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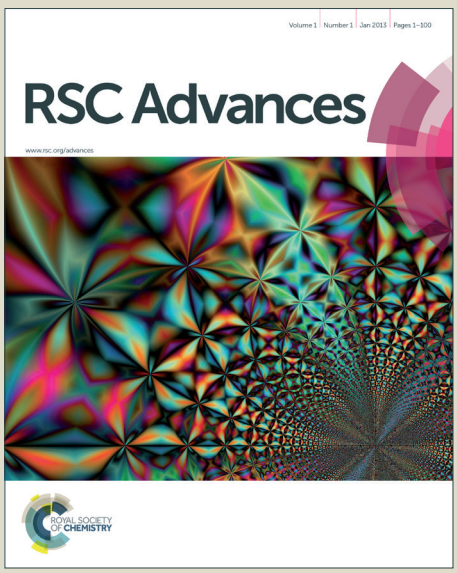




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# Nitrite and catalase levels rule oxidative stability and safety properties of milk: a review

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## Abstract

This review focus on recent evidences showing that various types of udder inflammation (mastitis) are associated with increased concentration of NO•-derived metabolites, nitrite and nitrate, and oxidatively modified organic components under commercial farming and experimental conditions. In milk, NO• constantly cycles through: i. Auto oxidation to nitrite; ii. Hydrogen peroxide-depended conversion of nitrite into $NO_2$• by lactoperoxidase; iii. Interaction of $NO_2$• with thiyl (RS•) radicals on proteins formed by NO• to generate S-nitrosothiols, and iv. Disintegration of NO• from S-nitrosothiols complete the cycle. The main mechanism, which restrains this cycle is the conversion of nitrite to nitrate by catalase in a hydrogen peroxide depended manner. The main source of hydrogen peroxide in milk derives from the oxidation of secreted hypoxanthine and xanthine by xanthine oxidoreductase. Formation of $NO_2$• have an important role in the glandular innate defense system because it has bactericidal effects towards major pathogens that infect the mammary gland. However, increased formation of $NO_2$• that occurs during mastitis and extended storage of milk for more than three days, even when kept in cold-dark conditions, induces nitrossative stress on milk organic components. Nitrossative stress in milk is reflected by a marked 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. The literature regarding the presence of nitrite and nitrate in milk under experimental, farm and marketed milk was reviewed and the potential implications were discussed. Relevant conclusions to improve safety of milk for human consumption were derived, and the particular importance in applying such recommendations for milk designated for the manufacturing of infant formulas was outlined.

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

## Introduction

Milk produced by farm animals, in particular by goats and cows, play a fundamental role in the nutrition of hundreds millions peoples over the world.[1] Nowadays, a bacterial infection associated with subclinical or clinical mastitis will be found in one or more mammary glands in 20 to 40% animals in given farms of cows, goats or sheep around the world.[1,2-8] Mastitis presents the most debilitating factor in the dairy industry and a lot of research is associated with understanding the interactions between bacterial and immune response, and milk quality.[1,2,6,10] However, to the best of our knowledge, the impact of changes in milk composition that occur during mastitis or milk storage on its safety properties for human consumption has not been considered as far as the milk delivered by dairy farms meets the present standards for accepting it by dairies in western countries. Nowadays, dairy plants grade milk upon reception according to its hygienic quality. In most of the countries, these standards comprise limits on maximal transportation temperature, maximal bacterial count, absence of antibiotic residues and somatic cell count.[11,12] However, these standards cannot reveal whether unfavorable changes caused by oxidative stress affected milk composition and that recording the oxidative modified changes in milk is important from the food safety point of view.

Modern findings have shown that nitric oxide (NO•) is produced by mammary epithelial cells and milk somatic cells.[13,14] The increase in released NO• is reflected by accumulation of nitrite and nitrate (NOx) in milk (Table 1). As elaborated below, it is proposed that measuring nitrite concentration and catalase activity in milk is critical for 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 hoped that this critical review would contribute to persuade researchers and food safety policy makers to be more aware to the importance of the routine measuring of nitrite and catlase in milk.

