BRAZILIAN JOURNAL OF MICROBIOLOGY

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Food Microbiology

Antimicrobial effect of copper surfaces on bacteria isolated from poultry meat

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ARTICLE INFO

Article history:
Received 19 December 2017
Accepted 5 June 2018
Available online 24 August 2018

Associate Editor: Elaine De Martinis

Keywords:
Antimicrobial copper-alloys
Foodborne pathogens
Salmonella
Listeria monocytogenes
Poultry meat

ABSTRACT

Poultry meat is a food product that usually carries high rates of microbial contamination, including foodborne pathogens. The poultry industry has established different systems to minimize these hazards. In recent years, extensive literature has demonstrated the antimicrobial activity of different contact surfaces made of copper to effectively reduce microbial loads. The aim of the present study was to evaluate the antibacterial effect of copper surfaces on the transmission of two foodborne pathogens – *Salmonella enterica* and *Listeria monocytogenes* – and a poultry native microbiota bacterial species – *Enterobacter cloacae*. We also evaluated the impact of the poultry meat matrix on the antimicrobial activity of a copper surface. Our results indicated that copper surfaces reduced the bacterial load quickly (<than 4 min) when the microorganisms were exposed to polished copper surfaces. Even when bacteria were inoculated on copper surfaces soiled with the organic matrix (washing water from poultry carcasses) and survival rates were significantly higher, an antimicrobial effect was still observed. Survival rates of two microorganisms simultaneously exposed to copper did not show significant differences. We found an antimicrobial effect over pathogenic and non-pathogenic microorganisms. Results suggest a potential role for copper surfaces in the control of microbiological hazards in the poultry industry.

Introduction

Foodborne diseases are an important public health problem resulting in significant social and economic burden worldwide.[1–3] Poultry meat is recognized as an important reservoir of pathogenic microorganisms, and it is one of the food products most frequently associated with foodborne diseases.[4,5] Illnesses caused by the consumption of poultry and poultry products cause annual economic losses of over $ 2.4 billion in the United States.[6] During poultry processing, carcasses are highly susceptible to microbial contamination. The major sources of contamination are the high bacterial loads, which include foodborne pathogens, of the intestine and cloacal and contamination in the food processing plant

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https://doi.org/10.1016/j.bjm.2018.06.008

environment.[7–10] Moreover, environmental conditions such as high humidity and bacterial biofilm formation may contribute to the persistence of pathogenic and non-pathogenic microorganisms in poultry processing plants.[11,12] Therefore, the poultry industry sets strict microbiological limits and controls to reduce the risk of contamination with pathogens for increased shelf life.[11,13] Accordingly, efforts to reduce microbial contamination, avoid biofilm formation, and prevent cross contamination are needed.

In 2008, the Environment Protection Agency of the United States (EPA) approved the use of copper alloys as clinical contact surfaces due to confirmed antimicrobial properties. Copper alloys have since been tested for multiple uses. The antibacterial activity of copper depends on the close contact between bacteria and the surface releasing ionic copper. The copper contact killing effect can be modified by different factors including temperature, characteristics of the copper alloy, humidity, bacterial species, type of contact between the bacteria and the surface, and the oxidization state of the copper, among others.[14–16] Regarding the mechanisms that explain the antimicrobial effect of copper, it has been proposed that copper ions released from surfaces induce membrane bacteria damage generating a loss of membrane potential and cytoplasmic content. In addition, reactive oxygen species produced by copper ions induce greater damage to cellular structures and even DNA degradation.[17,18]

Copper surfaces have been demonstrated to reduce the bacterial load of foodborne pathogens such as *Escherichia coli* O157:H7,[19] *Salmonella enterica*,[20] and *Listeria monocytogenes*.[21] However, studies have only considered direct effects of the microorganisms, without considering the influence of both the food matrix or the presence of other microorganisms. *Enterobacter cloacae* has been frequently isolated from poultry products, and is considered part of the native microbiota of poultry.[22] The effect of copper surfaces on this bacterial species, however, has not been evaluated.

