health.

INTRODUCTION

Clinical and epidemiological research indicates that a disturbance in the pro-oxidant/antioxidant balance in favour of the former lies at the root of diet-dependent metabolic diseases. The pathogenesis of atherosclerosis, neurodegenerative diseases and cancer is linked with free radical reactions and oxidative stress (Kaliora *et al.* 2006; Zhao 2009; Hybertson *et al.* 2011). When the pro-oxidant/antioxidant balance is maintained, free radicals and ROS are deactivated by endogenous and exogenous antioxidants (Poljsak *et al.* 2013). When free radical processes are too intense, high levels of prolonged oxidative stress become very harmful to cells and can induce permanent changes in the structure of biologically active macromolecules (proteins, lipids, DNA, sugars and other). Those changes disrupt biological functions of macromolecules, which leads to perturbations in cell metabolism (Valko *et al.* 2007).

Vitamins and phytocompounds found in cereal products, fruit and vegetables are the main sources of exogenous antioxidants in the diet (Palafox-Carlos *et al.* 2011). Those compounds protect the water-based environment of cells against oxidative stress. However, they are not active in a lipophilic environment, that is, in cell membranes and blood plasma lipoproteins. In the Western diet, reduced consumption of animal fats and their replacement with vegetable oil and margarine lowers the concentrations of vitamins A, E, D_3 and other lipophilic antioxidants (CLA, coenzyme Q_{10}, phospholipids). High consumption of vegetable oils, in particular oils rich in polyunsaturated fatty acids (PUFAs), increases the demand for lipophilic antioxidants by even more than 10-fold (Niki 2014). Low consumption of antioxidants, in particular lipophilic antioxidants, is the main (but not only) cause of the growing incidence of cardiovascular diseases, cancer, neurological and neurodegenerative diseases (Nicco *et al.* 2005).

LIPOPHILIC ANTIOXIDANTS

The major antioxidants in milk fat are conjugated linoleic acid (CLA), vitamins A and E, β-carotene and coenzyme Q_{10}. Other compounds with antioxidant properties include vitamin D_3, phospholipids, ether lipids and, possibly, 13-methyl-tetradecanoic acid (German and Dillard 2006; Baldi and Pinotti 2008).

NATURAL *TRANS* ISOMERS OF FATTY ACIDS

The most active antioxidant in fat milk is CLA, a unique component of milk and the meat of

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ruminants, which is characterised by conjugated double bonds. CLA occurs in the form of 28 positional and geometric isomers that differ in the location of double bonds and spatial configuration of *cis* and *trans* isomers (*cis–cis*, *trans–trans, cis–trans, trans–cis*). Isomers with high biological activity include *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Badr El-Din and Omaye 2007; Wang and Lee 2015).

Natural *trans* isomers of fatty acids (vaccenic acid and CLA) are found in ruminant lipids (cattle, sheep, goats), where they account for 4–6% of all fatty acids (FAs). *cis* FAs can be transformed to *trans* FAs by rumen bacteria (*Butyrivibrio fibrisolvens, Clostridium lochheadi* and *Cellobioparum)* that synthesise isomerases and hydrolases, including linoleic acid, α-linolenic acid and oleic acid. Free fatty acids in the *cis* configuration are released when feed triglycerides are hydrolysed by rumen lipases, and they are transformed by bacteria into bioavailable forms. Biohydrogenation and isomerization lead to the production of separate *cis* and *trans* isomers of α-linolenic acid, linoleic acid, oleic acid and vaccenic acid. Vaccenic acid (*trans*-11-octadecenoic acid) is transformed to CLA when a *cis* bond is introduced in position 9 by Δ^9-desaturase (Wang and Lee 2015). Dairy products (70% of total intake) and beef (25% of total intake) are the most abundant sources of CLA in the human diet (Huth 2007). The CLA content of sheep milk is twice higher in comparison to cow's milk, whereas goat milk is a less abundant source of CLA than cow's milk (Tsiplakou *et al.* 2009). CLA concentrations in cow's milk vary widely, from 2 up to as much as 37 mg/g of fat when vegetable oil is added to feed (Grega *et al.* 2005; Collomb *et al.* 2006). Cows' milk is the most abundant source of (85–90%) of *cis*-9, *trans*-11 CLA, known as rumenic acid (Table 1).

Table 1 Content of CLA and *cis*-9 and *trans*-11 isomers in milk and dairy products

Product	*CLA (mg/g fat)*	*Isomer cis*-9, *trans*-11 CLA (% CLA)	*Product*	*CLA (mg/g fat)*	*Isomer cis*-9, *trans*-11 CLA (% CLA)
Full fat milk	3.4–6.8	82–97	Cheddar	4.0–5.3	78–82
Milk 2% fat	4.1	–	Feta	4.9	81
Condensed milk	6.3–7.0	82	Cottage	4.5–5.9	83
Ice cream	3.6–5.0	76–78	Mozzarella	3.4–5.0	78–95
Butter	4.7–9.4	78–88	Parmesan	3.0	90
Buttermilk	5.4–6.7	–	Ricotta	5.6	84
Yogurt	3.8–8.8	83–84	Romano	2.9	92
Low-fat yogurt	4.4	86	Cheddar	3.6	92
Cream	4.6–7.5	78–90	Processed cheese	4.1–10.7	75

Source: Kritchevsky 2000; Dhiman *et al.* 2005.