## Nitric oxide metabolism 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 by the reaction of NO• with oxygen, superoxide or hydrogen peroxide.[15] RNOS formed by 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 milk serum (see detailed discussion below). Due to the extremely short physiological half-life of NO•, alternative strategies for detecting the reaction of NO• by assaying its biochemical products have been developed.[16] The quantification of RNOS in biological samples provides valuable information regarding the in vivo NO• production, bioavailability, and metabolism.[15,17] The established paradigm of NO• biochemistry from assembly by nitric oxide synthases to the formation of nitrite to eventual oxidation of nitrite ($NO_2^-$) to nitrate ($NO_3^-$) may only represent part of NO•'s in vivo effects on systemic fluids[15,17] and milk.[18-23] The interaction of NO• and RNOS with protein thiols, secondary amines, and metals to form S-nitrosothiols (RSNOs) and N-nitrosamino acids were found as important physiological determinants that affect milk antibacterial competence[18] and milk quality.[19,20,22]

Nitrite is a central homeostatic molecule in NO• biology in systemic fluids[24] and milk.[18,19,21,22] NOx in blood have been widely used as an index of endothelial nitric oxide synthase activity and as routine indirect measures of NO• levels.[24] However, it is clear that nitrite level in systemic fluids[16,24] and milk[18,21,22] represents a better direct index of NO• formation than nitrate. Thus, there is a lot of emphasize on the therapeutic application of nitrite, especially in cardiovascular diseases, using nitrite as marker as well as an active agent.[25,26] Recent studies on

bovine milk highlighted the importance of the evaluation of nitrosative stress induced during milk storage[21] and mastitis (Fig. 1).[22,23]

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-1 alpha ([NO•] = 100-300 nM), phosphorylation of p53 ([NO•] > 400 nM), and nitrosative stress (1 μM).[26] Milk nitrite concentration varies over the same range and it can even increase to the range of tenth μM (Table 1).[21,22] 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 concentrations above 1 μM are associated with nitrosative stress.[18,21-23]

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

Nitric oxide is a gaseous radical originally found to be released by endothelial cells. 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.[27,28] Flare-up of research on NO• metabolism revealed that NO• has many more biological functions. Especially relevant to the subject of this review were findings that NO• and NO•-derivatives are toxic molecules of the immune system, which contribute to the control of microbial pathogens and tumors.[29] For functioning in this line, NO• is produced in high amounts by inducible nitric oxide synthases by various leukocytes of the innate immune system.[30] Because NO• has extremely short physiological half-life (~1 s),[31] its effects on extracellular components is limited while being produced within the leukocyte's cells. However, nitrate and nitrite are abundant food components and the major source of exposure of nitrite and nitrate is from consumption of nitrate-enriched vegetables.[32] Nitrite and nitrates in the digestive

tract and blood are precursors of nitric oxide in an $NO_3^- \longrightarrow NO_2^- \longrightarrow$ NO• axis.[33] Whereas high intake of nitrate, and particularly nitrite, may induce toxicity and formation of various types of cancer, above all in infants,[32] there are plenty of evidences that their consumption within regulatory limits have positive effects, particularly on the cardiovascular functions.[34,35]

There are convincing evidences that NO• and its metabolites in the blood system and systemic fluids in humans[36] and goats[37] 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,[38] goats and sheep[3,4,39] by the tight junctions between the epithelial cells composing the alveoli. Under mammary inflammatory situations, the concentration of milk nitrite and nitrate exceeds by far their levels in the systemic fluids, indicating that they are produced locally.[18,22] Thus, it can be safely assumed that consumption of nitrate and nitrite through food, or increased secretion of NO• into the blood under inflammatory response in the cardiovascular fluid does not affect the chemistry of NO• 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.[18-23] Xanthine oxidase (XO), lactoperoxidase (LPO) and their respective substrates, xanthine/hypoxanthine, and NO• are components of milk in different mammalian species[19] and functions as constituents of the mammary innate immune system by interactively inducing an effective bactericidal environment against mammary gland pathogens. Hydrogen peroxide ($H_2O_2$) and NO• are constantly surged from the surrounding epithelial cells and milk leukocytes (Fig. 1). NO• cycles in milk through its auto-oxidation to nitrite, making it the best indirect estimate of NO• formation.

