A Critical Evaluation of Nitric Oxide Activity in the Milk of Dairy Ruminants: Nitrite and Catalase Levels Rule its Oxidation Stability and Safety Properties

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ABSTRACT

In this review we provided evidence that mastitis is associated with increase in the concentration of NO• -derived metabolites, nitrite and nitrate, under commercial farming and experimental conditions. NO• constantly cycle in milk through it auto oxidation to nitrite, conversion of nitrite to the potent free radical NO_2•, interaction of NO_2• with thyles radicals formed by NO• to form S-nitrosothiols and disintegration of NO• from S-nitrosothiols complete the cycle. Formation of NO_2• have an important role in the glandular innate defend system because it has bactericidal effects towards major pathogens that inflect the mammary gland. However increase formation of NO_2• that occurs during mastitis and extended storage for more than 3 days, even when kept in cold-dark conditions, induce nitrossative stress on milk organic components. Nitrossative stress in milk is reflected by mark increase in the concentration of 3-nitrotyrosine, carbonyl and lipid peroxides. Thus, it is possible that the current criteria for accepting milk by dairy plants oversight important information on milk safety for consumption by humans. Relevant conclusions to improve safety of milk for human consumption were derived. And the particular importance in applying such recommendations for milk designated for making infant formulas was outlined.

Key Words: milk, safety, free radical, nitric oxide, nitossative stress, oxidatively modified molecules

INTRODUCTION

Milk produced by farm animals, in particular by goats and cows play fundamental role in the nutrition of hundred millions peoples over the world (Silanikove et al., 2010). Nowadays, in 20 to 40% of the animals in a given cow, goat or sheep farm around the world, a bacterial infection will be found in one of their mammary glands (Halasa et al., 2007; Silanikove et al., 2010). Bacterial infection in cows, goats and sheep is associated with subclinical or clinical mastitis (Leitner et al; 2004a,b,c, 2006, 2007, 2008a). Mastitis presents the most debilitating factors in the dairy industry and a lot of research is associated with understanding the interaction between bacterial infection and immune response and milk quality (Halasa et al., 2007; Leitner et al.,2006, 2011, 2012; Silanikove et al., 2010). However, to the best of our knowledge, the impact of changes in milk composition that occur during mastitis or milk storage in the dairy farm storage tank on its safety properties for human consumption has not been considered so far as long as the milk delivered by dairy farms meet the present standards for accepting milk by dairies in western countries. High-quality raw milk constituents are needed for producing milk with long shelf-life and good-quality dairy products such as yogurt and

cheese (Auldist and Hubble, 1998; Barbano et al., 2006; Merin et al., 2008; O'Brien et al., 2001). Nowadays, dairy plants grade milk upon reception according to its hygienic quality. In most countries, these standards comprise limits on maximal transporting temperature, maximal bacterial count, absence of antibiotic residues and somatic cell count ((McLaughlin, 2006; PMO, 2007). However, as elaborated below, these standards cannot inform if unfavorable changes caused by oxidative stress affect milk composition and that recording the oxidative modified changes in milk is important from the point of view of food safety.

Relatively modern findings have shown that nitric oxide (NO•) is produced by mammary epithelial cells and milk somatic cells (Bouchard et al., 1999; Boulanger et al., 2001; Table 1). Increase NO• release is reflected by the presence of nitrite + nitrate (NOx) in milk and their concentration increases during mammary gland inflammation (Table 1).

As elaborated below, it is proposed that measuring of nitrite concentration and catalase activity in milk is critical to understanding the effects of NO• on milk composition during

mastitis and milk storage and the impact of these changes on milk safety. Therefore, it is hope that this critical review would contribute to make researchers and food safety policy makers more aware to the importance of measuring routinely nitrite and catlase in milk.

NITRIC OXIDE METABOLISIM AND CHEMISTRY IN RELATION TO DAIRY SCIENCE

A concise picture of relevant nitric oxide reactions

The chemistry of NO• in biological systems is broad and complex. Many effects of NO• do not involve NO•, but rather are mediated by reactive nitrogen oxide species (RNOS) formed from the reaction of NO• with oxygen, superoxide or hydrogen peroxide (Wink et al., 1996). RNOS formed from NO• can mediate either nitrosative or oxidative stress. It was shown that in milk, the extracellular effects of free radicals are basically nitrosative as a consequence of lack of superoxide production in the extracellular compartment of milk (see detailed discussion below). Due to the extremely short physiological half-life of NO•, alternative strategies for the detection of reaction of NO• by assaying its biochemical products have been developed (Bryan and Grisham, 2007). The

quantification of RNOS in biological samples provides valuable information with regard to in vivo NO•production, bioavailability, and metabolism (Wink and Mitchell, 1988; Wink et al., 1996). The established paradigm of NO• biochemistry from assembly by nitric oxide synthases to formation of nitrite to eventual oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) may only represent part of NO•'s effects in vivo in systemic fluids (Wink and Mitchell, 1988; Wink et al., 1996) and milk (Silanikove et al., 2005, 2006, 2007, 2009, 2012; Titov et al., 2010). The interaction of NO• and RNOS with protein thiols, secondary amines, and metals to form S-nitrosothiols (RSNOs), N-nitrosamines (RNNOs) were found as important physiological determinant that affect milk antibacterial competence (Silanikove et al., 2005) and milk quality (Silanikove et al., 2006, 2007, 2012).