The antimicrobial activity of copper suggests this metal could be used as a food-processing surface. We hypothesized that copper surfaces could reduce microbial load in the presence of the food matrix. In this study, we evaluated the antimicrobial effect of copper surfaces over two foodborne pathogens frequently associated with poultry meat: *S.* Enteritidis and *L. monocytogenes*. Our study considered the impact of the poultry meat matrix and the presence of poultry native microbiota, represented by *E. cloacae*, on the antimicrobial activity of copper.

Materials and methods

Bacterial strains

S. Enteritidis (S410), *L. monocytogenes* (L452), and *E. cloacae* (E11) were obtained from our culture collection which were originally isolated from poultry meat. Bacterial identity was confirmed by specific PCR reactions using primers INVA1-(5′-ACAGTGCTCGTTTACGACCTGAAT-3′) and INVA2 (5′-AGACGACTGGTACTGATCGATAAT-3′)[23] and Salm-gyrF (5′-GGTGGTTTCCGTAAAAGTA-3′) and Salm-gyrR (5′-GAATCGCCTGGTTCTTGC-3′) for *Salmonella* spp. confirmation and primers lmo3F (5′-GTCTTGCGCGTTAATCATTT-3′) lmo4R (5′-ATTTGCTAAAGCGGGAATCT-3′) for *L. monocytogenes* confirmation. *E. cloacae* identity, representing native microbiota, was confirmed through biochemical tests: TSI, LIA, MIO, Citrate, Phenylalanine and Urea. Strains were recovered in overnight cultures in Tripticase Soy Agar (TSA) (Oxoid Ltd, Basingstoke, Hampshire, England) and in Tripticase Soy Broth (TSB) (Oxoid Ltd, Basingstoke, Hampshire, England).

Determination of minimum inhibitory concentration of copper

To characterize strains' susceptibility to copper, we determined the minimum inhibitory concentration for copper (MIC-Cu), with the salt copper sulfate Cu_2SO_4, for the three strains according to the methodology described in Reyes-Jara et al.[24]

Pre-treatment of copper surfaces

Copper surfaces used in the study were 89% copper and 11% tin. Stainless steel surfaces were used as controls, and all surfaces were cut as coupons (2.5 cm × 2.0 cm). Previous to exposure assays, the coupons were pre-treated. Cleansed copper surfaces: copper coupons were treated with ethanol 70% for 2 min, to eliminate microbiological contamination and organic residues, and rinsed with distilled sterile water and dried at room temperature. Treated copper surfaces: copper coupons were cleaned as described in the cleansed copper surface group and then placed for 2 min in poultry carcass rinse water which had been previously sterilized by repeated freezing and thawing (10 times), exposure to ultraviolet radiation for 5 min (twice), and a final filtration step (0.4 μm). After treatment, coupons were dried and placed in sterile Petri dishes until completely dry. All coupons were used only once.

Exposure of bacteria to copper surfaces

To determine the antibacterial effect over more than one microorganism simultaneously, *S.* Enteritidis or *L. monocytogenes* were exposed in a mixed culture with *E. cloacae*, which represented the microbiota present in poultry carcasses. *S.* Enteritidis and *L. monocytogenes* were exposed to copper surfaces (cleansed and treated) as monocultures or in a mixture with *E. cloacae*. An overnight culture for each bacterium was refreshed with a same sterile medium for adjusting to an $OD_{600\,nm}$: 0.05, and grown until it reached an exponential growth phase ($OD_{600\,nm}$: 0.5). Bacteria were harvested, re-suspended and adjusted to 1×10^{10} CFU/mL in sterile phosphate-buffered saline (PBS), and then 40 μL of the suspension were disposed as a drop over copper and stainless steel coupons (control surface). To test bacterial mixtures (*S.* Enteritidis–*E. cloacae* or *L. monocytogenes*–*E. cloacae*), 40 μL of each bacterial suspension were mixed and disposed on coupons. Inoculated coupons were placed in Petri dishes at 25 °C during exposure times as described by Espírito Santo et al.[25]

