Are reactive oxygen species (ROS) the main mechanism by which copper ion treatment degrades the DNA of Mycobacterium avium subsp. paratuberculosis suspended in milk?

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Abstract

Aim: the aim of the present study is to show what is the effect of copper ions, and ROS generated in response to oxidative stress, on the damage to MAP DNA when exposed to a copper ion challenge in cow’s milk.

Methods and Results: spiked milk with different MAP bacterial loads was supplemented with blocking agents. These were either the copper chelators EDTA and BCS or the reactive oxygen species (ROS) quenchers D-mannitol, gallic acid and quercetin. DNA protection, MAP viability and ROS production generated after exposure to a copper challenge were then measured. In a bacterial load of $10^4$ cells mL$^{-1}$, both copper chelators and all ROS quenchers offered significant protection to MAP DNA. In a concentration of $10^2$ cells mL$^{-1}$, only D-mannitol and a mix of quenchers significantly protected the viability of the bacteria, and only at a concentration of $10^6$ cells mL$^{-1}$ was there a lower production of ROS when supplementing milk with gallic acid, quercetin and mix of quenchers.

Conclusion: based on these findings, it may be concluded that MAP DNA damage can be attributed to the combined effect of the direct copper ions and ROS generated. Nevertheless, taking into account the antioxidant environment that milk provides, the direct effect of copper could play a prominent role.

Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causal agent of paratuberculosis, a chronic and incurable infectious disease that is widespread among domestic ruminants (Fecteau 2018). This infectious disease has a great economic impact (Rasmussen *et al*. 2021) and MAP poses a potential threat to public health, due to its potential zoonotic link (Eslami *et al*. 2019). Control of this infection in herds has not been fully achieved (Whittington *et al*. 2019) and the spread of the pathogen through milk has been a neglected issue that could, in part, explain the failure of control programs (Steuer *et al*. 2020). The pasteurization of milk, as a decontamination tool at cattle herd level, has not been widely used, due to its high cost and, in the case of MAP, uncertainty of its overall effectiveness (Grant *et al*. 1996, 1998). Alternatively, the use of copper as a decontamination tool seems promising, as it exhibits an intrinsic antimicrobial effect (Salah *et al*. 2021). The antibacterial efficacy of copper is very well documented in different bacterial models (Vincent *et al*. 2018; Salah *et al*. 2021), including members of the *Mycobacterium* genus such as *Mycobacterium tuberculosis* (Mehtar *et al*. 2008), *Mycobacterium avium* (Lin *et al*. 1998) and recently in *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Steuer *et al*. 2018, 2020, 2021). Steuer *et al*. (2020, 2021) have provided *in vitro* evidence for copper-ion-induced inactivation of MAP cells in raw cow’s milk. However, the mechanisms of copper toxicity that would explain the bacterial inactivation are not entirely clear. A widely accepted mechanism is the oxidative damage of important cellular components, such as lipids, proteins, cell membranes and DNA, through ions ($\text{Cu}^+/\text{Cu}^{2+}$) (Lemire *et al*. 2013; Chatterjee *et al*. 2014) and/or the generation of reactive oxygen species (ROS) (Salah *et al*. 2021) which are highly reactive molecules formed by the incomplete degradation of oxygen. Biologically relevant ROS are superoxide anion ($\text{O}_2^{-}$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and the hydroxyl
radical (-OH) (Solioz 2018). Within a cell, the production and elimination of ROS is well balanced, but under stressful circumstances, such as copper-induced stress, this balance can be disturbed, triggering a state of oxidative stress that alters many cellular functions and structures (Yang et al. 2019). In the particular case of MAP, evidence has recently been shown that ionic copper in a standard liquid matrix affects DNA and cellular proteins, while generating a significant increase in ROS production (Tejeda et al. 2022b). This would suggest that in the case of this particular pathogen, we could point to ROS as a relevant agency for the antibacterial effects generated by copper. However, exactly how copper exerts its antibacterial effect in a complex biological matrix such as milk is still unknown. Although some authors refer to the generation of ROS as the main bactericidal mechanism (Salah et al. 2021), it is not clear how significant this is in milk, which raises the question of how important the capacity of direct redox copper ions could be in this natural liquid matrix. Therefore, the aim of the present study is to show what is the effect of copper ions, and ROS generated in response to oxidative stress, on the damage to MAP DNA when exposed to a copper ion challenge in cow's milk.