Nitrite is a central homeostatic molecule in NO• biology in systemic fluids (Bryan, 2006) and milk (Silanikove et al., 2005, 2006, 2009, 2012). NOx in blood have been widely used as an index of endothelial nitric oxide synthase activity and as routine indirect measures of NO • levels (Bryant, 2006). However, it is clear that nitrite level in systemic

fluids (Bryan, 2005; Bryan and Grisham, 2007) and milk (Silanikove et al., 2005, 2009, 2012) represents much more direct index of NO• formation than nitrate. Thus, there is much emphasizing on the therapeutic application of nitrite, especially in cardiovascular diseases, using nitrite as marker as well as an active agent (Bryan, 1996; Thomas, et al., 2008). Recent studies in bovine milk highlighted its importance for the evaluation of the nitrosative stress induced during milk storage (Silanikove et al., 2009) and mastitis (Silanikove et al., 2012; Titov et al., 2010).

Five basic distinct concentration levels of NO• activity inside cells have been proposed: cGMP-mediated processes ([NO•] < 1–30 nM), Akt phosphorylation ([NO•] = 30–100 nM), stabilization of HIF-1α ([NO•] = 100–300 nM), phosphorylation of p53 ([NO•] > 400 nM), and nitrosative stress (1 μM and above) (Thomas et al., 2008). Milk nitrite varies over the same range and it concentration can even increases to the range of tenth μM (Silanikove et al., 2009; 2012; Table 1). Whereas, there is no information regarding the biological significance of variations of NO•/nitrite in milk in the sub- μM range, it was

found that nitrite concentration in the range of 1 μM and above are associated with nitrosative stress (Silanikove et al., 2005, 2009, 2012; Titov et al., 2010).

Nitric oxide concentration in the systemic extracellular fluid is unrelated to its levels in milk

Nitric oxide is a gaseous radical that originally found to be released by endothelial cells (Ignarro et al., 1987; Palmer et al., 1987). The original concept was that the small quantities of NO• generated in a pulsative fashion by constitutive nitric oxide synthases has powerful vasodilator activity required for normal homeostatic function of the vasculature ((Ignarro et al., 1987; Palmer et al., 1987). Explosion of research on NO• metabolism revealed that it has many more biological functions. Especially relevant to the subject of this review were finding that NO, nitric •, NO• derivatives and reactive oxygen intermediates are toxic molecules of the immune system, which contribute to the control of microbial pathogens and tumors (Bogdan et al., 2000). For functioning in this line, NO• is produced in high amounts of by inducible nitric oxide synthases by various leukocytes of the innate immune system (Leone et al., 1991). Because NO• has

extremely short physiological half-life (~1s) (Hetrick and Schoenfisch, 2009), its effects on extracellular components is limited while being produced within the cell of leukocytes. However, nitrate and nitrite, are abundant food components and the major source of exposure of nitrite and nitrate comes from the consumption of nitrate-enriched vegetables (IARC, 2010). Nitrite and nitrates in the digestive tract and blood are precursors for nitric oxide in an NO_3^- ⟶ NO_2^- ⟶ NO• axis (Lidder and Webb, 2013). Whereas high intake of nitrate and particularly nitrite may induce toxicity and formation of various types of cancer, particularly in infants (IARC, 2010), there is much evidence that there consumption within regulatory limits have positive effects, particularly, on the cardiovascular function (McKnight and Duncan, 1999; Milkowski et al., 2010).

There are convincing evidence that NO• and its metabolites in the blood system and systemic fluids in human (Wennmalm et al., 1993) and goats (Nielsen et al., 2001) are completely separated from the mammary gland lumen. Under normal non-inflammatory conditions, the mammary gland lumen is effectively separated from the systemic fluids

in cows (Shamay et al., 2003), goats and sheep (Shamay et al., 2002; Leitner et al, 2004a,b) by the tight junctions between the epithelial cells composing the alveoli. Under mammary inflammatory situations, the concentration of milk nitrite and nitrate exceeds their levels in the systemic fluids, indicating that they are produced locally (Silanikove et al., 2005; 2012). Thus, it can safely assume that consumption of nitrate and nitrite through food, or increased secretion of NO• into the blood under inflammatory response in the systemic fluids does not affect chemical of NO•-depended changes in milk.

