

## Experimental assessment of the efficacy of copper ion treatment against penicillin G contained in UHT milk and PBS

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**ABSTRACT.** Antibiotics are widely used in animal production to treat bacterial infections and to improve performance and animal welfare. Their misuse poses a threat to public and animal health because of the possible development of antibiotic-resistant microorganisms. Among the many strategies that have been considered to address this problem are methods to degrade antibiotic residues, especially those from the human and animal food chain. This study describes the effect of copper ion treatment on the detection of penicillin G in a liquid matrix. An *in vitro* experimental study was designed using both commercial milk and PBS spiked with three different concentrations of penicillin G. Each sample was treated for 30 min with copper ions. All samples were tested for antibiotics before and after treatment using a commercial enzyme-linked receptor binding assay. Additionally, pH, copper concentration, and temperature were evaluated. Antibiotic residues were detected in all spiked PBS and milk samples before treatment with copper ions. However, after 30 min of treatment, no antibiotic residues were detected in any sample at any concentration tested. In conclusion, treatment of penicillin-contaminated milk and PBS samples with copper ions affects antibiotic detection, which would potentially reduce antibiotic levels.

**Keywords:** antibiotics, copper ion treatment, penicillin G, milk, PBS.

## INTRODUCTION

Antibiotics are widely used in animal production to treat bacterial infections and to improve animal performance. Although antibiotics play an important role in reducing the mortality rate, increasing production, and improving animal welfare, their misuse has raised concerns about public health and food safety owing to the potential development of antibiotic-resistant microorganisms (Ricci *et al.*, 2017).

Intramammary infections are one of the most frequent bacterial infectious diseases in dairy cows and are the main reason for antibiotic use in adult cattle (Stevens *et al.*, 2016). Waste milk (WM) is a term used to describe milk from cows with intramammary infections treated with antibiotics but may also refer to milk contaminated with other drugs, milk from cows with clinical mastitis, milk with high somatic cell counts (SCC), and post-colostrum transition milk (Ricci *et al.*, 2017).

Although the use of WM in calf feed is controversial because of its role in pathogen transmission (Selim & Cullor, 1997) and the risk of selection for antibiotic-resistant bacteria (Ricci *et al.*, 2017), it is widely used by dairy farmers (Calderon-Amor & Gallo, 2020).

The spread of antibiotic-resistant bacteria to the environment and their subsequent transmission to humans are considered one of the main threats to global public health (WHO, 2014).

Therefore, several methods have been evaluated to degrade antibiotic residues in WM, such as incubation with  $\beta$ -lactamases, electrochemical oxidation, heat, or pH treatment. However, none of these methods have been applied in the field (Ricci *et al.*, 2017).

It has been demonstrated that the complexation of different metal ions can affect the properties and stability of  $\beta$ -lactam antibiotics (Deshpande *et al.*, 2004). Among the metal ions, copper in the ionic form ( $\text{Cu}^{\text{II}}$ ) has a greater catalytic effect on the degradation of penicillins than  $\text{Zn}^{\text{II}}$ ,  $\text{Ni}^{\text{II}}$ , or  $\text{Co}^{\text{II}}$  (Gensmantel *et al.*, 1980). The degradation of  $\beta$ -lactams by  $\text{Cu}^{\text{II}}$  can occur via hydrolysis, oxidation, or both, depending on the type of  $\beta$ -lactam. For example, the degradation of penicillin G in water occurs via hydrolysis, followed by oxidation of the products of this hydrolysis (Chen *et al.*, 2016). Under mildly acidic conditions, both penicillin G and V were hydrolyzed by the cupric ion into penicilloic acids (Niebergall *et al.*, 1966).

Recently, our research group evaluated an antibacterial principle based on copper ions for decontaminating bovine milk. This principle was capable of significantly decreasing the load of viable pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *S. aureus*, and *E. coli* in an *in vitro* study (Steuer *et al.*, 2018).

Taking all these considerations into account, the present study aimed to evaluate the effect of copper ions on

the detection of penicillin G in milk by adding three concentrations of penicillin G sodium to ultra-high-temperature (UHT) milk and PBS.