Materials And Methods

Study design

In order to establish the capacity of direct ionic copper and the effects of ROS generated in response to oxidative stress by bacteria after a copper ion challenge on the integrity of MAP DNA in milk, an in vitro experiment was carried out under controlled conditions. The study design set out to block the direct pathway of the ionic copper or the action of ROS generated by copper ions by adding a blocking reagent. The experimental unit was set at 500 mL of commercial UHT milk containing a known concentration of MAP cells and the treatment consisted of the supplementation of a blocking reagent to each unit - either copper chelators or ROS quenchers and which -was then challenged with copper ions for 30 minutes. The experiment took in a total of 21 experimental units. Each experimental unit was separately inoculated with a different concentration of MAP cells as follows: A) 10^2 cells mL^{-1}; B) 10^4 cells mL^{-1}; and C) 10^6 cells mL^{-1}. The treatments were assigned with a number as follows: (I) Ethylenediaminetetraacetic acid (EDTA); (II) Batocuproin (BCS); (III) EDTA plus BCS; (IV) D-mannitol; (V) gallic acid; (VI) quercetin; and (VII) D-mannitol plus gallic acid and quercetin. In addition, three positive control units were included, which consisted of milk containing each separate MAP cell concentration (10^2, 10^4 and 10^6 cells mL^{-1}), and which were challenged with copper ions but without a blocking reagent. Additionally, in order to rule out a direct effect of the supplemented reagents on MAP, negative controls were carried out for each chelator/quencher agent, which were incubated with MAP cells for 30 minutes at room temperature without a copper ion challenge. Each experimental unit was run in 3 replicates. The experimental work was carried out at the Laboratorio de Enfermedades Infecciosas, Instituto de Medicina Preventiva Veterinaria (UACH).

MAP strains and inoculum preparation.
MAP strain ATCC 19698 was cultured in enriched broth 7H9, as reported by Tejeda et al. (2022a). In short, MAP growth was monitored weekly, using a Helios Gamma1 spectrophotometer (Thermo Scientific). When the absorbance at 600 nm reached a value of 1.0, it was estimated to be in late exponential growth at a concentration of ~ $10^{8}$ MAP cells mL$^{-1}$ with minimal dead cells present (Shin et al. 2007). MAP cell numbers were more precisely estimated taking into account the genome equivalent principle (Dzieciol et al. 2010) according to a published protocol (Steuer et al. 2018). MAP cultures were declumped by vortexing with sterile 3 mm glass beads. Then, ten-fold serial dilutions of MAP were made in sterile water and three dilutions ($10^{6}, 10^{4}$ and $10^{2}$ cells mL$^{-1}$) were used to spike the milk.

**Reagents**

Milk supplementation with copper chelators or ROS quenchers was carried out using powder reagents. The concentrations used were: 20 mM ethylenediaminetetraacetic acid (EDTA) (Titriplex® III, Merck); 20 µM batocuproin (BCS) (Sigma-Aldrich); 20 mM D- mannitol (Calbiochem®); 0.1 mg mL$^{-1}$ gallic acid (Sigma-Aldrich); and 10 µM quercetin (Sigma-Aldrich). Quercetin was dissolved in 5 mL of absolute ethanol before being added to the milk. All other reagents were directly added.

**Copper ion challenge**

MAP exposure to copper ions was performed using the same treatment protocol reported by Steuer et al. (2020, 2021), which used a device containing two high-purity copper plates (99%) that were stimulated with a low-voltage electrical current (24 V and 3 A) for 30 minutes, in order to stimulate a greater release of copper ions than without an electrical current. The device was inserted into a glass container (Pyrex® beaker) that contained 500 mL of UHT retail milk, spiked with a known MAP concentration, under constant agitation. Aliquots were obtained from the liquid matrix before and after the copper ion challenge for subsequent tests.