Nitric oxide metabolism in milk and its effects on milk oxidative stability

Recent studies have shown that enzymes linked to the metabolism of NO•affect the milk composition of inflamed mammary glands (Silanikove et al., 2005, 2006, 2007, 2009, 2012; Titov et al., 2010). Xanthine oxidase (XO), lactoperoxidase (LPO) and their respective substrates xanthine/hypoxanthine and NO• are components of milk in different mammalian species (Silanikove et al., 2006) and functions as component of the mammary innate immune system by interactively inducing an effective bactericidal environment towards major mammary gland pathogens: H_2O_2 and NO• are being

constantly surged from the surrounding epithelial cells and milk leukocytes (Figure 1). NO• start cycling in milk through its auto-oxidation to nitrite making it the best indirect estimate of NO•formation.

These autooxidation reactions of NO•can be described as follow (Ignarro et al., 1993):

$$NO\bullet + O_2 \longrightarrow OONO$$

$$OONO + NO\bullet \longrightarrow 2NO_2^- + O_2NNO_2\ (N_2O_4)$$

$$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+$$

Accordingly, both nitrite (NO_2^-) and nitrate (NO_3^-) can be theoretically performed from NO• gas in the presence of oxygen and water. However, classical study in NO• chemistry has shown that in biological aqueous solution, NO• did not yield significant quantities of nitrate (Ignarro et al., 1993); this finding is now considered as fundamental knowledge in NO• chemistry (Bryant, 2006).

Despite the fact that nitrite is the first product of NO• autoxidation, in milk, as in blood, the NO•-derived species accumulate mainly in the form of nitrate (Silanikove et al.,

2005, 2009, 2012), which is much less active than nitrite (Ellis et al., 1998). In blood, residual amounts of NO• react with water to form nitrite, which, in the presence of heme groups in proteins such as myoglobin or hemoglobin, rapidly oxidizes to nitrate and the corresponding met-heme protein (Sharma et al., 1987). However, milk does not contain hemoglobin or myoglobin; therefore, it is obvious that the equivalent mechanism in milk is different (silanikove et al., 2005, 2009).

Catalases are enzymes that are ubiquitously found in all kinds of living organisms and are best known for catalyzing the decomposition of hydrogen peroxide to water and oxygen. However, catalase contains porphyrin heme (iron) groups in its center and can thus oxidize various acceptors, including nitrite, by functioning as peroxidase according to classical 3-step reactions of peroxidases, according to the following reactions:

$$\text{Catalase-compound I} + NO_2^- \longrightarrow \text{Catalse-Compound II} + NO_3^-$$

$$\text{Catalse-Compound II} + NO_2^- \longrightarrow \text{Catalase} + NO_3^-$$

$$\text{Catalase} + H_2O_2 \longrightarrow \text{Catalase-compound I} + H_2O$$

It has been shown that the conversion of nitrite into nitrate by catalase is the main function of milk catalase and that it serves as a basic mechanism for the prevention of excessive nitrosative stress in milk (Silanikove et al., 2005, 2009; 2012). The increase in nitrate from few μM in high-quality milk to impressive levels at the low hundred of μM levels and even to the low mM levels in one report (Table 1) highlight the essential importance of catalase in maintaining the oxidative stability of milk under various inflammatory and storage conditions.

It is known that challenge with endotoxin (lipopolysacharide) and inflammatory mediators (cytokines) burst the secretion of NO• and xanthine/hypoxanthive) into milk by mammary gland cells (Boulanger et al., 2001; Silanikove et al., 2005, 2007). In addition to the use of hydrogen peroxide by catalase, it was shown that such burst is reflected by accumulation of NO•-derived metabolites that in turn impaired the oxidative stability of proteins and lipids in bovine milk. The main bactericidal effect of NO• in milk may be related to the conversion of nitrite into NO_2• in a hydrogen peroxide-dependent manner by LPO, according to the following reactions:

$LPO + H_2O_2 \longrightarrow LPO\text{-}Compound\ I + H_20$

$LPO\text{-}Compound\ I + NO_2^- \longrightarrow LPO\text{-}Compound\ II + NO_2\bullet$

$LPO\text{-}Compound\ II + NO_2^- \longrightarrow LPO + NO_2\bullet$

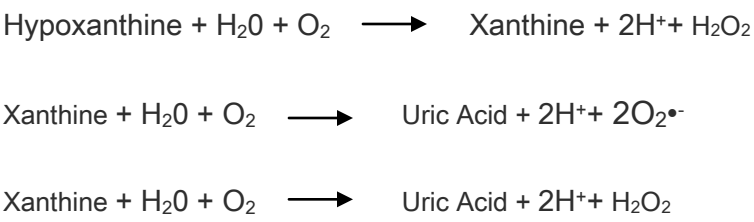
This proposition was further supported by the increased LPO activity and large increase in the content of the RNNOs, nitrotyrozine (3-Nitro-L-Tyrosine; 3-Nitrotyrosine; herby abbreviated as Ntyr) in whey proteins under inflammatory response (Silanikove et al., 2005, 2012). Ntyr cannot be produced directly by NO•, but it can be formed by interaction with NO_2• (Johnston and DeMaster, 2003; Sala et al., 2004).