These auto-oxidative reactions of NO• can be described as follows:[40]

$NO\bullet + O_2 \longrightarrow OONO$

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

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

Accordingly, both $NO_2^-$ and $NO_3^-$ can be theoretically preformed from NO• gas in the presence of oxygen and water. However, classical studies in NO• chemistry have shown that in biological aqueous solution, NO• did not yield significant quantities of nitrate,[40] a finding which is now considered as fundamental knowledge in NO• chemistry.[24]

Despite the fact that nitrite is the first product of NO• auto-oxidation in milk as in blood, the NO•-derived species accumulate mainly in the form of nitrate,[18,21,22] which is much less active than nitrite.[41] In blood, residual amounts of NO• react with water to form nitrite, which in the presence of heme- groups in proteins, such as hemoglobin, rapidly oxidizes to nitrate and the corresponding met-heme protein.[42] However, milk does not contain hemoglobin or myoglobin; therefore, it is obvious that the equivalent mechanism in milk is different.[18,21]

Catalases are enzymes that are ubiquitously found in all kinds of living organisms and are best known for catalyzing the decomposition of $H_2O_2$ to water and oxygen. However, catalase contains porphyrin heme (iron) groups in its center and thus can oxidize various acceptors, including nitrite, by functioning as peroxidase according to a classical 3-step reaction of peroxidases:

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

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

Catalase + $H_2O_2$ $\longrightarrow$ Catalase-compound I + $H_2O$

It has been shown that the conversion of nitrite to nitrate by catalase is the main function of milk catalase and that it serves as a basic mechanism in the prevention of excessive nitrosative

stress in milk.18,21,22,43 The increase of nitrate from few μM in high-quality milk to notable levels at the low hundred of μM (Table 1) highlights 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 (lipopolysaccharide) and inflammatory mediators (cytokines) cause burst of NO• release and secretion of xanthine/hypoxanthive into milk by mammary gland cells.[14,18,20] In addition to the use of $H_2O_2$ by catalase, it was shown that NO• 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:

$$\text{LPO} + H_2O_2 \longrightarrow \text{LPO-Compound I} + H_2O$$

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

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

This proposition was further supported by the increase in LPO activity and the large increase in the content of nitrotyrosine (3-Nitro-L-Tyrosine, or 3-Nitrotyrosine; [Ntyr]) in whey proteins under inflammatory response.[18,22] Ntyr cannot be produced directly by NO•, but it can be formed by interaction with NO2•.[44,45] Because mastitis causing pathogens such as *Escherichia coli* possesses nitrate reductase activity, the possibility that bacteria contribute to the conversion of nitrate to nitrite and to NO• in infected milk deserves consideration.

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 (Fig. 1), [18,21,22] as also found in other tissues and cells.[46] The formation of $NO_2$• has important function in the innate immune system of the mammary gland due to its bactericidal activity towards major

pathogens that are involved in the etiology of mastitis, i.e., *Staphylococcus aureus* (a gram positive bacteria) and *E. coli* (a gram negative bacteria).[18]

From the above discussion it appears that $H_2O_2$ has two functions in milk: i. In the catalase-depended oxidation of nitrite to nitrate, and ii. In the LPO-depended conversion of nitrite to the potent free radical $NO_2$•. Hydrogen peroxide is formed in milk from the activity of xanthine oxidase.[18,20-22] The distribution of xanthine oxidoreductase (XOR) and its two forms, xanthine oxidase and xanthine dehydrogenase (XD) in milk fractions have shown that it is associated with phospholipids of milk membranes.[47] 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 forms of XO and the XO in solution are free to reacts with its precursors, xanthine and hypoxanthine.[47]