**Selective separation of MAP in milk samples**

In order to select, concentrate and separate MAP from other non-target bacteria and inhibitors possibly present in milk, subsamples of this matrix were subjected to peptide-mediated magnetic separation (PMS) from aliquots obtained before and after the copper ion challenge. Peptide-mediated magnetic separation was performed on 1 mL aliquots of milk in a BeadRetriever™ (Invitrogen) according to the protocol published by Foddai et al. (2010).

**MAP total quantification and viability assessment**

In order to estimate the MAP bacterial load in milk before and after the copper ion challenge, MAP DNA was extracted according to a published protocol (Salgado et al. 2014) and then quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Subsequently, the obtained template was subjected to a qPCR protocol, based on the IS900 sequence detection, in a QuantStudio™ 3 system (ThermoFisher) (Salgado et al. 2014). The probe and primer sequences used were the same as those reported by Steuer et al. (2018). The number of DNA copies derived from the qPCR was calculated using a standard curve and expressed as bacterial cell equivalents (BCE) according to the protocol published by
Dzieciol et al. (2010). To assess the viability of MAP in milk for each treatment after the copper challenge, a magnetic phage separation (PhMS) was performed, according to a published protocol (Foddai and Grant 2020). This technique captures and concentrates MAP cells using activated paramagnetic beads attached to mycobacteriophage D29 and exploits the ability of the virus to naturally infect, replicate, and then lyse only viable mycobacterial cells, thus providing DNA for molecular confirmation by IS 900 qPCR.

Evaluation of the production of reactive oxygen intermediates (ROS)

The production of ROS generated by MAP cells was evaluated in each of the different treatments. The non-polar fluorescent 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (Sigma-Aldrich) was used according to a published protocol (Choi and Hu 2008) modified by Tejeda et al. (2022b). DCFH-DA was converted by cellular esterase to DCFH and then potentially oxidized by intracellular ROS and other peroxides, generating DCF. The fluorescent DCF production was measured by a spectrofluorometer with excitation wavelengths of 485 nm and emission of 530 nm. ROS concentration is expressed by fluorescence intensity. Each determination was run in 3 replicates.

Evaluation of physicochemical milk properties

The physicochemical variations in the milk, produced by the effect of the treatments and copper ion challenge were studied through the measurement of pH (Orion, model 420A), electrical conductivity (EC) (Hanna Instrumental, edge ™) and concentration of dissolved oxygen ([O₂]) (Oxy 730 InoLab). Each determination was run in 3 replicates.

Assessing copper concentration in milk

To determine the total copper concentration, the milk was calcined in a laboratory muffle (Barnstead Thermolyne™) to reach 450°C for a total of ten hours and then digested with concentrated HCL (37% w/w) and HNO₃ (eq L⁻¹) according to a modified protocol (Jorhem et al. 2000). The reading was taken by an atomic absorption spectrophotometer (AAS) and the result was reported in mg L⁻¹.

Statistical analysis

The reported response variable was the percentage of DNA protection (% DNA_P) calculated from the final bacterial load (post copper challenge), taking into account the initial bacterial load (prior to copper challenge) for each experimental unit. The result of each treatment (from I to VII) was compared with the corresponding positive control in order to determine its protective effect on MAP. To determine viability, the response variable was reported as the percentage of protection of viable cells (% viability). The calculation and comparison were similar to those detailed for DNA protection. To determine significant differences, a one-way ANOVA was used in the response variable % DNA_P, and a Mann Whitney U test in the response variable % viability. For the analysis of ROS production, the logarithmically transformed variable was used. Comparison of ROS production before and after the copper ion challenge was
performed with a paired t-test and the analysis of ROS production in each treatment compared to the positive control after the copper ion challenge was performed using t-student. For the copper concentration and the physicochemical properties of the milk in each treatment before and after the challenge with copper, a descriptive analysis was performed. The analyses were performed using the R program version 3.1.2 (R Development Core Team 2015). A P-value of < 0.05 was considered significant.