The above-described results are also consistent with the proposition that increased NO_2• production is also responsible for the accumulation of carbonyls and oxidized fat in milk (Silanikove et al., 2005 and 2009, 2012, Figure 1), as also found in other tissues and cells (Jung et al., 2007). The formation of NO_2• has important function in the innate immune system of the mammary gland due to its bactericidal activity toward major pathogens that are involved in the etiology of mastitis, *S. Aureus* (a gram positive bacteria) and *E. coli* (a gram negative bacteria) (Silanikove et al., 2005).

From the above discussion, it appears that hydrogen peroxide has two functions in milk: i. in the catalase-depended oxidation of nitrite to nitrate and ii. in the LPO-depended conversion of nitrite into the potent free radical NO_2•. Hydrogen peroxide is form in milk from the activity of xanthine oxidase (Silanikove et al., 2005, 2007, 2009, 2012). The distribution of xanthine oxidoreductase (XOR) and its two forms, xanthine oxidase (XO) and xanthine dehydrogenase (XD) in milk fractions have shown that it associated with milk phospholipid membranes (Silanikove and Shapiro, 2007). XOR was found to be distributed among an intra-membranous pool in which it takes the form of a mixture of XO and XD, with a clear predominance of XD, and a free pool of XO, of which ~50% is found in the outer surface of milk phospholipids membrane, and the remaining in solution. Thus, both the membrane-bound form of XO and the XO in solution are free to reacts with its precursors, xanthine and hypoxanthine (Silanikove and Shapiro, 2007).

The conversion of xanthine + hypoxanthine into uric acid is stoichiometrically linked to superoxide and H_2O_2 formation (Fridovic, 1970):

$$\text{Hypoxanthine} + H_20 + O_2 \longrightarrow \text{Xanthine} + 2H^+ + 2O_2\bullet$$



Whether superoxide or hydrogen peroxide would be coupled to xanthine + hypoxanthine oxidation at the molybdenum active site of XO depends on the number of electrons produced during the reaction, which depends on the reaction conditions. Fridovic (1970) has originally shown that under physiological conditions, ~80% H_2O_2 and ~20% $O_2^{\bullet -}$ are produced, whereas the production of 100% $O_2^{\bullet -}$ requires an environment of 100% O_2 at pH 10, which is quite unphysiological. In accordance, it was shown that superoxide is not produced by milk XO (Silanikove et al., 2005) and is scarcely produced by XO attached to the apical surface of bovine aortic endothelial cells (Kelley et al., 2010), whereas hydrogen peroxide is the main product of XO oxidation under both conditions. The lack of evidence for $O_2^{\bullet -}$ production in milk is consistent with the fact that milk is much more hypoxic in comparison with blood. As the production of peroxynitrite (ONOO-) requires the formation of $O_2^{\bullet}$ and $NO^{\bullet}$ in close proximity, the lack

of O_2• formation in milk also explains why milk is protected from the formation of ONOO-, the most powerful oxidant molecule in biological fluid (Godber et al., 2000). This molecule is highly instable and split very fast to the powerful radicals, NO_2• and the hydroxyl radical (•OH^-), or form the •CO_3^- radical by interacting with dissolved CO_2 (Wink et al., 2008). Thus, milk is effectively protected against the formation of the most devastating oxidant in biological fluids.

In systemic fluid, XO is considered frequently as a source for the formation of harmful radicals, such as peroxynitrite and superoxide. The situation in milk is quite complicated. On the one hand, it provides H_2O_2 for the conversion of NO• into NO_2•, which is essential for the glandular defense, but impairs milk composition. On the other, it provides H_2O_2 for the conversion of nitrite into nitrate by catalase, which is essential for the resolution of inflammatory response and in maintaining milk quality during its storage in the udder and under commercial farming conditions (Silanikove et al., 2009).

Additional anti-oxidant system in milk is based on the formation of s-nitrosothiols instead of the more reactive Ntyr, as describe as follow:

$RSH + NO_2\bullet \longrightarrow RS\bullet + NO_2^-$

$RS\bullet + NO\bullet \longrightarrow RSNO$

The association between thyl radicals (RS•) and NO• yields s-nitrosothiols (RSNO). RSNOs are typically relatively unstable molecules, which results in a slow dissociation of NO• from the s-nitrosothiols (Silanikove et al., 2005). Thus, formation of RSNO in milk proteins is the main reason for the constant cycling and accumulation of NO•-derived species in milk (Silanikove et al., 2005, 2009; Figure 1). As the rate of $NO_2\bullet$ formation is second order with regard to NO•-nitrite-mediated oxidation, nitrosation reactions are limited by the availability of nitrite, which in milk is mainly derived as a product of NO• auto-oxidation. Thus, by maintaining a constant NO•-cycle, the ability to respond rapidly to a bacterial infection is preserved as rather small increase in NO• surge will exceed the capacity of RSNO formation and nitrite oxidation by catalase to restrain the formation of $NO_2\bullet$.