The conversion of xanthine and hypoxanthine to uric acid is stoichiometrically linked to superoxide and $H_2O_2$ formation:[48]

$$\text{Hypoxanthine} + H_2O + O_2 \longrightarrow \text{Xanthine} + 2H^+ + 2O_2\bullet$$

$$\text{Hypoxanthine} + H_2O + O_2 \longrightarrow \text{Xanthine} + 2H^+ + H_2O_2$$

$$\text{Xanthine} + H_2O + O_2 \longrightarrow \text{Uric Acid} + 2H^+ + 2O_2\bullet^-$$

$$\text{Xanthine} + H_2O + O_2 \longrightarrow \text{Uric Acid} + 2H^+ + H_2O_2$$

Whether superoxide or $H_2O_2$ 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[48] has originally shown that under physiological conditions, ~80% $H_2O_2$ and ~20% $O_2\bullet-$ are produced, whereas the production of

100% $O_2$•− requires an environment of 100% $O_2$ at pH 10, which is quite un-physiological. In accordance, it was shown that superoxide is not produced by milk XO[18] and that superoxide is scarcely produced by XO attached to the apical surface of bovine aortic endothelial cells,[49] whereas $H_2O_2$ is the main product of XO oxidation under both conditions. The lack of evidence for $O_2$•− production in milk is consistent with the fact that milk is much more hypoxic in comparison to blood. As the production of peroxynitrite (ONOO-) requires the formation of $O_2$• and NO• 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 fluids.[50] Peroxynitrite 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 $CO_2$.[51] Thus, milk is effectively protected against the formation of the most devastating oxidant in biological fluids.

XO can catalyze the reduction of nitrate to nitrite and nitrite to nitric oxide (NO), thus acting as a source of NO and peroxynitrite (Fig. 1),[52] though as mentioned above, peroxynitrite is not produced in milk. 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 hand, 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.[21]

Additional anti-oxidant system in milk is based on the formation of S-nitrosothiols instead of the more reactive Ntyr, described as follows:

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

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

**The association between NO•, RSNO and NO-derived reactive species**

RSNOs are typically relatively unstable molecules, which result in a slow dissociation of NO• from the S-nitrosothiols.[18] Thus, formation of RSNO in milk proteins is the main reason for the constant cycling and accumulation of NO•-derived species in milk (Fig. 1)[18,21] 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 $NO_2\bullet$, exceeds the ability of biologically regulated systems, such as milk, to prevent oxidative changes in proteins and other organic substances in that system. Recently it was shown that large increase in the contents of Nytr, carbonyl and oxidized fat can be formed in milk under acute mastitis[22] and prolonged storage of milk.[21] From these results, it may be concluded that $NO_2\bullet$ formation, which is associated with nitrite concentration that exceeds 1 μM, reflects nitrosative stress. Thus, a key question that arises is: to which extent nitrosative stress occur under typical dairy farm situations and whether bulk milk (i.e., milk from the dairy farm tank, or dairy silos), or milk reaching the market may contain proteins and other organic molecules that were modified by nitrosative stress?

## An overview on the concentration of nitrite, nitrate, or NOx in milk

A summary of available data from publications in the scientific literature regarding nitrite, nitrate or NOx concentrations in milk of cows is summarized in Table 1. These data include information obtained from milk sampled from farm animals, from milk sampled under experimental conditions where mastitis was induced experimentally and from bulk milk, either from the 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,[21,53] whereas nitrite level in bacteria free milk is in the low nM range.[22] Most of the analyses on the single animal level were 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 in the absence of nitrate reductase in the Griess reagent. Thus, most data on NOx actually reflect nitrate concentration in the samples. Carrying out colorimetric reaction in milk is quite problematic because of the scattering effect owing to the colloidal nature of the casein micelles and the emulsification nature of the fat globules, which require quite extensive pre-treatments to eliminate their effect.[54] An effort to overcome this problem by taking into account recovery and calibration with a reference method was taken only in a few works.[53] 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 study the potential biological role of nitrite in the sub-μM range, or to avoid the time consuming and potential inaccuracies associated with the pre-treatments for colorimetric reactions, fluorimetric assay such as that carried by the DAN reagent or alternative procedures (Table 1) can provide detection level at the low nM range without the need to pre-treat the samples.[18,22]