Vaccenic acid (C18:1 n-7 with *cis* and *trans* configuration) is a precursor of *cis*-9, *trans-11* CLA isomers. As a biologically active cell membrane lipid (present in phospholipids and glycolipids), vaccenic acid is responsible for the integrity and function of body organs and tissues. Similarly to CLA, vaccenic acid exhibits antisclerotic and anticarcinogenic activity (Jakobsen *et al.* 2006). The wide spectrum of health benefits delivered by CLA can be attributed to high levels of antioxidant activity (approximately 100-fold higher than α-tocopherol; Badr El-Din and Omaye 2007). *In vitro* and *in vivo* studies demonstrated that *cis*-9, *trans*-11 CLA can inhibit the development of tumour cells, delay sclerotic changes and type A diabetes, reduce fat tissue, improve bone mineralisation and exert immunostimulatory and bacteriostatic effects (Parodi 2003). Through its antioxidant properties, CLA protects structural lipids against free radicals and ROS (Palacios *et al.* 2003; Kim *et al.* 2005). Even at low concentrations, CLA is a more effective free radical scavenger than α-tocopherol, and its activity levels are comparable to butylhydroxytoluene (BHT), a synthetic antioxidant used in food products with a high fat content (Badr El-Din and Omaye 2007; Fagali and Catalá 2008).

Due to high levels of antioxidant activity, CLA delivers anti-inflammatory, antisclerotic and anticarcinogenic effects (Lee *et al.* 2009). CLA's high levels of antiproliferative activity were confirmed in numerous *in vitro* studies investigating various lines of cancer cells (Lee *et al.* 2005; Kelley *et al.* 2007). When incorporated into cell membrane phospholipids, CLA modifies membrane permeability, which increases the resistance of biological structures to mutagens and carcinogens (Corl *et al.* 2003). In a study of rats, dietary supplementation with 0.5% CLA decreased cholesterol and triglyceride concentrations and reduced lipid oxidation levels (lower concentrations of compounds reacting with TBARS; Kim *et al.* 2005). Dietary CLA bonds with lipids and, less frequently, with phospholipids, and it scavenges free radicals (Ali *et al.* 2012).

CLA is more effective in inhibiting neoplastic growth at each stage of the process (initiation, promotion and progression) than tocopherols and n-3 PUFAs derived from the fat of fish and marine mammals. CLA can reduce the production of eicosanoids and inhibit DNA synthesis and angiogenesis (Lee *et al.* 2005; Bhattacharya *et al.* 2006; Kelley *et al.* 2007). The results of experiments performed on animal models indicate that a daily CLA dose of 3.5 g is effective in cancer prevention, which is much higher than the amount of CLA supplied by the normal human diet (0.5–1.5 g/day; Varga-Visi and Csapó 2003). Epidemiological research demonstrated that breast cancer mortality rates are lower in countries with the highest consumption of CLA-rich ripened cheeses than in countries where those products

are less popular (Zlatanos *et al.* 2002; Larsson *et al.* 2005). The anticarcinogenic effects of natural CLA are higher than synthetic CLA (Ip *et al.* 1999). The health benefits of CLA in milk fat can be attributed to its synergistic interactions with other milk components (α-tocopherol, β-carotene, vitamins A and D_3, phospholipids, short-chain saturated FAs, vaccenic acid, coenzyme Q_{10}, ether lipids; Parodi 2003).

CLA is also an immunostimulant that enhances the production of immune system cells, mainly lymphocytes, and increases phagocytic activity (Corino *et al.* 2009; Menon *et al.* 2010). Randomised trials on healthy volunteers confirmed that dietary supplementation with a mixture of two CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in a 1:1 ratio) at a daily dose of 3 g for 12 weeks increased IgA and IgM levels and decreased IgE levels. CLA decreased the concentrations of proinflammatory cytokines (interleukin-1, IL-1; and tumour necrosis factor-alpha, TNF-α) and increased the levels of anti-inflammatory cytokines (IL-10; Song *et al.* 2005). CLA not only inhibits inflammatory processes in cells, but also prevents them by lowering the concentrations of arachidonic acid in the plasma phospholipid fraction by modifying the arachidonic acid pathway (Menon *et al.* 2010).

Numerous authors have demonstrated that CLA reduces hyperinsulinemia and free FA levels in the blood by sensitising bodily tissues to insulin. CLA decreases the activity of lipoprotein lipase (responsible for lipid accumulation in adipocytes) and stearoyl-CoA desaturase (SCD) that participates in the biosynthesis of monounsaturated FAs. CLA activates palmitate transferase and intensifies β-oxidation. Regardless of the above, *trans*-10, *cis*-12 CLA inhibits preadipocyte differentiation, thus regulating the production of adipocytes (Chung *et al.* 2005; Iga *et al.* 2009). Supplementation of obese subjects' diets with CLA (a mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in a 1:1 ratio) at a daily dose of 3.4 g for 12 weeks led to fat tissue reduction (Blankson *et al.* 2000). Similar results were reported in a randomised trial involving 60 patients with the metabolic syndrome who received 500 mL of milk supplemented with a mixture of CLA isomers at a daily dose of 3 g for 12 weeks. A decrease in body fat levels reduced abdominal obesity (Laso *et al.* 2007). In a group of patients with diet-controlled glycemia, dietary supplementation with a mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (1:1) at a daily dose of 3 g for 8 weeks improved the blood lipid profile by inducing a significant increase in HDL levels, decreasing the LDL/HDL ratio and reducing fibrinogen concentrations (Moloney *et al.* 2004).

CLA exerts multidirectional health benefits and prevents inflammation by controlling arachidonic acid transformations and promoting high levels of antioxidant activity. Those effects reduce the risk of atherosclerosis, cancer and neurological disorders. CLA plays an important role in regulating the blood lipid profile, preventing hypertriglyceridemia, obesity and type 2 diabetes (Crumb and Vattem 2011; Davoodi *et al.* 2013).

VITAMIN E

Milk is not a rich source of vitamin E. Vitamin E is found in fat globule membranes, and its concentrations are much higher in butter (18–35 μg/g fat) than in milk (0.30–0.83 μg/mL; Sunarić *et al.* 2012). The vitamin E content of dairy products, such as UHT milk, is reduced during long-term storage (Michlová *et al.* 2015). Despite low levels of vitamin E in milk and dairy products (below 1 mg per 100 g), vitamin E plays the role of an antioxidant that protects milk fat against autoxidation (Lindmark-Mansson and Akesson 2000; Sunarić *et al.* 2012).