Nitrosative stress can be defined as a condition in which the production of highly reactive nitrogen containing chemicals, such as NO_2•, exceeds the ability of biologically regulated systems, such as milk, to prevent oxidative changes in proteins and other organic substances in that system. It has been shown recently that large increase in the contents of Nytr, carbonyl and oxidized fat can be formed in milk under acute mastitis (Silanikove et al., 2012) and prolong storage of milk (Silanikove et al., 2009). From these results, it may concluded that NO_2• formation, which is associated with nitrite concentration that exceed 1 μM, reflect nitrosative stress. Thus, a key question that arise is: to which extent nitrosative stress occur under typical dairy farms situation and whether bulk milk (i.e., milk from the dairy farm tank, or dairy silos), or milk reaching the market may contained proteins and other organic molecules that were modified by nitrosative stress?

AN OVERVIEW ON THE CONCENTRATION OF NITRITE, NITRATE, OR NOx IN THE MILK OF DAIRY RUMINANTS

A summary of available data from publications in the scientific literature in regards to nitrite, nitrate, or NOx concentrations in milk of dairy ruminants is summarized in Table 1. These data include information obtained on milk sampled from farm animals, from milk sampled under experimental situations where mastitis was induced experimentally and from bulk milk, either from a dairy farm level, or from marketed (pasteurized) milk. Most of the data reported in Table 1 was obtained by using colorimetric assay, applying the Griess reagent. The lower limit of detection of this method is around 1 μM (Bintoro et al., 1996; Silanikove et al., 2009) whereas nitrite level in bacterial free milk is the low nM range (Silanikove et al, 2012). Most of the analysis on the single animal's level was reported as NOx without an effort to distinguish between nitrite and nitrate concentrations. Griess reaction detects nitrite, therefore, for the determination of nitrite by the Griess reaction, the tests should have been carried out without the presence of the enzyme nitrate reductase in the Griess reagent. Thus, most data on NOx in Table 1 actually reflect nitrate concentration in the samples. Carrying out colorimetric reaction in milk is quite problematic because of the scattering effect of lipid droplets and casein

micelle colloids, which require quite extensive pre-treatments to solve the problem (Silanikove and Shapiro, 2012). However, in only in few publications an effort was to approach this problem by taking into account recovery and calibration with reference method (e.g., Bintoro et al., 1996). It may be concluded that the Griess reaction is suitable to detect abnormal (> 1 μM) concentrations of nitrite in milk if done and calibrated properly. However, if the aim is to the study the potential biological roles of nitrite in the sub-μM range, or to avoid the time consuming and potential inaccuracies' associated with the pre-treatments for colorimetric reaction, fluorometric assay such as that carried by the DAN reagent can provide detection level at the low nM range without the need to pre-treat the samples (Silanikove et al., 2005, 2012).

The data in Table 1 strongly confirm the notion that NO• secretion into milk is accelerated during sub-clinical and clinical forms of mastitis and that the increase in NO• release into milk is associated with increase in the concentrations of nitrate and nitrite. The large variability in the reported results might be associated with analytical problem discussed above. In addition, it is known that the there is a specific interaction

between the type of bacteria infecting the mammary gland and the host immune system (Leitner et al., 2006). The data of Merin et al (2008) and Silanikove et al (2007) provide examples that subclinical mastitis associated with infection with *S. dysgalactiae* and *E. coli* is particularly devastating in terms of its effect on milk quality and being reflected by higher nitrate concentration in comparison with infection with S. aureus, which is considered as a highly pathogenic bacteria, but its effect on milk composition is milder. The results of Komie et al (2004) represent an example where acute clinical mastitis raised milk nitrate concentration to very high levels. It seems that much more is left to be learned with regard to the interaction between bacterial infection of the mammary gland and the immune response in relation to NO• metabolism in the mammary gland lumen and development of nitrosative stress in milk.