Results

Estimation of bacterial load of protected MAP cells

None of the reagents had any effect on DNA integrity by themselves (data not shown). When milk was inoculated with \(10^2\) MAP cells mL\(^{-1}\) and exposed to the copper challenge, gallic acid, quercetin and the ROS quenching mix composed of D-mannitol + gallic acid + quercetin demonstrated significant protection of MAP DNA, compared to the control (P < 0.05) (Fig. 1A). By contrast, all reagents, both chelators and quenchers, offered significantly greater protection compared to the control (P < 0.05) in the \(10^4\) MAP cells mL\(^{-1}\) milk concentration (Fig. 1B). Finally, in the \(10^6\) MAP cells mL\(^{-1}\) milk concentration, the chelating agent EDTA, and EDTA + BCS, and the quenching agents D-mannitol, gallic acid and quercetin provided significant DNA protection compared to the control (P < 0.05) (Fig. 1C).

When comparing the treatments with each other, significant DNA protection (P < 0.05) was only observed in the bacterial concentration of \(10^4\) cells mL\(^{-1}\) when the ROS quenching mix was used, in contrast to D-mannitol alone. In all other cases, no significant differences were detected between treatments (P > 0.05).

Estimation of viable bacterial load

Regarding MAP viability after exposure to copper ions, in the \(10^2\) cells mL\(^{-1}\) bacterial load concentration, a greater protection than in the control was observed when D-mannitol and the mix of three quenchers were used (P < 0.05) (Fig. 2A). There were no significant differences with the control (P > 0.05) in all other experimental units. Interestingly, viability in controls corresponding to the \(10^4\) and \(10^6\) cells mL\(^{-1}\) loads was not completely eliminated (Fig. 2). The viability was completely eliminated after exposure to copper ions only in the control corresponding to the \(10^2\) cells mL\(^{-1}\) load.

ROS production

For all three bacterial concentrations used, a significant increase in ROS production was observed after exposure to a copper challenge compared to no challenge, for all treatments, including the control (P < 0.01). In the \(10^2\) and \(10^4\) cells mL\(^{-1}\) concentrations, the ROS levels were significantly higher than those in the control (P < 0.05) for all treatments, except for quercetin in a concentration of \(10^2\) cells mL\(^{-1}\) and quercetin and a mix of quenchers in a concentration of \(10^4\) cells mL\(^{-1}\) (P > 0.05) (Fig. 3A and 3B). In the \(10^6\) cells mL\(^{-1}\) concentration, there was greater ROS generation than in the control when BCS and EDTA + BCS supplemented the milk, and lower ROS generation when gallic acid, quercetin, and a quencher mix was added (P < 0.05) (Fig. 3C).
Physicochemical changes in milk treated with chelators/ROS quenchers and challenged with copper

The pH of commercial UHT milk has been standardized by the manufacturer at pH 6.6. Most of the added reagents did not modify the milk’s pH, with the exception of the EDTA reagent alone and EDTA + BCS, which caused a slight acidification when added to the MAP-inoculated milk prior to the copper challenge (Table 1). Likewise, EDTA and EDTA + BCS were the only reagents that slightly increased the electrical conductivity of milk before exposure to copper (Table 1). After the copper ion challenge, an increase in pH and a decrease in both electrical conductivity and the concentration of dissolved oxygen in the spiked MAP milk was observed in all the treatments as well as in the positive control (Table 1).

