

INFLUENCE OF REACTIVE OXYGEN SPECIES ON UDDER HEALTH AND MILK QUALITY: A REVIEW

Influência das espécies reativas de oxigênio na saúde do úbere e na qualidade do leite: uma revisão

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ABSTRACT

Mastitis is considered the most common disease affecting dairy herds. It causes relevant economic losses to producers and decreases milk quality and yield for the dairy industry. Regarding this matter, little is discussed about the formation of reactive species of oxygen and nitrogen in the mammary gland and the negative effects of these compounds on milk quality. Nevertheless, studies reveal that a series of chemical reactions happen in the mastitis-related inflammatory process, leading to enzyme alterations and toxic compound formation. Long-term consumption of those compounds in milk may pose consumer health risks. Thus, the present study outlines the deleterious effect of oxidative compounds on milk quality and its potential implications for udder health.

Keywords: antioxidant; mastitis; milk composition; oxidative stress; ROS.

RESUMO

A mastite é considerada a doença de maior importância nos rebanhos leiteiros.

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Recebido / Received: 07/10/2020
Aprovado / Approved: 15/01/2021

Ocasiona grandes perdas econômicas aos produtores e diminuição da qualidade e do rendimento do leite para a indústria. Pouco se fala a respeito da formação das espécies reativas de oxigênio e nitrogênio na glândula mamária e os efeitos negativos destes compostos na qualidade do leite. Porém, estudos demonstram que no processo inflamatório durante a mastite, ocorrem uma série de reações químicas que levam a alterações de enzimas e formação de compostos tóxicos, que quando consumidos frequentemente no leite e a longo prazo, podem trazer consequências negativas à saúde do consumidor. Assim, a presente revisão destaca o efeito deletério dos compostos oxidativos na qualidade do leite e suas possíveis implicações para a saúde do úbere.

Palavras-chave: antioxidante; mastite; composição do leite; estresse oxidativo; ERO.

INTRODUCTION

It is noteworthy that milk is one of the most nutritionally complete foods available in nature as an excellent source of many minerals, proteins, and essential fatty acids. The current milk quality parameters include the absence of veterinary drug residues and low somatic cell and bacterial counts (PICINI *et al.*, 2017). However, there are few studies on the presence of hazardous oxidative compounds in commercial milk and their implications for human health.

Despite years of research, mastitis remains a major issue for the dairy industry and dairy herds due to its high prevalence and the concerns associated with: a) reduced milk production and quality; b) treatment costs and additional labor; c) premature culling and death of dairy animals; d) milk disposal; e) public health; f) animal welfare, and g) image of the dairy sector (i.e., nutritious food from healthy animals) (VARGAS *et al.*, 2016; PIEPERS; DE VLIEGHER, 2018). However, the risks of the consumption of milk from cows affected by mastitis and, thus, presenting high somatic cell count (SCC), is rarely discussed.

Additionally, the literature lacks information on the impact of metabolites produced by microorganisms causing mastitis and toxic

compounds produced during the inflammatory response to infection. Among these toxic compounds, we highlight the reactive oxygen species (ROS). They are involved in immune system activation, and they have many other essential roles in the physiological process (ALFADDA; SALLAM, 2012). However, the excessive presence of these species can damage cellular components and even tissues, leading to oxidative stress.

Increased ROS production in dairy cows, especially during inflammation of the mammary gland, may affect milk quality resulting in technological problems, such as off-flavors and even risks to human health (OLIVER.; CALVINHO, 1995; PAIXÃO *et al.*, 2017). In this scenario, our research approaches the following aspects: the occurrence of these molecules in the mammary gland, methods of ROS detection in milk, and the impact on udder health and milk quality.

REACTIVE OXYGEN SPECIES (ROS)

ROS are highly reactive molecules produced mainly in the mitochondrial electron transport chain. Organic molecules, inorganic compounds, and atoms containing one or more unpaired, independently existing electrons may be considered ROS (HALLIWELL,

1994), which are chemically unstable and highly reactive but with a short half-life. They are commonly termed free radicals, even if ROS and reactive nitrogen species (RNS) present some non-radical reactive species. So, ROS may be classified into two groups: free oxygen radicals and non-radical species. Non-radical species do not have unpaired electrons, but they are unstable and may generate free radicals. The main ROS are singlet oxygen ($^1\text{O}_2$), superoxide anion ($\bullet\text{O}_2^-$), hydroxyl radical ($\bullet\text{OH}$), nitric oxide (NO), peroxynitrite (ONOO^-), and semiquinone radical (Q^\bullet). Although hydrogen peroxide (H_2O_2) is not considered a free radical, it is able to cross the nuclear membrane and induce DNA damage through enzymatic reactions (ANDERSON, 1996).