Nitrite and nitrate levels in bulk milk available in the market were reported for milk produced in Brazil, Indonesia, Poland (two studies) and Slovenia. Except for the milk in Slovenia, the data on nitrite concentration from the other countries showed that its concentration was well above the detection limit by the Griess reagent. Thus, this

information should be considered as reliable. The data available from Slovenia was available to us only from the abstract and provided information on the average level; thus, most likely maximum levels exceeded that (1 μM) value; i.e., they were clearly into range that can be considered nitrosative. In fact, the reported values for marketed on the concentration of nitrite in milk were in the same range and even exceeded considerably in one case the value obtained in acute clinical mastitis in single animals (Silanikove et al, 2012), which seems strange at first view. However, the results of Silanikove et al (2009) have shown that storing of raw milk for 3 to 4 days, which is common situation in many farming conditions in many countries, resulted in accumulation of nitrite concentration from the low nM range for up to 5 μM. The following explanation was provided: i. The NO•-cycle described in Figure 1 will continue to function as long as the relevant enzyme, mainly xanthine oxidase and lactoperoxidase, are active (i.e., the milk is not pasteurized) ii. However, at some stage metabolites that used as a source for hydrogen peroxide will exhaust, which will result in accumulation of NO•-autooxidation product, nitrite, in milk. In Silanikove et al (2009)

study, high quality milk from non-infected glands was used, hence, nitrite levels accumulated to 5 μM from initial levels at the low sub- μM range. It is well known that milk is particularly sensitive to oxidation and serious problem for the dairy industry is the lipid oxidation of milk fats, which gives rise to lipocatabolic odor (Lindmark-Månsson and Akesson, 2000) and may results in wasting large amounts of milk (Pal and Mulay, 1985). The phenomena of increase oxidatively modified molecules upon exposure to fluorescent light due to formation of singlet oxygen is well established (Scheidegger et al., 2010). However, in this review we provided evidence that formation of oxidatively modified products in milk, could also result from the formation of NO_2• during mastitis and thus, it is more likely an explanation to formation of lipocatabolic odor in tons of bulk milk. This conclusion is based mainly on research carried mainly in one laboratory, but it is substantiated by some publications: The study of Marenjak et al (2009) showed that low quality milk (i.e., with high in somatic cell count) contain more Lpx than high quality milk and in the study of Mannello et al (2009) it was found that nipple aspirate fluid collected from breast cancer women contains increased protein carbonyl concentration,

which suggested that oxidative stress in the mammary gland was involved in the etiology of the disease. The study of Bhat et al (1980) sustains our finding (Silanikove et al, 2009) that the content of Lpx and carbonyls may increase during milk storage. Similarly, Fonseca et al (2013) have shown that storing goat milk for more than 3 days resulted with higher formation of lipid-modified-components and with lower quality of the whole milk powder made from that milk. Thus, it is possible that if the initial levels of nitrite in milk immediately after milking are much higher than in Silanikove et al (2009), it could end up at the level reported in Table 1 after few days of storage.

According to the data from Brazil (Seraphim et al., 1998), bulk milk from farm level contained more nitrite and nitrate than marketed milk. However, this cannot be taken as evidence that pasteurization reduced the content of nitrite and nitrate in milk. It could have been simply a result of dilution with better quality milk on the dairy silo level. A poorer scenario for the disappearance of nitrite/nitrate level in raw milk during the period elapsed between transportation from farm to dairies plants, storage in dairy silo and pasteurizations is the potential formation of highly carcinogenic and difficult to detect

alkyl proteins (Druckrey, 1973; Bouchikhi et al., 1999). It could be a result of combined activity of α-hydroxylase activity, which may origin from bacterial contamination and indigenous oxidizing enzymes, such as xanthine oxidase and lactoperoxidase, according to scheme originally proposed by Druckrey (1973) and suggested to be of relevance to dairy products treated with nitrite (Bouchikhi et al., 1999).

INTEGRATIVE DISCUSSION

Milk and milk-derived dairy products, such as cheese and yogurt, along with grains, meats, vegetables and fruits are categorized as nutrient-dense foods, i.e., foods that deliver many nutrients and are relevant to health throughout the life cycle (Drewnowski and Fulgoni, 2008; Silanikove et al., 2010). Because of its special characteristic, such as high Ca-content in soluble form and the general resembles to protein and fat composition in human milk, ruminants milk, particularly bovine milk are used as the major source of nutrients for manufacturing infant (< 1 year old babies) and follow-up (> 1 year old babies) formulas. Formulas for babies need to be prepared according to the *Codex Alimentarius* , which is a collection of internationally recognized standards, codes

of practice, guidelines and other recommendations relating to foods, food production and food safety by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). However, upon browsing the *Codex Alimentarius*, no relevant information regarding the issues reviewed here could be found in regard to milk as well as for infant and follow-up formulas.

Thus, the critical question that arise from the discussion and information reviewed above is it to which extend it is relevant to food safety, or in other words, does the current food safety regulation are insufficient in regards to marketed milk, particularly when it comes to milk that indented to be used for babies food?

Compared with other foods, milk is extremely compound matrix. Though its structure appears to be continual and homogeneous, milk is composed of at least five physically and functionally discrete phases (Silanikove, 2008; Silanikove et al., 2006). About 70 enzymes, which are unevenly and specifically associated with one or more of the milk physical fractions, were identified so far in milk. Milk enzymes have an important biological role and so far have been found to be involved in the control of milk secretion,

developmental stage (involution), the gland innate immune, both by producing bactericidal free radicals and preventing by oxidative damage to its essential nutrients. These milk enzyme cascades function at all time during the 6 to 12 hours milk is stored in the mammary gland and during the 2 to 6 days it might be stored before being pasteurized in dairy plants.