The data in Table 1 strongly confirm the notion that NO• secretion into milk is accelerated during clinical and subclinical forms of mastitis and that the increase in NO• release into milk is associated with increased concentrations of nitrate and nitrite. The large variability in the results reported in Table 1 might be due to analytical problems, as discussed above. In addition, it is known that there is a specific interaction between the type of bacteria infecting the mammary gland and the host immune system.[6] It was shown that subclinical mastitis associated with infections with *Streptococcus dysgalactiae* and *E. coli* is particularly devastating in terms of its effect on milk coagulation properties and quality.[20,55] These changes were reflected by higher concentration of nitrate in comparison with infection with *S. aureus*, which is considered as a highly pathogenic bacteria, but its effect on milk composition is milder.

Acute clinical mastitis induced by staphylococcal enterotoxin C, a toxin produced by *S. aureus*, raised milk nitrate concentration to very high (mM) levels.[56] Thus, it seems that much more is left to be learned regarding 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, Morocco, New Zealand, Poland (two studies), Taiwan and Slovenia (Table 1). Except for the milk marketed in Taiwan, 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; therefore, most likely the maximum levels exceeded that (1 μM) value; i.e., they were clearly in the range that can be considered nitrosative. In fact, nitrite concentration in marketed milk were in the same range and even exceeded considerably the value obtained in acute clinical

mastitis in single animals in 4 cases (Table 1), which seems strange at first view. However, storing raw milk for 3 to 4 days, which is a common practice in many farming conditions in many countries, resulted in accumulation of nitrite concentration from the low nM range up to 5 μM.[21] The following explanation was provided: i. The NO•-cycle described in Fig. 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 a certain stage metabolites used as a source for $H_2O_2$ are exhausted, thus resulting in accumulation of NO•-autooxidation product, nitrite, in the milk. In the latter study,[21] high quality milk from uninfected glands was used, hence, nitrite levels accumulated to 5 μM from its initial levels at the low sub-μM range. As explained below, it is possible that if the initial levels of nitrite in milk immediately after milking are much higher than those reported,[21] it could end up at the level reported in Table 1 in bulk milk after few days of storage.

It is well known that milk is particularly sensitive to oxidation and a serious problem for the dairy industry is lipid oxidation of milk fat, which gives rise to lipocatabolic odor[57] (rancidity) and thus may result in discarding large amounts of milk.[58] The phenomenon of increased oxidatively modified molecules upon exposure to fluorescent light due to formation of singlet oxygen is well established.[59] 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 udder infllammation and thus, it is more likely an explanation to the formation of rancid flavor in large volumes of bulk milk. This conclusion is based on research carried mainly in one laboratory, but it is substantiated by some publications: The study of Marenjak et al. [60] showed that low quality milk (i.e., with high somatic cell count) contain more Lpx than high quality milk and in the study of Mannello at al.[61] it was found that nipple aspirated fluid collected from breast cancer women contains increased the content of carbonyls on proteins, which suggested that oxidative stress in

the mammary gland was involved in the etiology of the disease. The study of Bhat et al.[62] sustains our findings[21] that the content of Lpx and carbonyls may increase during milk storage. Similarly, Fonseca et al.[63] have shown that storing goat milk for more than 3 days resulted in higher formation of lipid-modified-components and with lower quality powder produced from that milk.

According to the data from Brazil,[64] bulk farm milk 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 the 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 the farm to dairy plants, storage in dairy silos and pasteurization is the potential formation of highly carcinogenic and difficult to detect alkyl proteins.[65,66] It could be a result of combined activity of α-hydroxylase activity, which may originate from bacterial contamination and indigenous oxidizing enzymes such as xanthine oxidase and lactoperoxidase, according to a scheme originally proposed by (Druckrey)[65] and suggested to be of relevance to dairy products treated with nitrite.[66]

## Integrative discussion

Milk and milk-derived dairy products, such as cheese and yogurt, along with grains, meat, 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.[1,67] Because of its special characteristic, such as high Ca-content in a soluble form and the general resemblances to protein and fat composition in human milk, ruminants milk and 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 food, 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 regarding milk, as well as infant and follow-up formulas.