**MATERIAL AND METHODS**

**Experimental design**

To fulfil the proposed aim, an *in vitro* experimental study was conducted. Phosphate saline buffer (PBS) and UHT commercial milk were used as liquid matrices. Volumes of 500 mL were taken from each matrix, into which potassium penicillin G (Merck KGaA, Darmstadt, Germany) was added to reach concentrations of 20, 200, and 1,000 ppb. Each sample was treated for 30 min with a copper treatment device consisting of a glass receptacle, in which two high-purity copper plates were immersed. The copper plates were stimulated with a low-voltage (24 V) electric current (3 A) to quickly release copper ions (Steuer et al., 2018). PBS and milk samples without antibiotics were used as negative controls. In addition, pH controls were added using PBS and milk contaminated with 1,000 ppb penicillin and incubated for 30 min at pH 11.5. All samples were

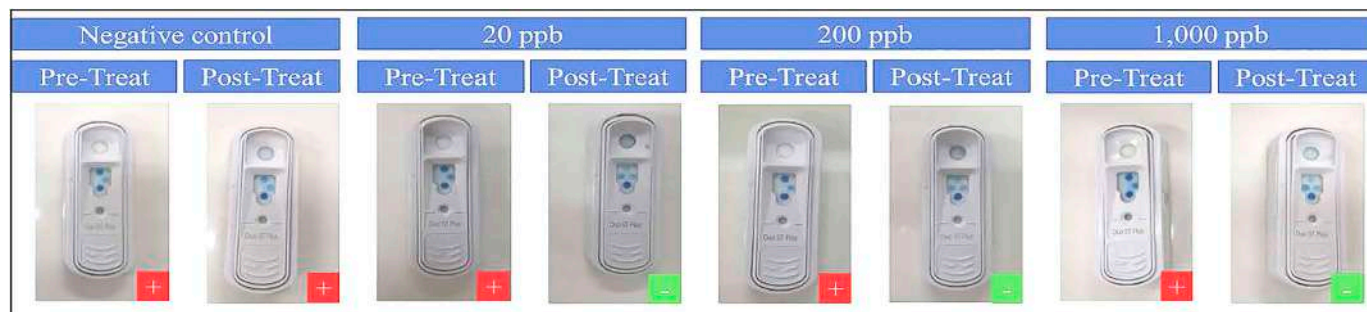
tested for antibiotic detection before and after treatment with the commercial enzyme-linked receptor-binding assay IDEXX SNAPduo ST Plus Test (Idexx Laboratories Inc., Westbrook, ME, USA). The pH, Cu concentration, and temperature of the samples were also evaluated.

**Statistical analysis**

All analyses were performed using R Statistical Software (v4.2.1; R Core Team 2022) and Excel 2016 (Microsoft Corp., Redmond, WA). The Wilcoxon signed-rank test was used to determine significant differences in each response variable (Cu concentration, pH, and temperature) before and after Cu treatment. Differences were considered statistically significant at  $p < 0.05$ .

**RESULTS AND DISCUSSION**

In all PBS and milk samples where the three concentrations of penicillin (20, 200, and 1,000 ppb) were added, antibiotic residues were detected before treatment with copper ions. However, after 30 min of treatment, no antibiotic residues were detected in any of the samples (Figure 1).



**Figure 1.**  $\beta$ -Lactam detection in milk with SNAPduo ST Plus Test before and after 30 min treatment with copper ions. The blue dot on the left side of the reading window indicates no detection of  $\beta$ -lactam antibiotics in the sample (-). If the blue dot was missing, the sample contained a  $\beta$ -lactam antibiotic residue (+).

Interestingly, when samples were incubated at an alkaline pH as a treatment, antibiotic residues were detected before and after treatment.

In the PBS, the average pH was 8.06 (SD 0.06) in pre-treated samples and 11.74 (SD 0.37) in post-treated samples ( $p < 0.05$ ). The average copper concentration was 0.03 mg/L (SD 0.03) in pretreated samples, and 525.91 mg/L (SD 07.39) in post-treated samples ( $p < 0.01$ ). The average temperature was 20.80 °C (SD 1.28) in the pre-treated samples and 27.53 °C (SD 1.36) in the post-treated samples ( $p < 0.05$ ) (Table 1).

In milk samples, the average pH was 6.58 (SD 0.03) in pretreated samples and 9.10 (SD 0.159) in post-treated samples ( $p < 0.05$ ). The average copper concentration was 0.40 mg/L (SD 0.25) in the pretreated samples and 1,698.82 mg/L (SD 162.06) in the post-treated samples ( $p$

$< 0.01$ ). The average temperature was 19.98 °C (SD 1.04) in pretreated samples and 33.98 °C (SD 1.24) in post-treated samples ( $p < 0.05$ ) (Table 1).

For dairy farmers, WM implies economic loss because it cannot be marketed for human consumption. This loss has been estimated to range from US\$3.7 to US\$23 per cow per year (Hogeveen et al., 2011). However, most concerns relate to the effects of its use as calf feed on the selection and transmission of antibiotic-resistant bacteria (Ricci et al., 2017).