Table 1
Mean values for pH, electrical conductivity (EC) and the concentration of dissolved oxygen ([O₂]) for each treatment in milk artificially contaminated with MAP before (No Cu) and after (with Cu) being challenged with copper ions for 30 minutes.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>pH</th>
<th>EC</th>
<th>[O₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Cu</td>
<td>With Cu</td>
<td>No Cu</td>
</tr>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td>EDTA</td>
<td>5.3 ± 0.0</td>
<td>9.2 ± 0.1</td>
<td>7.1 ± 0.0</td>
</tr>
<tr>
<td>BCS</td>
<td>6.6 ± 0.0</td>
<td>9.7 ± 0.0</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>EDTA + BCS</td>
<td>5.3 ± 0.0</td>
<td>9.6 ± 0.1</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>D-MANNITOL</td>
<td>6.7 ± 0.0</td>
<td>9.5 ± 0.0</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>GALLIC ACID</td>
<td>6.6 ± 0.0</td>
<td>9.3 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>QUERCETIN</td>
<td>6.8 ± 0.1</td>
<td>9.6 ± 0.0</td>
<td>4.9 ± 0.0</td>
</tr>
<tr>
<td>D-MANNITOL + GALLIC ACID + QUERCETIN</td>
<td>6.7 ± 0.0</td>
<td>9.2 ± 0.0</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>CONTROL (+)</td>
<td>6.6 ± 0.1</td>
<td>9.7 ± 0.0</td>
<td>5.3 ± 0.3</td>
</tr>
</tbody>
</table>

EC: Electrical conductivity expressed in mS cm⁻¹

[O₂]: Concentration of dissolved oxygen expressed in mg l⁻¹

No Cu: No challenge with copper ions in the MAP-contaminated milk

With Cu: Copper plates immersed in the MAP-contaminated milk and stimulated with a low voltage (24V) electrical current (3 Amperes) for 30 minutes.
Determination of copper concentration in milk

The concentration of copper in the milk treated with chelators or quenchers prior to the copper challenge was on average 0.721 mg mL\(^{-1}\). After the copper challenge, the concentration of this metal in the milk supplemented with chelators EDTA and EDTA + BCS was higher than it was in the control (Table 2). The copper concentration in the positive control was higher than in the experimental units that were supplemented with ROS quenchers and BCS (Table 2).

Table 2

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>No Cu</th>
<th>With Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1,11</td>
<td>846,56</td>
</tr>
<tr>
<td>BCS</td>
<td>0,48</td>
<td>544,06</td>
</tr>
<tr>
<td>EDTA + BCS</td>
<td>0,77</td>
<td>1255,82</td>
</tr>
<tr>
<td>D-MANNITOL</td>
<td>0,50</td>
<td>278,28</td>
</tr>
<tr>
<td>GALLIC ACID</td>
<td>0,19</td>
<td>378,47</td>
</tr>
<tr>
<td>QUERCETIN</td>
<td>0,82</td>
<td>205,23</td>
</tr>
<tr>
<td>D-MANNITOL + GALLIC ACID + QUERCETIN</td>
<td>0,42</td>
<td>156,90</td>
</tr>
<tr>
<td>CONTROL (+)</td>
<td>1,47</td>
<td>624,98</td>
</tr>
</tbody>
</table>

No Cu: No challenge with copper ions in the MAP-contaminated milk

With Cu: Copper plates immersed in the MAP-contaminated milk and stimulated with a low voltage (24V) electrical current (3 Amperes) for 30 minutes.

Discussion

Although we previously had some information about the antibacterial mechanism, some things still remained unclear. The present study shows evidence that explains the role of the direct effect of copper ions, as well as ROS generated in response to oxidative stress, on damaged DNA from MAP cells suspended in milk. It does this through the use of ionic copper blocking agents and ROS blocking agents to determine whether the interference caused by these blocking agents protected MAP DNA in milk.

The copper chelators used (EDTA and BCS) have previously shown selective chelation of Cu\(^{+2}\)/Cu\(^{+1}\) (Warnes and Keevil 2011, 2016), which was protective for the DNA of Gram (+) bacteria. The use of mannitol, a sugar alcohol, was justified due to its powerful quenching effects on -OH (Dai et al. 2017).
the other hand, quercetin and gallic acid were selected for their distinctive antioxidant capacity against prooxidative copper (Apak et al. 2007, 2008). Quercetin is a flavonoid with an important antioxidant capacity against O$_2^-$ (Yuting et al. 1990) and gallic acid exhibits a protective effect against oxidative damage induced by H$_2$O$_2$, even though it does not seem to be selective (Bai et al. 2020; Sroka and Cisowski 2003).