Thus, the critical question that arises from the discussion and information reviewed above is: to which extent are they relevant to food safety, or in other words, does the current food safety regulations are insufficient regarding marketed milk, particularly when it comes to milk that is indented to be used as baby food?

Compared to other foods, milk is an extremely compound matrix. Though its structure appears to be continual and homogeneous, milk is composed of at least five physically and functionally discrete phases.[19,68] About 70 enzymes, which are unevenly and specifically associated with one or more of the milk physical fractions were identified to date in milk. Milk enzymes play 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 immunity, by producing bactericidal free radicals as well as by preventing oxidative damage to its essential nutrients. These milk enzymes cascades function at all time during the 6 to 12 hours that the milk is stored in the mammary gland (between milking) and during the 2 to 4 days it might be stored before being pasteurized in the dairy plant.

In the present 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 evidences that an increase in the concentration of NO•-derived metabolites, Ntyr, carbonyls and lipid peroxides in milk is a

universal response under nitrosative stress (nitrite concentration > 1 μM). The biochemical responses occurring in the mammary gland in respond to bacterial infection dictate to a large extent the quality of milk for manufacturing cheese and yogurts[55] and as discussed, might negatively affect its marketing quality in terms of safety for human consumption.

As mentioned above, there are no specific recommendations in the *Codex Alimentarius* regarding permissible levels of nitrite and nitrate in milk. However, The joint FAO/WHO Expert Committee on Food Additives[69,70] confirmed the previous acceptable daily intake of 0–3.7 mg/kg body weight per day for nitrate ions and average daily intake of 0–0.06 mg/kg body weight per day for nitrite ions. These levels were also endorsed in the more recent IARC[32] monograph. However, it was noted that these average daily intake levels do not apply to infants under the age of 3 months. Bottle-fed infants under 3 months are most susceptible to methaemoglobinaemia following exposure to nitrate and/or nitrite in drinking-water.[69] 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. However, three recent deaths from nitrite tainted milk in China[71] show that carelessness and lack of requirements to measure the level of nitrite in milk may prove to be tragic.

Even drinking a cup of milk (240 mL) composed of mastitic milk will not bring nitrite/nitrate levels to the upper permissible intake level. However, information reviewed above show that inflammatory reactions in the mammary gland during mastitis and extended milk 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 signals formation of nitrosative stress in milk. The data in Table 1 show that in 6 different countries around the globe, one of them (New Zealand) being the largest exporter of milk, the concentration of nitrite in

marketed milk exceeds considerably this upper critical level and thus it may contain oxidatively modified molecules.

In the present review, it is proposed that these relatively high concentrations of nitrite are a consequence of using milk of poor quality. According to present regulations in Western countries, low quality milk from cows having mastitis can still enter the food chain: Typically, nowadays, the somatic cell count (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.[72] Mastitis usually infects a single gland and typically has SCC of ~ $1 \times 10^6$/mL and above.[73] Thus, according to the current SCC-based hygienic criterion, such milk in small quantities but with high SCC may perhaps enter the BMT, and owing to mixing, 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 coagulation propertied that was not related to SCC.[74] It was suggested that this variability was related to mixing milk from post-clinical infection when the milk appears normal and from subclinically infected udders with the general milk. However, mixing of milk from infected glands with milk from uninfected 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.[8] Indeed, the results of recent studies[10,20,74-76] indicate that it is important for the dairy industry to develop analytical tools that will 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.[21]

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.[77] Food is considered as a major source for intake of such compounds and high exposure to them may increase the probability of developing cancer.[78,79] However, as far as we are aware of, there are no known regulations for higher permissible intake of any kind of oxidatively modified molecules. According to Tricker,[80] total human exogenous exposure to N-nitrosamines (a group of carcinogenic substances that are formed under conditions similar to those forming Ntyr) is estimated to be 1.10 μmol/day; the major exposure sources is the diet (0.79 μmol/day). In studies of Silanikove et al.,[21,22] Ntyr was determined only in whey protein to avoid interference from casein to the color reaction of 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.[81] 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.,[21] consumption of a cup (240 mL) of low quality milk may be associated with intake of 2.5 μmol/day of Ntyr [240 mL × 30 g/L (protein concentration) × 350 nM/g (Ntyr concentration in protein)], which is 3.6 time higher than the average exposure to N-nitrosamines from diet according to the study of Tricker.[80] According to the information in Table 1, marketed milk can contain much higher nitrite concentration than those reported by Silanikove et al.[21] and therefore the exposure might be even higher. Furthermore, Ntyr is only one substance of many nitrosative compounds that may be formed under similar conditions, such as di-tyrosine, various N-nitrosamines in addition to carbonyls and Lpx. Thus, if this scenario is a real situation, obviously the penetration of such milk to the daily human diet is undesirable.