Until the last decade, ROS was believed only to cause oxidative damage to biomolecules, contributing to the development of a variety of diseases. However, recent evidence suggests that intracellular ROS are critical for the maintenance of many normal physiological functions, including immune defense and antibacterial action (ALFADA; SALLAM, 2012; SENA; CHANDEL, 2012; DI MEO *et al.*, 2016; SIES; JONES, 2020). One of the first lines of defense against pathogens is the production of ROS in the respiratory burst by activated phagocytes (ALFADDA; SALLAM, 2012).

The organism presents a complex system of enzymatic (e.g., superoxide dismutase and catalase) or non-enzymatic (e.g., vitamin E and selenium) antioxidants to control the presence of ROS (SHARMA *et al.*, 2011). Excessive production of ROS or an imbalance between the generation thereof and the action of antioxidant systems may conduct to pathological situations, so-called oxidative stress (SHARMA *et al.*, 2011; ALFADA; SALLAM, 2012).

Moreover, the deleterious effects of ROS may arise from the secondary for-

mation of highly reactive species. Unlike the primary species (i.e., $\bullet\text{O}_2^-$, H_2O_2) and NO (nitric oxide), the secondary species (i.e., $\bullet\text{OH}$, ONOO^- , and HOCl – hypochlorous acid) are highly toxic and, if they are not controlled, may cause irreversible damage to the organism. The secondary compounds are usually formed by reversible reactions between the primary compounds and cellular targets, such as proteins and fatty acids (WEIDINGER; KOZLOV, 2015).

OXIDATIVE STRESS

Oxidative stress occurs when there is an accumulation of ROS. The term has been coined to denote changes in redox signaling and control (SIES; JONES, 2020). Chemical reactions occur between an oxidizing agent and a reducing agent, which may be part of a structural cellular component or even a secondary messenger. The consequences of oxidative stress include lipid peroxidation and protein reactions (SHARMA *et al.*, 2011; JÓŹWIK *et al.*, 2012), resulting in cellular and tissue damage. The production of ROS has been associated with a variety of human diseases, such as liver disorders, cancer, insulin resistance, diabetes mellitus, cardiovascular diseases, atherosclerosis, and aging (ALFADDA; SALLAM, 2012; LIGUORI *et al.*, 2018).

In dairy cows, ROS is largely involved in the etiologies of many disorders, especially during increased metabolic demand periods, such as the peripartum or transition period (SHARMA *et al.*, 2011). From the clinical perspective, oxidative stress is the primary cause of most metabolic disorders in cows during the transition period (CASTILLO *et al.*, 2013). Due to the physiological stress during calving associated with the decrease in feed intake (and antioxidants ingestion) and the negative energy balance (SHARMA *et al.*, 2011), the increased oxygen consumption at

higher metabolic demands results in increased ROS production and lower availability of antioxidant defenses. Oxidizing substances in plasma and erythrocytes, as well as antioxidants products, vary at this stage and lead to changes in oxidative status during early lactation (BERNABUCCI *et al.*, 2002; CELI, 2010), especially in high producing dairy cows. Oxidative stress enhances the susceptibility to mastitis. Besides this negative effect on animal health, ROS accumulation, and oxidative stress can impact milk quality (SORDILLO *et al.*, 1997; PAIXÃO *et al.*, 2017).