When MAP was present in a concentration of $10^2$ cells mL$^{-1}$ in milk, the blocking of all ROS except -OH by D-mannitol offered the highest protection against DNA compared to the (+) control. In the same context, the lack of significant differences between the control and treatments I-IV may be explained by the fact that working with bacterial loads close to the detection limit of the qPCR technique for extractions from milk and by-products makes it subject to errors (Donaghi et al. 2011).

On the other hand, when MAP is present in milk at a concentration of $10^4$ cells mL$^{-1}$, all blocking agents offered significant DNA protection, compared with the (+) control and there were no significant differences between them. This fact can be considered evidence that the DNA was damaged by the direct action of both copper ions and the ROS generated. Warnes and Keevil (2011, 2016) obtained similar results using copper surfaces on other bacterial models.

Also, when the milk contained $10^6$ cells mL$^{-1}$, the lack of significant DNA protection in treatments II and VII may be due to the fact that the BCS chelator and mix of quenchers were not present in sufficient concentrations to provide significant DNA protection against the higher bacterial load.

The production of ROS in the milk increased significantly after the copper challenge, indicating that the constant application of electrically stimulated copper favored ROS production. This is consistent with the fact that ROS are generated endogenously in aerobic bacteria as part of the microbial metabolism, and that eukaryotic cells are also present in milk (Ryman et al. 2015), the production of which can drastically increase in the face of external stressors such as copper and overcome cellular antioxidant systems, causing oxidative stress (Lam et al. 2020). The most biologically important ROS are O$_2^-$, H$_2$O$_2$ and -OH (Salah et al. 2021). Their importance is due to the fact that they are known to act as direct antimicrobials and are even capable of preventing and breaking down the formation of biofilms, and some direct applications of ROS have been explored in recent years (Dryden 2018). Thus, the excess of ROS in the early stages of cell death seems to be involved in the bactericidal activity of copper (Warnes and Keevil 2016), for example, through the oxidation of cellular macromolecules such as DNA promotes cell death through apoptotic pathways (Circu and Aw 2010). This idea is consistent with previous findings by Steuer et al. (2021) where MAP DNA was not detected in most milk samples analyzed after exposure to copper ion treatment.

Interestingly, O$_2^-$ quenching by quercetin produced a significant decrease, or prevented a significant increase, in ROS production compared to the (+) control in all bacteria concentrations studied, perhaps due to the fact that O$_2^-$ is a primary ROS, from which H$_2$O$_2$ and -OH are subsequently generated dependently or independently of Fenton-type reactions (Solioz 2018; Salah et al. 2021).
determination of ROS production using the nonspecific DCFH-DA probe was able to detect total fluorescence generated by the aforementioned ROS and potentially to a lesser extent by other ROS and NOS (Rastogi et al. 2010), but the selective quenching of ROS by means of the quenchers used conferred specificity by virtually subtracting the fluorescence contributed by each of them. In short, the significant increase in ROS production witnessed in most of the treatments compared to the respective control (+) could be directly related to the survival of MAP detected by magnetic phage separation/qPCR in all treatments at bacterial concentrations of $10^4$ cells mL$^{-1}$ and $10^6$ cells mL$^{-1}$, and in most at $10^2$ cells mL$^{-1}$. This is supported by the fact that oxidative stress response mechanisms are crucial for survival (Vaishampayan and Grohmann 2021). If the bacterial cell continues to be viable and metabolically active, then it is capable of regulating the gene expression of antioxidant systems to prevent damage caused by oxidative stress for some period of time, so that the mechanism of ROS production and antioxidation is maintained (Cheng et al. 2020). In MAP, these strategies would include adaptations such as enzymes of the pentose phosphate pathway, as well as KatG, SodA and GroEL, which deactivate free radicals, even protecting DNA against DNase or -OH (Basu et al. 2009; Weigoldt et al. 2013). The survival of MAP seen at $10^4$ cells mL$^{-1}$ and $10^6$ cells mL$^{-1}$, and most treatments at $10^2$ cells mL$^{-1}$ concentrations could also be explained by its ability to mount homeostatic responses to control copper levels, like other bacteria (Argüello et al. 2013).