In a recent study, the content of oxidatively modified lipid in the form of 4-hydroxynonenal and 4-hydroxyhexenal was found to be considerably higher in milk formula than in human milk, [82] which may suggest that low quality milk may have been used for making those baby formulas. Altogether, there is dearth of information regarding the significance of the presence of oxidatively modified substance in foods.[21,82]

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 precursors in the food chain. These molecules 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.[21] for references). Epidemiological studies have 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.[21] Nevertheless, there is also 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.[83,84] Currently, the National Health and Medical Research Council of Australia,[85] and the American Academy of Pediatrics[86] 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 the present review, which show that bovine milk contains free radicals, its 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 reactive nitrogen species formed from NO• reactions[87] 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.

Catalase was identified in the present review as the most important factor in milk that maintains its 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.[88] 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 be seen from the limited existing information, nitrate levels in milk cannot be use as predictive of nitrite levels, hence potential activity of $NO_2$• and formation of oxidatively modified molecules. A requirement for measuring of most oxidatively modified products in milk on a routine basis would expose the dairy plants to quite significant burdens. However, 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. Analysis of nitrite and its calibration against formation of oxidative modified products may provide a practical solution for that need.

Based on the information reviewed, monitoring nitrite levels in milk is of immediate priority in the following circumstances, in order to potentially improve the safety of milk for human

consumption: 1. Nitrite levels in milk indented for producing infant formulas; 2. Nitrite levels in milk stored for more than 3 days; 3. Nitrite levels in cows recently exposed to clinical mastitis, particularly those caused by *E. coli*; 4. Nitrite levels in countries which use nitrite containing substances in disinfectants used in dairy operations (cleaning milking machines, pipes, tanks etc.), or for preservation of low quality milk to ensure that nitrite concentration does not accumulate to toxic levels in marketed milk as happened in China.

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

## Abbreviations

| | |
|---|---|
| BMT | Bulk milk tank |
| LPO | Lactoperoxidase |
| NO• | Nitric oxide |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| NOx | Nitrite + nitrate |
| Ntyr | Nitrotyrosine (3-Nitro-L-Tyrosine, or 3-Nitrotyrosine) |
| ONOO- | Peroxynitrite |
| RNNOs | N-nitrosamines |
| RNOS | Reactive nitrogen oxide species |
| RSNOs | S-nitrosothiols |
| SCC | Somatic cell count |
| XD | Xanthine dehydrogenase |
| XO | Xanthine oxidase |
| XOR | Xanthine oxidoreductase |

**Legend - Figure 1: A schematic presentation of factors triggering inflammatory response in the mammary gland and accelerating the NO-cycle in milk.**

Schematic model that describes the simultaneous activation of the plasmin (PL) system and the nitric oxide NO-derived cycle in subclinically infected mammary glands. The increased activity of PL causes release of peptides from the casein micelles, which in turn down-regulate milk secretion and casein micelles clotting.[9,22] The release of peptides that are rich in phosphates impairs the coagulation of milk by reducing $Ca^{+2}$ availability.[3,4,99] In parallel, the pro-inflammatory peptides released by PL up-regulates the NO-cycle rate in milk.[22] The increased release of NO into milk is associated with up-regulation of formation of bactericide radical (nitric dioxide), which is associated with formation of nitrotyrosine, carbonyls and lipid peroxide.[18,21,22] Hydrogen peroxide plays an important role in the NO-cycle by its use as a substrate for LPO in forming nitric dioxide and as a substrate for CAT in conversion of nitrite to nitrate. The latter reaction is the main mechanism which restrains the NO-cycle in milk. The source of hydrogen peroxide in milk is oxidation of xanthine and hypoxanthine by XO, which results in accumulation of uric acid as the end product of the xanthenes oxidation.[18,21,22,48] The increased content of oxidized components in milk most likely increases their susceptibility to proteolysis of milk proteins.[6,19]