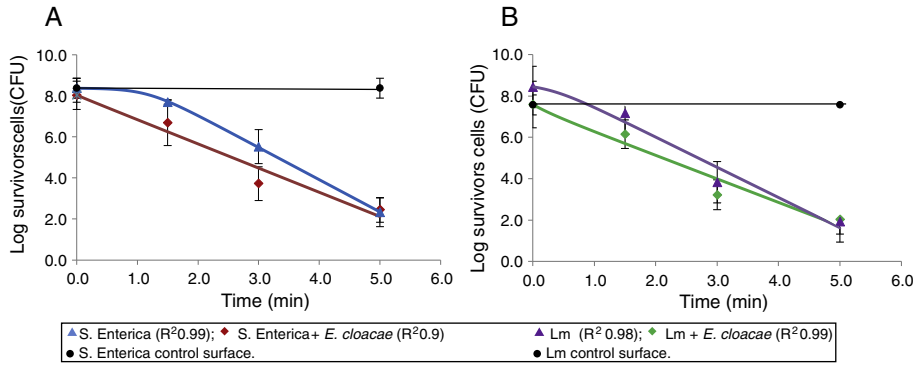

Fig. 1 – Inactivation kinetics curves for (A) S. Enteritidis S410 and (B) *Listeria monocytogenes* L452 on polished copper surfaces. The pathogens were exposed individually and in a mixture with *Enterobacter cloacae* E11. The average of 3 repetitions is shown. Control surfaces were stainless steel coupons. Error bars depict standard error.

Bacterial count

Bacteria were recovered by introducing each coupon in a tube containing 3 mL of PBS and ten 5 mm sterile glasses beads. The tube was vigorously vortexed for 1 min. Then, aliquots were taken to determine bacteria number by plate counting in TSA and on XLD agar media (Oxoid Ltd, Basingstoke, Hampshire, England) for S. Enteritidis and Palcam agar (Oxoid Ltd, Basingstoke, Hampshire, England) for *L. monocytogenes*. All cultures were carried out at 37 °C, and experiments were repeated at least three times.

Data analysis

Inhibition curves were obtained by plotting total bacterial counts (Log 10 CFU) over time. Since each experimental condition was run in triplicates, three curves were available for each treatment. Inhibition curves were fit to the Log Linear + Shoulder model using the GInaFit v1.7 plugin for Microsoft Excel.[26,27] A regression analysis confirmed the model was the best fit for each dataset ($R^2 > 0.9$).

T(4D) reduction values (the time required to reduce bacterial counts in 4 logs), calculated by GInaFit v1.7, were used to compare experimental conditions. Statistical analyses were performed using the software RStudio.[28] First, a Shapiro–Wilk test was run to verify that data followed a normal distribution, and then, a three-way ANOVA was used to analyze possible differences among the treatments. To determine statistical significance, a p-value ≤ 0.05 was set.

Results

Minimum inhibitory concentration of copper

The MIC-Cu was determined for all three microorganisms isolated from poultry meat. *Salmonella* S410 and *E. cloacae* E11 showed MIC-Cu values of 2 mM and *L. monocytogenes* L452 had a value of 4 mM.

Table 1 – Reduction time T(4D) for S. Enteritidis and *Listeria monocytogenes* exposed to copper surfaces under different experimental conditions.

Microorganism	*Enterobacter cloacae*	T(4D) (min)	
		Polished	Treated
S. Enteritidis	–	3.8 ± 0.7[a]	19 ± 1.8[b]
S. Enteritidis	+	3.3 ± 0.9[a]	17.5 ± 3.3 [b]
L. monocytogenes	–	3.1 ± 0.3[a]	30.6 ± 1.2[c]
L. monocytogenes	+	3.4 ± 1.1[a]	33.3 ± 1.5[c]

Different letters indicate significant differences p-value <0.05.