One of the most complex and highly prevalent disease in dairy herds is mastitis. The disease has a huge economic importance; however, we must draw attention to the implications for public health (PRAKASHBABU *et al.*, 2020). Mastitis occurrence enhances the production of ROS due to the increase in the population of polymorphonuclear leukocytes (PMNLs) from blood, especially neutrophils with high microbicidal capacity, but also to the production of molecules that activate or generate reactive nitrogen intermediates (ABD ELLAH, 2013). PMNLs produce proteolytic enzymes and ROS, which can damage the mammary tissue if not correctly suppressed. Therefore, considering that milk with a high SCC has an important presence of ROS related compounds, mastitis must be strictly controlled to minimize the negative impact of these compounds on public health, a fact that is generally neglected in the milk supply chain (TAO, 2015; KHAN *et al.*, 2019).

INFLUENCE OF ROS ON THE MAMMARY GLAND

The pathogen invasion into the mammary gland is followed by phagocytosis and inactivation of the pathogen by PMNLs in a process called the respiratory burst.

Excessive stimulation of such a process may result in mammary epithelial cells injury and decreased milk secretion (ALNAKIP *et al.*, 2014). According to Chew (1996), the process by which a particle binds the surface of PMNLs activates the NADP-oxidase system. It results in the sequential conversion of an oxygen molecule to superoxide anion, or H_2O_2 , then myeloperoxidase, a highly bactericidal component, generates hypochlorite ions (OCl^-). A second important pathway involves a bactericidal reaction with superoxide H_2O_2 to generate $\bullet OH$ and atomic oxygen (O). Both $\bullet OH$ and O_2 radicals are unstable and react with bacterial lipid hydroperoxides to form bactericides. However, such ROS, when in the extracellular environment, may be harmful to immune cells and surrounding tissues (CHEW, 1996).

During inflammation, macrophages and epithelial cells of the mammary gland produce significant amounts of nitric oxide (NO), an inflammatory mediator in mastitis (ABD ELLAH, 2013; ALNAKIP *et al.*, 2014; IBRAHIM *et al.*, 2016). The antimicrobial property of NO is ascribed to $ONOO^-$, a reactive metabolite derived from the oxidation of NO (ABD ELLAH, 2013; IBRAHIM *et al.*, 2016). In severe mastitis, $ONOO^-$ is excessively produced, which may result in changes in antioxidant balance (ABD ELLAH, 2013).

Silanikove *et al.* (2014) have studied the effects of subclinical mastitis by non-*aureus staphylococci* (NAS) on the milk quality and total goat milk antioxidant capacity by checking the existence of the NO cycle. All metabolite concentrations were tested, and the enzyme activity was significantly higher in mammary glands experimentally infected by NAS than in control glands, according to data shown in Table 1. Indeed, a positive correlation between somatic cell count (SCC) and NO concentrations was observed (ABD ELLAH, 2013).

Table 1 – Concentration increase rate of metabolites and enzymes in the mammary gland of goats experimentally infected by non-aureus staphylococci (SILANIKOVE *et al.*, 2014)

Metabolite/Enzyme	Increase (relative to 1)
Nitrite	2,3
Nitrate	8,7
Uric acid	2,2
S-nitrosamines	2,0
Xanthine oxidase	2,3
Lactoperoxidase	5,3
Catalase	3,6

ROLE OF ANTIOXIDANTS

The continuous ROS production during metabolic processes led to the development of physiological antioxidant defense mechanisms to reduce the intracellular levels of these compounds and body damage (SIES, 1993). Antioxidants are agents responsible for inhibiting and reducing the injuries caused to cells by ROS. According to Sies; Stahl (1995), an antioxidant is any substance that, when present in low concentrations compared to an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. According to Sies (1993), the agents that protect cells against the excessive ROS effect may be classified as enzymatic or non-enzymatic antioxidants (Table 2). Ascorbic acid is the most important water-soluble antioxidant, while tocopherol is the most important lipid-soluble antioxidant (ABD ELLAH, 2013; PEHLIVAN, 2017).

In addition to vitamins, some minerals, including zinc and copper, also are described as displaying antioxidative properties, and they have additional specific functions in dairy cows' metabolism (YANG; LI, 2015). Selenium is also important since it makes part of the structure of the enzyme glutathione

peroxidase (GSH-Px), which is involved in the cellular antioxidant system (SHARMA *et al.*, 2011).

Table 2 – Main non-enzymatic and enzymatic antioxidants (SIES, 1993).