In this study, milk and PBS samples were contaminated with penicillin G to assess the effect of copper ion treatment on residue detection. Penicillin is one of the most common antibiotic residues detected in WM (Selim & Cullor, 1999; Pereira et al., 2014a). Furthermore, it is commonly used in studies as an antibiotic model to

simulate WM (Pereira *et al.*, 2014b; Pereira *et al.*, 2016). The three concentrations of penicillin used in the present study were chosen based on the range of concentrations found in WM (Pereira *et al.*, 2014a) and the detection limit of the SNAP test (2 ppb). The sensitivity of the test used for the penicillin, cephalosporin, and tetracycline groups was 4, 30, and 18 ppb, respectively. The SNAP test detects beta-lactam antibiotics, cephalosporins, and tetracyclines by binding to enzyme-linked receptors.

Among the methods that have been described for degrading antibiotic residues contained in WM, heat treatment, pH treatment, and electrochemical methods can be highlighted. Heat treatment is widely used as a method to reduce bacterial populations in WM, but it can also reduce the concentrations of certain antibiotics. Heat treatment of milk at 120 °C for 20 min degraded 47% amoxicillin, 84% ampicillin, 53% cloxacillin, and 61% penicillin G (Roca *et al.*, 2011). Recently, Garzon *et al.* (2020) reported that heating milk at 92 °C for 20 min could degrade 35.24% of the initial concentration of ceftiofur. In the same study, the authors described that alkalinizing milk to pH 10 resulted in a 95.72% degradation of the initial concentration of ceftiofur. In the present study, one effect of copper ion treatment was to increase the pH of all milk and PBS samples to 11.74 and 9.10, respectively. The latter is complemented by what was published in Božić *et al.* (2018), where it was established that penicillin G did not show any coordinative or redox interaction with Cu<sup>2+</sup> in phosphate buffer at a physiological pH of 7.4.

However, in the samples spiked with 1,000 ppb of penicillin and alkalinized to pH 11.5, β-lactam residues could be detected before and after treatment, showing no effect of pH on residue detection. This difference could be explained by the difference in the structure of the antibiotics (ceftiofur – penicillin) and detection methods (HPLC-SNAP).

In experimental trials, there is evidence of the efficacy of electrochemical oxidation of metals for the degradation of antibiotics in waste milk. Kitazono *et al.* (2017) demonstrated the degradation of chlortetracycline and cephalozine with ions produced by the electrical stimulation of a titanium cathode, while Elmola & Chaudhuri (2009) demonstrated the degradation of amoxicillin, ampicillin, and cloxacillin using the Fenton reaction, which involves the use of iron ions and hydrogen peroxide. Electrochemical oxidation is a process in which organic substances are oxidized and converted into nontoxic substances under the action of an electric current (Wang & Zhuan, 2020). Kitazono *et al.* (2012), who found an 83% reduction in the concentration of oxytetracycline after a 6-hour treatment, used electrochemical oxidation of metals for the degradation of antibiotics in WM. In a later study, Kitazono *et al.* (2017) demonstrated the degradation of chlortetracycline and cephalozine with ions produced by electrical stimulation of a titanium cathode. The degradation of antibiotics in other aqueous matrices in the presence of various metal compounds has also been demonstrated (Wang &

Zhuan, 2020).

A study by Riediker *et al.* (2004) showed that milk with penicillin G, amoxicillin, and ampicillin stored for six days at 4 °C experienced a degradation of more than 50%. However, no degradation was observed in milk spiked with ceftiofur and stored for 14 d at 4 °C (Karageorgou & Samanidou, 2010).

The widespread use of WM in calf feeding represents a challenge in the development of efficient methods for antibiotic removal. The results of this study suggest that electrochemical oxidation with copper ions affects the detection of penicillin in milk and could potentially be a method for degrading penicillin G residues in waste milk to avoid environmental contamination. If this treatment is confirmed to be effective for treating waste milk, we should monitor the possible effect of copper on the health of calves. Steuer *et al.* (2021), reported no evidence of copper toxicity after the application of copper ion to control MAP in milk intended to feed calves, based on the plasma activity of the liver enzymes evaluated, and hepatic copper concentrations were also normal. However, the Cu concentration in milk after treatment was significantly lower than that reported in the present study.

Further trials with other antibiotic detection techniques and evaluations of their post-treatment biological activities are needed.

#### Competing interests statement

The authors declare that they have no conflicts of interest.

#### Ethics statement

No experimental animals or animals for clinical research were used in this study

#### Author contributions

FU and MS conceived the ideas of the study. FU, MV, and CT performed laboratory assays. FU and MS led the writing of the manuscript. MS obtained the funding for this study. All the authors revised the manuscript, critically contributed to the drafts, and approved the final version for publication.

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