The vitamin E content of milk is determined by tocopherol levels in feed. The milk of cows fed green forage contains much higher levels of vitamin E than the milk of cows fed a total mixed ration – (TMR); (Grega *et al.* 2005). In feed and in foods of plant origin, vitamin E is present in the form of eight chemical compounds, including four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). Milk fat contains only α-tocopherol which characteristically has the highest antioxidant activity (Akoh and Min 2008; Kalač 2012). According to Akoh and Min (2008), α-tocopherol demonstrates 100% activity, whereas the biological activity of γ- and δ-tocopherols is 10 and 100 times lower, respectively. The biological activity of vitamin E is observed only in α-tocopherol, whereas the remaining isomers, even when fully absorbed, are not converted to active α-tocopherol in the human body. The above can be attributed to the presence of a specialised protein (α-tocopherol transfer protein, αTTP) in the liver that binds, transports and supplies only α-tocopherol to various sites in the body (Azzi 2007; Manor and Morley 2007). The remaining tocopherols and tocotrienols, which are derived mainly from vegetable fats in the human diet, are excreted with bile. αTTP participates in the process of binding α-tocopherol with VLDLs and releasing them into the bloodstream. In the blood, VLDLs are transformed into LDLs and HDLs. α-tocopherol is thus present in all lipoproteins, which contributes to the distribution and efficacy of vitamin E in the body (Schneider 2005).

Vitamin E is the most active lipophilic antioxidant in the human body due to its chemical structure which includes a chromane ring with a head and a tail (isoprenoid side chain; Munne-Bosch 2005; Schneider 2005). The presence of a hydrophobic isoprenoid chain promotes fat solubility, whereas the aromatic ring is responsible for the polar character of vitamin E. The chemical structure of vitamin E facilitates its location in the lipid bilayer of cell membranes, where the hydrophobic chain is inserted between the hydrocarbon chains of lipids and the polar head is exposed to the hydrophilic environment (DellaPenna 2005; Wu and Croft 2007; Zingg 2007a,b).

The main biological functions of vitamin E in the body are the prevention of oxidation of cell membrane lipids and plasma lipoproteins, and scavenging of free radicals and ROS. Vitamin E also enhances the activity of antioxidant enzymes dependent on GSH (peroxidase, transferase, glutathione reductase). The antioxidant effects of α-tocopherol, in particular its ability to disrupt lipid peroxidation chain reactions, have been well researched (Niki 2014). During lipid peroxidation (in biological membranes and lipoproteins), α-tocopherol interacts with peroxide radicals to form relatively low-reactive and stable tocopheryl radicals (DellaPenna and Pogson 2006). The tocopheryl radical (TOC-O$^{\bullet}$) can interact with other free radicals or bind with peroxide radicals, which terminates free radical reactions.

In addition to maintaining the liquidity and integrity of cell membranes, vitamin E influences enzyme activity (phospholipase A2, cyclooxygenase, protein kinases B and C), participates in signal transmission and gene expression control, and exerts immunomodulatory and neuroprotective effects (Zingg and Azzi 2004; Munteanu and Zingg 2007; Wu and Croft 2007). Vitamin E assists vitamin A in deactivating ROS, and enters into synergistic interactions with selenium to protect cell membranes against the negative effects of lipid peroxidation. Under exposure to oxidative stress, vitamin E protects vitamin A and regenerates β-carotene. The tocopheryl radical is regenerated to the metabolically active form of vitamin E in the presence of glutathione, vitamin C, coenzyme Q_{10} (ubiquinone) and β-carotene (He *et al.* 2010; Niki 2014). Low levels of the above antioxidants increase the demand for vitamin E.

As a powerful antioxidant, vitamin E contributes to the optimal structure and permeability of cell membranes, which enhances the function of cells and tissues that are most exposed to oxidative stress (erythrocytes, pulmonary alveoli). Vitamin E deficiency can accelerate ageing and increase the risk of atherosclerosis, cardiovascular diseases, cancer and neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis; Farris and Zhang 2003; Berman and Brodaty 2004; Gohil *et al.* 2007; Niki 2014).

It should be noted that the intake of synthetic vitamin E is associated with an increased risk of myocardial infarction and other cardiovascular diseases. The above was confirmed by a meta-analysis of 19 independent trials (1993–2004) performed on a total of 136 000 patients. Daily vitamin intake of 400 IU increased mortality by around 10%. Synthetic vitamin E may exert toxic effects because it has greater affinity for cell membrane structures, and it ousts natural dietary antioxidants (Miller *et al.* 2005).

CAROTENOIDS AND VITAMIN A

Dairy products are a rich source of carotenoids and bioavailable vitamin A in the daily diet. The highest levels of vitamin A (0.150–1.5 mg/100 g) are found in ripened cheeses and butter, whereas milk, cottage cheese and yoghurt contain significantly lower amounts of vitamin A (0.015–0.150 mg/100 g). Vitamin A and β-carotene concentrations are determined by the bovine diet. Vitamin A precursors are carotenoids supplied with bovine feed (Nozière *et al.* 2006), in particular β-carotene (Calderón *et al.* 2007). Similarly to other carotenoids, β-carotene is decomposed by intestinal carotene dioxygenase to retinal (aldehyde) which is reduced to retinol or retinoic acid (DellaPenna and Pogson 2006). Esterified retinol is transported with blood to the mammary gland. Carotene dioxygenase is characterised by low levels of activity, and when large amounts of β-carotene are ingested with feed, it appears in milk in an unchanged form.

Retinal and retinoic acid are the major biologically active forms of vitamin A. Retinal contributes to vision health, and all-trans retinoic acid regulates cell division, differentiation and growth. Retinoic acid directly affects the epithelium, and it regenerates the skin, cornea and mucous membranes in the gastrointestinal, urinary, reproductive and respiratory systems. Retinoic acid stimulates the immune system and the production of white blood cells – granulocytes and leucocytes (Kim 2011).