Non-enzymatic	Enzymatic
α -tocopherol (Vitamin E)	Superoxide dismutase
β -carotene (Vitamin A)	Catalase
Ascorbic acid (Vitamin C)	NADPH quinone oxireductase
Flavonoids	Repair enzymes
Plasmatic Proteins	
Selenium	
Reduced glutathione	
Chlorophylline	
L-cysteine	
Curcumin	

Dairy cow nutrition may play an important role in oxidative stress and, consequently, in mastitis frequency and milk quality. For example, a high energy diet rich in starch enhances the risk of oxidative stress to some extent, because a high level of blood glucose stimulates the production of ROS (JÓŻWIK *et al.*, 2012). In a significant manner, micronutrient deficiencies are related to mastitis resistance, with consequent diminution of the defenses against the bacterial infections or decrease in the integrity of the teat tissue by causing alterations on the keratin layer or impairing the integrity of epithelial cells (SORDILLO *et al.*, 1997). On the other hand, supplementation with antioxidants has a negative correlation to mastitis frequency and severity (ABD ELLAH, 2013). In dairy cows, vitamin supplementation (combinations of vitamins A, D, and E) reduces the incidence of clinical mastitis (ABD ELLAH, 2013;

CASTILLO *et al.*, 2013) and could also increase milk quality (CASTILLO *et al.*, 2013). According to Castillo *et al.* (2013), vitamin E supplements may increase milk oxidative stability, indirectly and directly, i.e., by improving udder health and increasing the tocopherol content in milk, respectively. Ascorbic acid is produced in the cattle liver (MATSUI, 2012); however, milk ascorbic acid concentration decreases during mastitis, so vitamin C administration may also contribute to the mastitis treatment (WEISS *et al.*, 2004; ABD ELLAH, 2013).

Other nutrients have positive effects on ROS in dairy cows. Osorio *et al.* (2014), while researching the effects of the inflammatory process in the cow during postpartum, surprisingly discovered positive responses on liver function, inflammation, and oxidative stress status of cows utilizing a ruminal protector. Smartamine M[®] (polymer-coated methionine to pass through the rumen, and to be released in the abomasum, and absorbed in the small intestine) or MetaSmart[®] (specific methionine) were used.

Similarly, Basiricò *et al.* (2017) found a positive effect using conjugated linoleic acid

(CLA) on the antioxidant response of lipid peroxidation in BME-UV1 cells. Mayasari *et al.* (2017) demonstrated that the absence of the dry period might lead to an elevation in the cholesterol, ROS, and ceruloplasmin levels, and reduction in the plasma bilirubin and paraoxonase concentration. Additionally, they have also reported reduced hepatic function index and augmented occurrence of clinical issues in the cows under study.

METHODS FOR ROS DETECTION IN COW MILK

The SCC is globally considered as one of the most important parameters for assessing the quality of milk. Milk SSC is a known indicator of mastitis occurrence, but it may be an indicator of oxidative stress as well since it is positively correlated with the malondialdehyde (MDA) content (ZIGO *et al.*, 2019).

There are other alternative methods for assessing milk quality, such as the measurement of ROS levels or the detection of secondary compounds. Currently, ROS detection is most accurately carried out

Table 3 – Effects of micronutrients on the mammary gland immunity (YANG; LI, 2015)

Micronutrient	Observations
Vitamin A	Adequate levels led to a decrease in milk somatic cell count.
β-carotene	Increase the bactericidal function of phagocytes. Increased proliferation induced by lymphocyte mitogens.
Vitamin E	Increase in the bactericidal activity of neutrophils and decrease in the incidence of clinical mastitis.
Selenium	Deficiency leads to a decrease in neutrophils efficiency. Increased intake enhances the bactericidal capacity of neutrophils and decreases the severity and duration of mastitis.
Copper	Deficiency diminished neutrophils' phagocytic capacity and caused more susceptibility to bacterial infection.
Zinc	Deficiency leads to diminished leucocytic function and augments susceptibility to bacterial infection.

using spectrophotometric techniques in the UV region (VILLAMENA, 2016). As this author mentioned, however, there are other techniques for ROS identification in cells and biological tissues, namely: 1) fluorescence spectroscopy and microscopy; 2) electron paramagnetic resonance (EPR); 3) immunochemical techniques; 4) mass spectroscopy; 5) electrochemical spectroscopy; and 6) nuclear magnetic resonance imaging.