In this review we unveiled the mechanism by which increased purge of NO• from surrounding epithelial cells and milk leukocytes during bacterial infection is used to form an effective bacteriostatic environment in milk and presented evidence that an increase in the concentration of NO•-derived metabolites (nitrate and nitrite) in milk is a universal response in different experimental and farming situations. The biochemical responses that occurs in the mammary gland in response to bacterial infection dictate to large extend milk quality for processing into cheese and yogurts and as discussed may negatively affect it marketing quality in terms of safety for human consumption.

As mentioned above, there is no specific recommendation in the *Codex Alimentarius* regarding permissible levels nitrite and nitrate in milk. However, The joint FAO/WHO

Expert Committee on Food Additives (WHO, 2003a,b) confirmed the previous acceptable daily intake of 0–3.7 mg/kg body weight per day for nitrate ion and average daily intake of 0–0.06 mg/kg body weight per day for nitrite ion. These levels were also endorsed in the more recent IRAC (2010) monograph. However, it was noted that these average daily intake do not apply to infants under the age of 3 months. Bottle-fed infants under 3 months of age are most susceptible to methaemoglobinaemia following exposure to nitrate and/or nitrite in the drinking-water (WHO, 2003a). Taking into consideration the knowledge available at that time, and considering the data reported in Table 1, the lack of relation to nitrite/nitrate ion levels in milk seems reasonable. Except for the upper limit of nitrate levels sheep and goat milk found in a single study, even drinking a cup of milk (240 ml) composed from mastitic milk will not bring nitrite/nitrate levels to the upper level of permissible intake. However, research carried in the last few years have shown that inflammatory reactions in the mammary gland, mastitis and extended storage are associated with induction of nirosative stress that modified the oxidative stability of milk organic components and is reflected by formation of Ntyr, Lpx

and carbonyls. A nitrite level of around 1 μM (~ 50 ppm) was identified as the critical level that signal formation of nitrossative stress in milk. The data in table 1 show that in 4 different countries around the globe, the concentration of nitrite in marketed milk exceeds considerably this upper critical level and thus it may contain oxidatively modified molecules. In this review, t was proposed that these relatively high concentrations of nitrite resulted as consequence of using milk that is initially of low quality. According to present regulation in Western countries, low quality milk from cows having mastitis can still enter into the food chain: Typically, nowadays, the somatic cell content (SCC) in the bulk milk tank (BMT) in most European Union countries is around 250 000/ml, whereas the upper permitted level is 600 000/ml (EEC Council Directive 94/71/EC, 1994). Mastitis usually infects a single gland and typically has SCC of 1 × 106/ml and above (Rainard and Riollet, 2006). Thus, according to the current SCC-based hygienic criterion, such milk in small quantities but with high SCC may perhaps enter the BMT, and the milk will still meet the above-described criteria. The amount of low-quality milk that can enter the BMT without violating these criteria is inversely

related to the SCC level. Analysis of the quality of bulk milk in 11 tanks of Israeli dairy farms has shown large variability in milk quality for curdling that was not related to somatic cell count (SCC) (Leitner et al., 2008). It was suggested that this variability was related to mixing milk from post-clinical infection when the milk appears normal and from sub-clinically infected udders with the general milk. However, mixing of milk from infected udders with milk from non-infected ones could not be detected by measures such as determination of SCC, proteose peptone content and % of casein, which worked well at the individual cow level as predictors of udder inflammation (Leitner et al., 2008). Indeed, the results of recent studies (Silanikove et al., 2007; Leitner et al., 2008b, 2012; Forsback et al., 2009, 2010) indicate that it is important for the dairy industry to develop analytical tools that will allow to prevent combining low-quality milk, such as milk rich in somatic cells and nitrite, with high-quality milk in order to ensure optimal yield and quality of curd from milk designated for cheese production. Furthermore, we also showed that even high-quality milk with nitrite level at the low nM range will deteriorate if stored for a period of 3 to 4 days (Silanikove et al., 2009).