Antioxidant activity is one of the most important biological functions of carotenoids, in particular β-carotene, and vitamin A. Antioxidant properties prevent the oxidation of the LDL fraction and inhibit lipid peroxidation in tissues with low partial pressure of oxygen. Under such conditions, carotenoids, in particular β-carotene, scavenge singlet oxygen and lipid peroxides more effectively than vitamins A, E and C. At high partial pressure of oxygen, for example in respiratory epithelium, β-carotene and vitamin A can exert pro-oxidative effects, and their autoxidation initialises the peroxidation of structural lipids (Müller *et al.* 2011; Fiedor and Burda 2014).

As low molecular weight antioxidants, β-carotene and vitamin A are important dietary components that prevent atherosclerosis and cancer. Their anticarcinogenic effects can be attributed to the presence of many conjugated double bonds in their molecules (in particular in carotenoids), which absorb singlet oxygen. This is an important consideration in preventing certain types of skin cancer induced by UV radiation. Due to their ability to absorb singlet oxygen, carotenoids effectively protect DNA against oxidation. Vitamin A, in particular retinoic acid, protects the body against early stages of cancer, whereas β-carotene prevents neoplastic progression (Palozza *et al.* 2002; Sharoni *et al.* 2012; Tanaka *et al.* 2012).

VITAMIN D_3

Similarly to α-tocopherol, β-carotene and retinol, vitamin D_3 is part of milk's nonenzymatic antioxidant system. The

most active form of vitamin D_3, 1,25-dihydroxycholecalciferol, exerts antioxidant effects by inhibiting lipid peroxidation (Mutlu *et al.* 2013; Saedisomeolia *et al.* 2013). The vitamin D_3 content of cow's milk is low at less than 0.002 mg/100 of milk, and it is higher in summer than in fall and winter. Milk fat (butter, ripened cheese, cream) is a much more abundant source of dietary vitamin D_3 (Jakobsen and Saxholt 2009). In the human body, vitamin D_3 is responsible mainly for regulating the calcium and phosphorus balance and maintaining calcium homeostasis. Vitamin D_3 affects the absorption of calcium and phosphorus in the small intestine, and together with parathormone and calcitonin, it is responsible for the resorption of calcium and phosphorus in bones. Vitamin D_3 decreases the amount of calcium and phosphorus excreted with urine, which promotes healthy mineralisation of bones and teeth (Zhang and Naughton 2010; Hossein-nezhad and Holick 2013). Vitamin D_3 is required for the healthy function of parathyroid glands and kidneys. Vitamin K_2, which is present only in the milk of cows fed green forage, is involved in the synthesis of osteocalcin, bone protein that binds calcium ions (Ca^{+2}). The vitamin K_2 content of fermented dairy products on the European market ranges from undetectable to 1100 ng/g, depending on the product's specific microflora (Manoury *et al.* 2013).

Endogenous synthesis of vitamin D_3 in the human body, which takes place in the presence of UVB radiation (256–313 nm wavelength), plays an equally important role in the dietary intake of vitamin D_3. Its precursor is 7-hydrocholesterol, which is secreted by sebaceous glands on the surface of the skin. Cholecalciferol produced in the skin is transported by blood to the liver and the kidneys, where it is transformed to 1,25-dihydroxycholecalciferol. $1,25(OH)_2D_3$ may also be synthesised in immune system cells – macrophages. Vitamin D_3 controls the production of cytokines by Th1 lymphocytes, induces monocyte differentiation and the production of antibodies by B cells and inhibits the proliferation of T cells. Autoimmunisation is probably caused by defective secretion of $1,25(OH)_2D_3$ by macrophages (Schleithoff *et al.* 2006). According to epidemiological research, the prevalence of colorectal, ovarian, breast and prostate cancer is inversely proportional to vitamin D_3 concentrations in the blood serum, and it varies with latitude (Zhang and Naughton 2010). In subjects with $1,25(OH)_2D_3$ concentrations higher than 20 ng/mL, the risk of prostate and colorectal cancer was reduced by 30–50% (Ahonen *et al.* 2000). The prevalence of multiple sclerosis and rheumatoid arthritis was found to be significantly higher in regions with less exposure to sunlight. A prospective study revealed that daily vitamin D_3 intake in daily doses higher than 400 IU significantly reduced the risk of the above autoimmune disorders (Merlino *et al.* 2004; Munger *et al.* 2004). Vitamin D_3 is used in the treatment of psoriasis, and it inhibits demyelination of nerve tissue in multiple sclerosis and reduces damage to splenic β-cells in diabetic patients (Zhang and Naughton 2010).

COENZYME Q_{10}

Milk fat contains small amounts of coenzyme Q_{10} (Table 2). Coenzyme Q_{10} transports electrons in the electron transport chain and participates in ATP synthesis, which improves energy efficiency in cells and tissues. The heart muscle is most sensitive to coenzyme Q_{10} deficiency (Bank *et al.* 2011). Coenzyme Q_{10} is a highly active antioxidant, and its reduced form (ubiquinol) protects cell membranes and LDLs against peroxidation more effectively than α-tocopherol and β-carotene. Ubiquinol prevents the initiation and progression of peroxidation of lipid and phospholipid PUFAs in mitochondrial membranes. By binding with proteins, coenzyme Q_{10} stabilises mitochondrial membranes and ensures their optimal liquidity (Borekova *et al.* 2008).

Ubiquinol promotes the antioxidant effects of vitamin E which inhibits lipid peroxidation in membranes only at the propagation stage. Ubiquinol also participates in the regeneration of the reduced form of vitamin E (reduces the tocopheryl radical to tocopherol). Despite the above, the antioxidant potential of coenzyme Q_{10} is not determined by vitamin E. It is believed that direct antioxidant effects in cells can be attributed to the reduced form of coenzyme Q_{10}, whereas indirect effects follow from the regeneration of vitamin E (Modi *et al.* 2006; Littarru and Tiano 2007).