In addition, bovine milk has been widely studied due to its antioxidant properties, which is mainly due to non-enzymatic components such as sulfur amino acids, phosphate, vitamin A and E, carotenoids, minerals such as zinc and selenium, and enzyme systems such as superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) (Khan et al. 2019). For these reasons, milk, as a matrix, probably hinders the action of ROS on cellular targets. Evidence of the above is the degree of protection that milk has been shown to provide some lactobacilli against the effect of ROS when $H_2O_2$ is added (Castro et al. 2021).

In this scenario, copper could be more relevant than ROS, even surpassing components in milk, such as casein subunits, which have shown effects that favor the autoxidation of metals such as iron ($Fe^{+2}$ and $Fe^{+3}$), resulting in an inhibitory effect on the toxicity of this metal on certain cellular targets (Cervato et al. 1999).

Finally, the concentration of copper in the milk after 30 min of exposure was similar to the concentration in phosphate buffered saline (PBS), as reported by Tejeda et al. (2022a) using the same copper treatment, demonstrating a constant and efficient release of copper in milk, even though the physicochemical characteristics of cow's milk differ from PBS.

**Conclusions**

From the results reported in this investigation, it seems that copper may exert its antibacterial effect against MAP suspended in milk through DNA damage brought about by the complementary actions of ionic copper and the ROS produced in response to the oxidative stress generated by exposure to this metal. Considering the protective and antioxidant environment of milk, together with the results obtained
through the use of ROS chelators and antioxidants, it seems that ionic copper and its oxide reduction potential have a greater relative importance in this context.

**Declarations**

**ACKNOWLEDGEMENTS**

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**CONFLICT OF INTEREST** No conflict of interest declared

**AUTHORS’ CONTRIBUTION**

MV: design of the study, laboratory work, data analysis and draft writing; CT: design of the study, laboratory work and data analysis; RU: laboratory work; ECI: data analysis; MS: design of the study, data analysis and draft writing. All authors read and approved the final manuscript.

**References**


calves an overlooked item in paratuberculosis control programs?. *Trop Anim Health Prod* **52**, 89–94.


**Figures**

**Figure 1**

Percentage of DNA protection (% DNAp) in milk artificially contaminated with: A)10^2 MAP cells mL^-1, B)10^4 MAP cells mL^-1 and C)10^6 MAP cells mL^-1, and supplemented with I) EDTA, II) BCS, III) EDTA + BCS,
IV) D-mannitol, V) Gallic acid, VI) Quercetin, and VII) D-mannitol + gallic acid + quercetin, after being challenged with copper ions for 30 minutes. The determination was made by PMS-qPCR. P < 0.05 and P < 0.01 are represented by * and **, respectively.

Figure 2

Percentage of protection of viable cells (% Viability) in milk artificially contaminated with: A)10^2 MAP cells mL\(^{-1}\), B)10^4 MAP cells mL\(^{-1}\) and C)10^6 MAP cells mL\(^{-1}\), and supplemented with I) EDTA, II) BCS, III) EDTA + BCS, IV) D-mannitol, V) Gallic acid, VI) Quercetin, and VII) D-mannitol + gallic acid + quercetin, after being challenged with copper ions for 30 minutes. The determination was made by PhMS-qPCR. P < 0.05 and P < 0.01 are represented by * and ** respectively.

Figure 3

ROS production (Fluorescence Intensity) in milk artificially contaminated with: A)10^2 MAP cells mL\(^{-1}\), B)10^4 MAP cells mL\(^{-1}\) and C)10^6 MAP cells mL\(^{-1}\), and supplemented with I) EDTA, II) BCS, III) EDTA + BCS, IV) D-mannitol, V) Gallic acid, VI) Quercetin, and VII) D-mannitol + gallic acid + quercetin, after being challenged with copper ions for 30 minutes. The determination was made using the DCFH-DA method. P < 0.05 and P < 0.01 are represented by * and ** respectively.