For detection of O₂ in food, Barba *et al.* (2020) suggested using Electron Spin Resonance (ESR) Spectroscopy, a highly sensitive technique for ROS detection. Verma; Ambatipudi (2016) showed that modified gas chromatography and mass spectrometry might be effective for determining free fatty acid, polyunsaturated fatty acid, and prostaglandin levels in the mastitis-affected cow milk. Chipilev *et al.* (2017) reported chromatographic and spectrophotometric methods for the detection of fatty acid peroxidation in bovine milk.

Recently, the use of analytical kits for assessing the oxidation level has been growing due to their uncomplicated handling, fast results, and lower prices when compared with other laboratory equipment (GUTIERREZ, 2014). This author reported the use of gas chromatography as another effective method for identifying volatile compounds produced by secondary oxidation.

EFFECTS OF ROS ON MILK QUALITY

A daily intake of dairy products is essential to maintain health and help prevent some diseases in humans. Milk and derived products thereof have beneficial effects on the control of some diseases. Many studies have been carried out with milk aiming to investigate the positive effects of milk on human health associated with the prevention of oxidative stress. Milk is a source of

lipophilic antioxidants (e.g., tocopherols, retinol, carotenoids), hydrophilic antioxidants (e.g., ascorbate, phenols, and low molecular weight thiols), and antioxidants derived from casein and whey proteins (NIERO *et al.*, 2018).

The study carried out by Choi *et al.* (2015) has demonstrated that consumption of milk and its derivatives showed a positive correlation with the increased glutathione concentration in the brain of older adults. It must be emphasized that, in the elderly, the brain is a major site of ROS-related injuries due to the decreased level of antioxidant defenses in the brain.

Studies have also revealed that dairy proteins can enhance antioxidants capabilities in cells. For example, bovine whey proteins exhibit antioxidant activity that is relatively resistant to processing methods, and it is increased by enzymatic hydrolysis. The action mechanism of milk proteins on oxidative stress and inflammation remains unclear; nonetheless, calcium may suppress the pro-inflammatory process and ROS production. Additionally, the milk protein-derived inhibitors of angiotensin-I-converting enzyme may also have effects on the anti-inflammatory process (FEKETE *et al.*, 2016).

Mastitis has a known negative impact on milk quality. Beyond the possible presence of pathogens in milk, the synthetic capacity of the mammary cells and vascular permeability is altered during the inflammatory process, which alters the milk composition. Both clinical and subclinical mastitis is associated with decreased total antioxidant capacity of milk (ABD ELLAH, 2013), and ROS produced by PMNLs during the inflammatory response may also react with milk components affecting organoleptic properties of milk. Tao (2015) reported that milk from infected cows is susceptible to enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids with multiple unsaturation. The oxidation products

of polyunsaturated fatty acids might exhibit pro-inflammatory effects. As a result, there is a high probability that the milk intake from mastitis-affected cows (SSC \geq 200,000 cells/mL) can cause several disorders; a balance between antioxidants and ROS is needed to maintain cellular homeostasis (LOBO *et al.*, 2010). Another negative point of lipid oxidation is the decrease in food quality. It may cause off-flavors and reduced nutritional values (KHAN *et al.*, 2019), in addition to the decreased shelf life of dairy products (TAO, 2015).

CONCLUSION

The present study highlights and briefly show the deleterious effect of oxidative compounds on the mammary gland and milk quality. Therefore, little is known about the presence of oxidizing agents in marketed milk and their implications for human health, especially over long-term consumption. In this regard, further studies are needed. The presence of ROS-oxidized polyunsaturated fatty acids reduces the shelf-life of these products and brings adverse effects to food quality by producing off-flavors and reducing the nutritional values.

Furthermore, the oxidizing substances present in milk from mastitis-affected ruminants support the fact that this disease threatens the positive image of milk as a high-quality, nutritious, and healthy product to humans. Altogether, these facts strengthen the importance of routine mastitis control practice on dairy farms. Finally, the use of antioxidants in dairy cattle nutrition, associated with mastitis prevention measures and programs, may also increase the chance to minimize the effects of ROS on animals and milk quality, to produce healthier milk for the consumers.

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