COMPONENTS OF THE BOVINE MILK FAT GLOBULE MEMBRANE

Nearly all milk fat is enclosed in lipid globules whose membranes are composed of phospholipids, glycosphingolipids, cholesterol and proteins. Milk phospholipids include phosphatidylcholine (lecithin), phosphatidylethanolamine (cephalin), sphingomyelin, phosphatidylinositol, phosphatidylserine and lysophosphatidylcholine (lysolecithin; Table 3). Milk phospholipids account for around 30% of lipids that make up the lipid globule membrane (Singh 2006). In addition to phospholipids, the membrane also contains sphingolipids with a predominance (30%) of sphingomyelin, as well as small amounts of lactosylceramide, glucosylceramide and gangliosides (Lopez *et al.* 2010; Smoczyński *et al.* 2012).

Phospholipids contain significantly higher amounts of unsaturated FAs than triglycerides, which is why they play an important role in metabolic processes in cells and organs. Unsaturated FAs impart liquidity and permeability to membranes, promote the transport of nutrients and metabolites, intensify cholesterol metabolism and determine the function of ion channel proteins (Kelley *et al.* 2007). Phospholipids are involved in cell–cell interactions, differentiation, proliferation and transmembrane transport as receptors for many enzymes, hormones and growth factors. They participate in

Table 2 Content of oxidised forms of coenzyme Q_{10} in dairy products (μg/g)

Unfermented products	*CoQ10 (μg/g)*	*Fermented products*	*CoQ10 (μg/g)*
Milk 3.5% fat	1.3	Yoghurt 1.5–1.6% fat	0.7–1.4
Milk 1.5–1.6% fat	0.7–1.2	Yoghurt 0% fat	to 0.1
UHT milk 3.5% fat	1.7	Fermented milk 3.2% fat	0.5–0.9
UHT milk 1.6% fat	1.2	Fermented milk 1.6% fat	0.5
UHT milk 0.5% fat	0.5	Kefir 3.5% fat	0.9
Cream 35% fat	0.9	Kefir 1.6%	0.7
Cream 20–22% fat	0.5–0.9	Emmentaler cheese	1.3
Butter	7.1	Edam cheese	1.2

Source: Mattila and Kumpulainen 2001; Strazisar *et al.* 2005; Pravst *et al.* 2010.

Table 3 The content of phospholipids in dairy product

Phospholipid fraction	*Cow milk (μg/mL)*	*Butter (mg/g fat)*	*Buttermilk (mg/g fat)*	*Cream (mg/g fat)*
Phosphatidylinositol (PI)	0.64	0.23	1.08	0.46
Phosphatidylserine (PS)	5.53	0.30	4.62	0.81
Phosphatidylethanolamine (PE)	33.37	0.60	15.02	1.59
Phosphatidylcholine (PC)	3.69	0.48	15.92	1.38
Sphingomyelin (SM)	3.23	0.33	8.21	1.09
Total content	46.21	1.95	44.85	5.32

Source: Avalli and Contarini 2005; Donato *et al.* 2011.

protein synthesis and cell regeneration, and they are a source of phosphates for ATP synthesis (Starks *et al.* 2008; Cohn *et al.* 2010; Schubert *et al.* 2011).

Phospholipids contain PUFAs and are able to bind cations; therefore, they can act as antioxidants that protect the gastrointestinal mucosa, brain, liver, spleen and kidneys against the toxic effects of ROS (Spitsberg 2005). Milk fat phospholipids contain optimal proportions of n-6 linoleic acid and n-3 α-linolenic acid (4:1 on average), which promotes the synthesis of eicosanoids, local hormones with immunostimulatory and anticarcinogenic properties, from n-3 α-linolenic acid (Patterson *et al.* 2012).

Milk phospholipids exhibit neuroprotective effects: they control brain activity, improve memory and resistance to stress, and reduce the risk of depression and neurodegenerative diseases. Phosphatidylcholine (lecithin) and sphingomyelin, the predominant fractions of milk phospholipids, are the only sources of choline, the precursor for the neurotransmitter acetylcholine, in young individuals. Choline enhances the function and structural integrity of cells, and it stimulates the nervous system, prevents hyperhomocysteinemia, improves concentration and memory and speeds up recovery after intense physical activity (Osella *et al.* 2008). Numerous authors have demonstrated the presence of correlations between phospholipid deficiency and neurological disorders (Oliveira and di Paolo 2010; Kosicek and Hecimovic 2013). In Alzheimer's disease, phosphatidylinositol, phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-phosphate, which are required for correct synaptic function, are present at reduced levels in brain tissue. The concentrations of phosphatidylcholine and phosphatidylethanolamine are also decreased, which leads to the degeneration of structural phospholipids in the brain. Phosphatidylserine, which plays a key role in nerve signal transmission (prevents neuronal death and dendrite degeneration), effectively counteracts brain ageing. Phosphatidylserine activates tyrosine hydroxylase (which synthesises neurotransmitters, including dopamine) whose deficiency is noted in Parkinson's disease. Butyrophilin, a glycoprotein that accounts for 40% of proteins in fat globule membranes, prevents multiple sclerosis (Spitsberg 2005; Oliveira and di Paolo 2010).

Phospholipids regulate gastrointestinal function, and they are effective in treating liver diseases (Cohn *et al.* 2009; Wat *et al.* 2009). They also prevent infections of the gastrointestinal tract by inhibiting the growth of pathogens such as *Listeria monocytogenes, Campylobacter jejuni, Escherichia coli, Salmonella Enteritidis* and *Helicobacter pylori* (similarly to saturated fatty acids C10, C12 and C18; Sprong *et al.* 2002). Phospholipids have antiviral properties, and they inhibit β-glucuronidase. By inhibiting pathogen growth and antioxidant activity, milk fat phospholipids exert anti-inflammatory effects and prevent carcinogenesis in the gastrointestinal tract (Lopez 2011).