As discussed and explained in detail, high levels of oxidized substances such as Ntyr, Lpx and carbonyls in milk under inflammatory conditions, or extended storage are associated with a high level of nitrite in that milk. Ntyr, Lpx and carbonyls are considered as causative agents and hallmarks of cancer, atherosclerosis and other inflammatory diseases (Ohshima et al., 2003). Foods are considered as a major source for intake of such compound and high exposure to them increase the probability of development of cancer (Eichholzer and Gutzwiller, 1998; Knekt et al., 1999). However, as far as we aware there are no known regulation for higher permissible intake of any kind of oxidatively modified molecules. According to Tricker (1997), total human exogenous exposure to N-nitrosamines is estimated to be 1.10 μmol/day; the major exposure sources are the diet (0.79 μmol/day). In Silanikove et al (2009, 2012) studies Ntyr was determined only in whey protein to avoid interference from casein to the color reaction in the ELISA method used to quantify them. However, there is no reason to assume that casein is resistant to Ntyr formation. Indeed, Chiappetta et al (2009) demonstrated that Ntyr forming-sites are distributed among all milk proteins, including

casein and major and minor whey proteins. Based on the data in Silanikove et al (2009), consumption of a cup (240 ml) of low quality milk may be associated with intake of 2.5 μmol/day of Ntyr [240ml x 30 g/l (protein concentration) x 350nM/g (Ntyr concentration on protein)], which is 3.6 time higher than the average exposure to N-nitrosamines from diet. According to the information in table 1, commercial milk can contain much higher nitrite concentration than in Silanikove et al (2009), and therefore the exposure might be even higher. If this estimation is a real situation, obviously, the penetration of such milk to the daily human diet is undesirable.

In a recent the study, the content of oxidatively modified lipid in the form of 4-hydroxynonenal and 4-hydroxyhexenal was found to be considerable higher in milk formula than human milk (Michalski et al., 2008), which may suggest that low quality milk may be used for making babies formulas. All and all, there is dearth of information regarding the significance of the presence of oxidatively modified substance in foods (Michalski et al., 2008; Silanikove et al., 2009).

Due to their lower body mass and higher surface-to-mass ratio than those of adults, infants are particularly sensitive to the presence of free radical products and their pre-cursors in the food chain that contribute to the total reactive oxidative load that infants have to deal with, and they are considered to be factors in the etiology of common infants' and preterm infants' pathogenesis, such as necrotizing enterocolitis, bronchopulmonary dysplasia and type I diabetes (see Silanikove et al for references).Epidemiological studies haves shown that bovine milk is a safe food that contributes positively to preventing obesity and metabolic syndromes, in addition to being an almost irreplaceable source of dietary calcium, particularly for adolescents and postmenopausal women (see, Silanikove et al., 2009). Nevertheless, there also is epidemiological evidence that consumption of cow's milk during the first year of life predisposed infants to type I diabetes, although the basis for that remained elusive (Gerstein, 1994; Akerblom et al., 2002). Currently, the National Health and Medical Research Council of Australia (NHMRC, 2003), and the American Academy of Pediatrics (Committee on nutrition, 1992) recommend that cow's milk should not be

used by infants aged less than 12 months, other than in small amounts in food. The information summarized in this review, which show that bovine milk contains free radicals, their precursors, and oxidative modified products, such as Ntyr, on the one hand, and the concept that type I diabetes is possibly due to the selective death of β-cells as a result of a nonspecific inflammatory attack by diabetogenic RNOS formed from NO• reactions (Kroncke et al., 1995) on the other hand, provide potential explanation for the link between consumption of cow's milk by infants under the age of 12 months and their susceptibility to develop type I diabetes.

In this review, we identified catalase as the most important factor in milk that maintain it oxidative stability. In humans, catalase gene polymorphism is a familiar problem, associated with a range of stress-related oxidative diseases such as atherosclerosis, diabetes, dyslipidemia, and neurodegenerative disease (Mates et al., 1999). We could not find equivalent information regarding potential polymorphism of catalase in bovines and increasing the knowledge in this respect appears to be important.

CONCLUSIONS

Based on the reviewed information, it can be argued that the lack of evaluation of nitrite or formation of oxidative modified products under routine farm practical situation put out of sight the occurrence of nitrosative stress on milk organic components, hence, its safety for human consumption. As can seen from the limited available existing information, nitrate levels in milk cannot be use as predictive of nitrite levels, hence potential activity of NO_2• and formation oxidatively modified molecules. A requirement for measuring of most oxidatively modified products in milk on routine basis, would expose the dairy plants to quite significant burden. On the other hand, analysis of nitrite level by fluorometric methods is fast and accurate. Thus, we would like to suggest that there is an urgent need to develop meticulous safety criteria standards that would limit the contents of radical precursors and radical-preformed oxidized substances in dairy products intended for human consumption and in particular for their use in milk designated to produce infants' formulas. Such criteria should be practical and knowingly

applied by the food industry. Analysis of nitrite and its calibration against formation of oxidative modified products may provide a practical solution for that need.

Currently, no information regarding catalase polymorphism in bovines appears to be available. Thus, in light of its essential contribution to maintenance of milk-quality and safety maintenance, it seems to be important to gain further knowledge on this aspect.

Figure Legend

Figure 1. Scenario of NO•- cycling and the formation of NO_2• oxidation products in bovine milk. See Silanikove et al (2005, 2009) and text for details and justification of the basic NO•-cycle.

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