Explanation of symbols and abbreviations used in the figure:

Casein-derived active peptides:  , casein micelle:  , CAT = catalase, LPO = lactoperoxidase, NO = nitric oxide; PA = plasminogen activator, PL = plasmin, PLG = plasminogen, XO = xanthine oxidoreductase

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Table 1. Summary of reports presenting data on the maximal* values (average + SD, or reported as such) concentrations of nitrite, nitrate, or nitrite + nitrate (NOx) in cow's milk under farm, experimental and in bulk milk under farm or marketed level.

| Nitrite µM | Nitrate µM | NOx µM | Analytical method | Source | Remarks | References |
|---|---|---|---|---|---|---|
| nm | | 12 | Colorimetric; Griess reaction | Dairy farm, SA | Subclinical mastitis | 89 |
| 1 | 73 | | Not available from the abstract | Bulk | The information refers to average values from the abstract. Marketed milk in Slovenia | 90 |
| nm | nm | 35 | Colorimetric; Griess reaction | Dairy farm, SA | Subclinical mastitis | 91 |
| 38 | 212 | | Colorimetric: derivation of Griess reaction | Bulk | Marketed milk in Indonesia | 53 |
| 29 | 589 | | Colorimetric: derivation of Griess reaction | Bulk | Marketed milk in Bialystok, Poland; Mean value of nitrite 2.61 µM | 92 |
| bdl | 12 | 12 | Colorimetric; Griess reaction | SAE | Acute mastitis induced by E. coli and endotoxin (LPS) | 93 |
| nm | nm | 25 | Colorimetric; Griess reaction | SAE | Acute mastitis induced be endotoxin (LPS) | 13 |
| 5 | 130 | | ion exchange LC with spectrophotometric detection | Bulk | Marketed milk in New Zealand | 94 |
| 7, 7, 14 | 40, 89, 94 | | Colorimetric; Griess reaction | Bulk | Marketed milk in 3 districts in Morocco | 95 |
| nm | nm | 13000 | Colorimetric; Griess reaction | SAE | Acute mastitis induced by combination of TNF-α and the enterotoxin C of *S. aureus* | 56 |
| nm | nm | 210 | Colorimetric; Griess reaction | Dairy farm, SAE | Subclinical sub- clinical and clinical mastitis by Clostridium perfringens | 96 |
| 18 | 108 | | Colorimetric; derivation of Griess reaction | Bulk | Marketed milk in various regions in Poland | 97 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | 338 | - | Colorimetric; Griess reaction | Dairy farms, bulk | From the tank of dairy farms in Brazil | 64 |
| 2 | 160 | | Colorimetric; Griess reaction | pasteurized milk | Marketed milk in Brazil | 64 |
| nm | | 54 | Colorimetric; Griess reaction | Dairy farm, SA | Subclinical mastitis with Strep. dysgalactiae and E. coli. | 20 |
| 5 | 14 | | Colorimetric; Griess reaction | Bulk | Simulation of storage of bulk (tank) milk for up to 4 days under cold and dark conditions | 21 |
| 5 | 100 | | Fluorometric, DAN reagent | Dairy farm, SAE | Acute mastitis induced by endotoxin (LPS) | 22 |
| 3 | 120 | | Novel enzymatic method developed by the authors | Dairy farm, SAE | Sub clinical mastitis | 23 |
| Below detection level; much below 1 μM | 7 | | Ion chromatography | Bulk | Marketed milk in Taiwan | 98 |

*basal levels in the experimental studies is not reported, but it was always significantly lower than in infected glands or animals

Abbreviations used: SA- single animal; SAE – single animal under experimental condition; nm – not measured; bdl- below detection level; LPS - lipopolysaccharide.