The anticarcinogenic properties of selected phospholipids, such as sphingomyelins, have been documented in numerous clinical trials. Sphingomyelin, applied in amounts of 0.025–0.1% in the diets of mice with chemically induced colorectal cancer and mice with transplanted human cancer cells, inhibited neoplastic growth in more than 50% after 4 weeks (Dillehay *et al.* 1994). Milk phospholipids contain ether lipids (alkylglycerols and alkylglycerol phospholipids) that stimulate the phagocytosis and apoptosis of cancer cells. Macrophages activated by ether lipids secrete more than 60 different substances that inhibit acute and chronic inflammations and, more importantly, recognise tumour cells. Ether lipids are characterised by high levels of antioxidant activity. They stimulate the phagocytosis and apoptosis of tumour cells, have immunostimulatory effects and protect tissues against the toxic effects of hydroxyl radicals generated during radiotherapy. Ether lipids exert multidirectional action and they are effective in very small concentrations (Magnusson and Haraldsson 2011).

HYDROPHILIC ANTIOXIDANTS

In addition to highly active and, in some cases, unique lipophilic antioxidants, milk is also a source of hydrophilic

antioxidants. In milk, hydrophilic antioxidants constitute a large group of nitrogen compounds, such as casein fractions, whey proteins (in particular lactoferrin and β-lactoglobulin), bioactive peptides, low molecular weight nitrogen compounds and uric acid. Another important group of antioxidant compounds are enzymes that neutralise free radicals in milk, including superoxide dismutase (SOD), catalase (CAT), lactoperoxidase (LPx) and glutathione peroxidase (GSHPx). Those enzymes form a synergistic system that increases the antioxidant potential of milk (Sukkar and Bounous 2004; Pihlanto 2006).

ANTIOXIDANT ACTIVITY OF CASEIN

Proteins (casein fractions and whey proteins) and peptides released by proteolytic enzymes have antioxidant properties. Model studies conducted with the involvement of pro-oxidants – ferric ions, ascorbic acids and lipoxygenase – demonstrated that all casein fractions can inhibit the autoxidation of arachidonic acid. Selected β-casein sequences (169–176 and 33–48) inhibit the oxidation of oleic acid *in vitro* (Rival *et al.* 2001b).

The antioxidant properties of caseins can be modulated by dephosphorylation of the protein chain. Dephosphorylated casein and β-casein are more hydrophobic, and they inhibit chemically induced peroxidation of linoleic acid more effectively than their native forms. Higher hydrophobicity increases the scope of interactions with linoleic acid (Rival *et al.* 2001a). Casein scavenges free radicals more effectively when the peroxidation of linoleic acid is chemically induced (with 2,2′-azobis(2-amidinopropane) dihydrochloride) than when oxidation is initiated enzymatically (Rival *et al.* 2001a).

Proteolysis affects casein's ability to inhibit lipid peroxidation. Regardless of concentration, casein hydrolysates demonstrate antioxidant activity due to a higher content of amino acids with antioxidant properties, including histidine, proline, lysine and tyrosine. Free radicals are deactivated by peptides containing hydrophobic amino acids (proline, histidine, tyrosine and tryptophan) and selected free amino acids (tyrosine and cysteine; Pihlanto 2006). A peptide composed of six amino acids (Tyr–Phe–Tyr–Pro–Glu–Leu) is particularly effective in eliminating peroxide radicals. Its antioxidant activity is highly influenced by the C-terminus (Glu-Leu; Irshad *et al.* 2015; Mohanty *et al.* 2015). Casein fragments containing valine (Val) or leucine (Leu) in the N-terminus are also highly effective in scavenging superoxide radicals. Peptide hydrophobicity was not found to be correlated with inhibition of peroxidation, which implies that it is not the sole cause of antioxidant activity. It is believed that the antioxidant activity of peptides is significantly enhanced by tyrosine (Tyr), a strong proton donor (Rival *et al.* 2001a).

Unlike casein hydrolysates, casein phosphopeptides disrupt autoxidation reactions, but at high concentrations, they can induce pro-oxidation processes (Diaz *et al.* 2003). Due to their specific structure, casein hydrolysates and low molecular weight casein sequences are more powerful radical scavengers than casein phosphopeptides. Casein hydrolysates are characterised by high concentrations of histidine, lysine, proline and tyrosine – amino acids that are free radical scavengers (Power *et al.* 2013). Together with β-carotene, casein sequences with estimated molecular weight of 3 kDa can deactivate peroxide, hydroxyl and DPPH radicals, proportionally to their concentrations in the reaction environment (Sakanaka *et al.* 2005). Tyrosine and phenylalanine reduce free radicals by releasing hydrogen ions. Histidine delivers antioxidant effects by chelating metal ions, absorbing active oxygen and scavenging free radicals. Casein and its fragments with phosphorylated serine (SerP–SerP–SerP–Glu–Glu) are highly capable of chelating calcium, iron and zinc cations (Diaz and Decker 2004).

ANTIOXIDANT ACTIVITY OF WHEY PROTEINS

Whey proteins, in particular lactoferrin and β-lactoglobulin, significantly influence the antioxidant properties of milk. Whey proteins demonstrate immunostimulatory, anticarcinogenic and chemopreventive effects *in vitro*. As a source of cysteine, they constitute the substrate in the synthesis of glutathione, an important systemic antioxidant. The antioxidant activity of whey proteins is correlated with their concentrations and with the content of histidine and other hydrophobic amino acids (Jiménez *et al.* 2012; Power *et al.* 2013; O'Keeffe and FitzGerald 2014).

Lactoferrin delivers a host of health benefits, and it is the most valuable protein in the human diet. Lactoferrin binds iron and it increases its bioavailability while blocking its pro-oxidant action. Despite the above, the antioxidant potential of lactoferrin decreases proportionally to the degree of iron saturation. The lactoferrin content of milk is estimated at 0.02–0.35 g/L and it is higher in colostrum (1.5–5 g/L; Liang *et al.* 2011a). The biological activity of lactoferrin is determined by various technological factors: parameters of the heat treatment, the fat content and the degree of saturation of iron (Wakabayashi *et al.* 2006; Considine *et al.* 2007).

Lactoferrin is not highly susceptible to digestive enzymes, trypsin and chymotrypsin, and it maintains its biological properties when ingested with food (Adlerova *et al.* 2008). It aso exerts immunostimulatory effects by preventing pathogen colonisation in the gastrointestinal tract and promotes the growth of beneficial gut microbiota. Lactoferrin attenuates the inflammatory response, increases the cytotoxicity of natural killer cells *in vitro* and inhibits the release of oxygen radicals by leucocytes at sites of inflammation (Steijns and van Hooijdonk 2000; Wakabayashi *et al.* 2006).

The antioxidant properties of milk are indirectly dependent on β-lactoglobulin which has protective effects on

retinol and α-tocopherol. By binding with β-lactoglobulin, vitamins undergo only minor oxidation during transfer from the stomach to the small intestine (Liang *et al.* 2011b). All peptides formed from β-lactoglobulin are free radical scavengers; their activity, measured by the ORAC (oxygen radical absorbance capacity) method and expressed as Trolox (a vitamin E analogue) equivalents, is determined in the range of 4.45–7.67 mM. The ability to absorb generated radicals is attributed to the presence and location of tryptophan (Trp), tyrosine (Tyr) and methionine (Met). The equivalent concentration of a mixture of the above amino acids is characterised by lower ORAC values, which testifies to the importance of the secondary structure of antioxidant peptides (Hernández-Ledesma *et al.* 2007). A peptide (Trp–Tyr–Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile) characterised by a greater ability to scavenge free radicals than butylated hydroxyanisole (BHA) and is a synthetic antioxidant used in the food industry, was isolated from β-lactoglobulin. Enzymatic hydrolysis of other whey proteins also leads to the formation of peptides with high levels of antioxidant activity. High molecular weight peptides (>45 kDa) deactivate peroxides more effectively. Met–His–Ile–Arg–Leu and Tyr–Val–Glu–Glu–Leu peptides are also powerful antioxidants due to the presence of tyrosine and methionine in their structure (Hernández-Ledesma *et al.* 2005).

A comparison of the antioxidant properties of various milk proteins indicates that casein is more effective in inhibiting the oxidation of linoleic acid (under model conditions) than whey proteins. The α_s-casein fraction is characterised by the highest levels of antioxidant activity (Cervato *et al.* 1999). The above was confirmed by Zulueta *et al.* (2009) who demonstrated that total antioxidant status of milk subjected to pasteurisation and UHT processing is determined mostly by caseins (α_s-, β- and κ-), which are characterised by greater thermal stability and a higher share of potentially antioxidant amino acids – tyrosine, tryptophan, histidine, lysine and methionine. Free amino acids are not highly active antioxidants, which indicates that secondary and tertiary protein structures play an important role in shaping their properties (Zulueta *et al.* 2009).

ANTIOXIDANT ENZYMES IN MILK

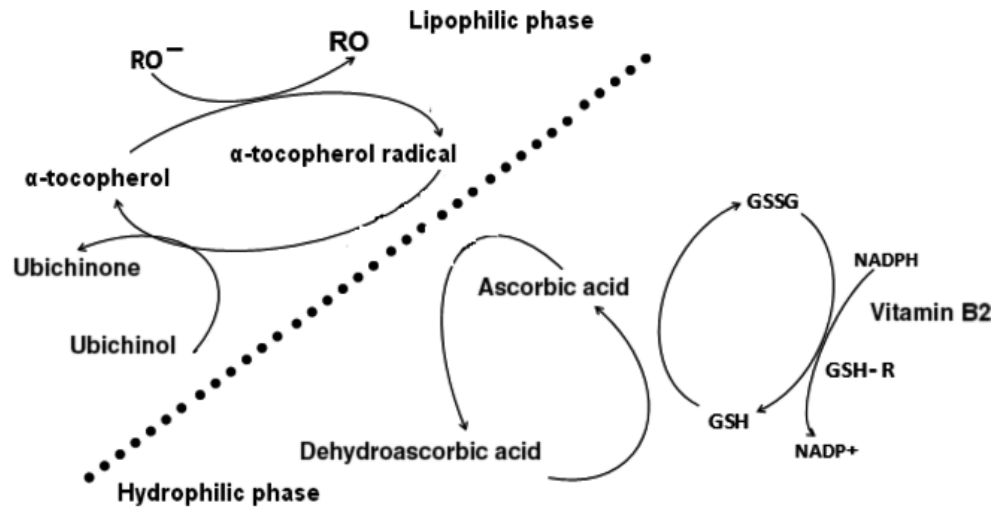
Regardless of casein, whey proteins and the resulting antioxidant peptides, enzymes that neutralise free radicals and ROS significantly enhance the antioxidant capacity of milk (Fang *et al.* 2002). Superoxide dismutase (SOD) present in milk is highly similar to SOD in erythrocytes. SOD transforms superoxide anion radicals to hydrogen peroxide with antimicrobial properties (Filipovic *et al.* 2003; Miller 2004). Catalase (CAT) is one of the most active milk enzymes. Its active centre contains haem-bound iron which breaks down hydrogen peroxide into water and oxygen. Most of the enzyme is bound to the membrane of fat globules, which is why 60% of catalase is found in cream (Fox and Kelly 2006). Lactoperoxidase (LPx) is a special milk enzyme. It catalyses oxidation reactions of various substances (aromatic acids, aromatic amines, thiocyanates, phenols and ascorbic acid) where H_2O_2 is the hydrogen acceptor. LPx exerts bacteriostatic and bactericidal effects by oxidising milk thiocyanates (SCN^-) that originate mainly from feed. LPx is highly resistant to gastric juice, and it remains biologically active in the gastrointestinal tract (Isobe *et al.* 2011). Similar to catalase, glutathione peroxidase (GSHPx) is capable of removing hydrogen peroxide. GSHPx is found in small quantities and it is a carrier of approximately 30% of selenium present in milk. Glutathione peroxidase, catalase and SOD protect cells against free radicals (Fox and Kelly 2006; Andrei *et al.* 2011).

INTERACTIONS BETWEEN ANTIOXIDANTS IN MILK

Despite differences in their location (lipophilic phase and hydrophilic phase) and biological activity levels, milk antioxidants interact effectively by forming an antioxidant network (Figure 1). Antioxidant synergy plays an important role in milk where components are effectively protected by lipophilic and hydrophilic antioxidants. The appearance of ROS in milk initiates enzymatic protective and repair mechanisms. Antioxidants enter into highly specific interactions, which boosts their activity and supports mutual regeneration (Skibsted 2012). In the lipophilic phase, α-tocopherol is regenerated from the tocopheryl radical in the presence of coenzyme Q_{10} in the following arrangement: oxidised form (ubiquinone)–reduced form (ubiquinol): (Bentinger *et al.* 2010; Pravst *et al.* 2010; Kapoor and Kapoor 2013).

Antioxidant synergy associated with interactions between lipophilic α-tocopherol (vitamin E) and hydrophilic ascorbic acid (vitamin C) has been extensively researched. Ascorbic acid contributes to antioxidant synergy by reducing the tocopheryl radical to α-tocopherol. Dehydroascorbate is reduced to ascorbate by reduced glutathione (GSH) with the involvement of NADPH (Choi *et al.* 2004; Duarte and Lunec 2005; Politis 2012; Szarka *et al.* 2012). Synergistic interactions between lipophilic and hydrophilic antioxidants impart high antioxidant potential to milk and effectively protect milk fat against oxidation (Lindmark-Mansson and Akesson 2000; Gaucheron 2011).

Conjugated linoleic acid (CLA) and vitamin E (α-tocopherol) are the most active antioxidants in milk fat. The antioxidant potential of vitamin E, manifested by its ability to deactivate singlet oxygen, is complemented by β-carotene and vitamin A which are much more active than vitamins E and C. Vitamins A, E and D_3 are characterised by high levels of thermal stability and they remain active in all dairy products regardless of the applied thermal processing method (Claeys *et al.* 2014).



RO* - organic free radical
RO - organic compound
GSSG - glutathione disulfide
GSH - glutathione
GSH-R - glutathione reductase
NADPH - nicotinamide adenine dinucleotide phosphate
NADP+ - oxidised form of nicotinamide adenine dinucleotide phosphate

Figure 1 Interaction between lipo- and hydrophilic antioxidants in milk.

Similarly to GSH, vitamin C does not effectively protect milk fat against oxidation because it acts mainly in the aqueous phase (Gaucheron 2011). Milk fat is protected against oxidation by antioxidant enzymes (SOD, CAT, GSHPx, Se-GSHPx), in particular the various casein fractions and/or peptides (Pihlanto 2006; Sharma *et al.* 2011). All casein fractions demonstrate antioxidant activity in milk. Studies of $n-3$ linolenic acid revealed that caseins are also able to inhibit liposome oxidation (Sharma *et al.* 2011). The antioxidant capacity of albumins, Fe^{+2}-binding lactoferrin and phosphopeptides, which block the formation of the most reactive hydroxyl radical, can be attributed to their ability to chelate transition metals (Wakabayashi *et al.* 2006; Adlerova *et al.* 2008).

Milk fat is effectively protected against oxidation by highly active lipophilic antioxidants and antioxidant proteins, including enzymes (Figure 1). Due to the thermal stability of milk fat antioxidants, cholesterol oxidation products (oxysterols) are not found in milk, including UHT milk and milk powder.

CONCLUSIONS

Numerous research studies indicate that the high antioxidant potential of milk is determined mainly by proteins (Zulueta *et al.* 2009; Mann *et al.* 2015). Milk fat antioxidants are responsible for maintaining the pro-oxidant/antioxidant balance in the human body. Hydrophilic antioxidants are not active in the lipid structures of cells, tissues and organs (Baldi and Pinotti 2008; Tijerina-Sáenz *et al.* 2009). Structures that are most prone to oxidative stress (cell membranes, brain, nervous, cardiovascular and respiratory systems) are built mainly of lipids. Milk fat antioxidants which are active in the lipophilic environment support the defence system in preventing oxidative stress. Unique milk fat components, including natural *trans* isomers (vaccenic acid, CLA), ether lipids, 13-methyl-tetradecanoic acid and antioxidant vitamins, have anti-inflammatory, antisclerotic, anticarcinogenic and neuroprotective properties (Gaucheron 2011; Claeys *et al.* 2014).

Lipophilic milk antioxidants are characterised by high levels of thermal stability and they remain active in all dairy products regardless of the applied thermal processing method. No other food product contains as many beneficial ingredients as milk fat. Its antioxidant content, in particular the content of lipophilic antioxidants, is largely determined by the composition of animal diets. Various authors observed that the content of β-carotene and fat-soluble vitamins in the milk of cows fed green forage is 2- or even fourfold higher than in the milk of cows fed a monodiet (Nozière *et al.* 2006; Baldi and Pinotti 2008; Butler *et al.* 2008). In a Swiss study, no significant differences in the SFA content of milk fat were determined between dairy cows raised in an organic farming system and cows fed a traditional high forage diet. However, organic milk was characterised by higher concentrations of branched-chain

PUFAs, in particular $n-3$ fatty acids and CLA (Collomb *et al.* 2008).

The growing incidence of diet-dependent metabolic diseases (obesity, type 2 diabetes, atherosclerosis, cancer, neurological and neurodegenerative diseases) around the world can probably be attributed to long-term deficiency of lipophilic antioxidants. The above results mainly from low consumption of milk fat (due to the aggressive marketing and advertising of vegetable fats and oils) and the widespread use of TMR diets in dairy cattle farms (Kaushik *et al.* 2001).

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