Antimicrobial activity of polished copper surfaces

The antimicrobial effect of copper surfaces over two pathogens was evaluated using cleansed, polished surfaces during different time courses. The survival rate of pathogens exposed to copper surfaces was lower in cleansed, polished copper surfaces when compared to the control (stainless steel coupons) independent from the presence of *E. cloacae* (Fig. 1). In cleansed copper surfaces, a 4-log reduction time (T(4D)) of *Salmonella* and *L. monocytogenes* was achieved in approximately 3 min (Table 1).

Antimicrobial activity of treated copper surfaces

To mimic the effects of food residue on copper surfaces, we treated the surfaces with sterilized carcass rinse water and then exposed bacteria to it. Inactivation kinetic curves demonstrated that bacterial load reduction took longer in treated surfaces than on cleansed copper surfaces, not only in monocultures, but also in mixtures of the pathogens and *E. cloacae* (Fig. 2). The time required to reach reduction rates of T(4D) in the presence of organic material (treated surfaces) was significantly longer for both pathogens (Table 1). *L. monocytogenes* survived greater than 30 min in treated copper surfaces. We observed significant differences in T(4D) reduction times between both pathogens under different study conditions (Table 1). *L. monocytogenes* almost doubled the T(4D) value observed for S. Enteritidis in treated copper surfaces.

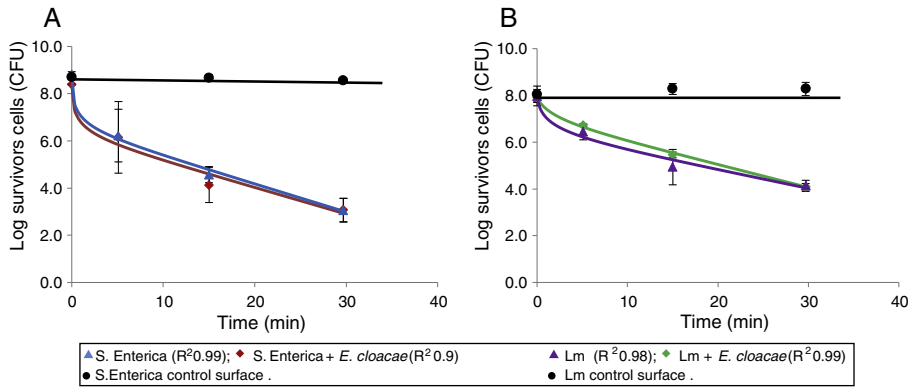

Fig. 2 – Inactivation kinetics curves for (A) S. Enteritidis S410 and (B) *Listeria monocytogenes* L452 on treated copper surfaces with poultry carcass rinse water. Pathogens were exposed individually and in a mixture with *Enterobacter cloacae* E11. The average of 3 repetitions is represented on the graph. Error bars depict standard error.

Antimicrobial activity of copper surfaces on E. cloacae

We evaluated the antimicrobial effect of polished and treated copper surfaces on *E. cloacae*. The results showed that *E. cloacae* inactivation was faster than the other two microorganisms when exposed to copper surfaces (supplemental Fig. 1). The T(4D) of *E. cloacae* in polished surfaces was lower than 1.5 min when exposed individually or in the presence of a pathogenic bacteria. The T4(D) increased to 5 ± 0.8 min when *E. cloacae* was exposed to treated copper surfaces by itself (supplemental Fig. 1).

Discussion

The high demand for poultry meat and other poultry products has led the industry to commercialize millions of tons each year. As a consequence, companies must control hazards related to these products, focusing on microorganisms that can cause serious diseases and/or economic losses for the industry.

The antimicrobial effect of copper surfaces has been previously studied. Warnes et al.[29] analyzed the antimicrobial effect of a 99.9% copper alloy surface for *Salmonella* Typhimurium observing a 7 log reduction after a 5-min exposure. Similar results were showed by Espírito Santo et al.[30] who showed a 9 log reduction of *E. coli* after 1 min of exposure to surfaces which were 99% copper. In both cases, bacterial death occurred in a few minutes, similarly to what we observed when we exposed *Salmonella* and *Listeria* to surfaces that were 89% cleansed copper. Conversely, Wilks et al.[21] exposed *L. monocytogenes* to high copper content surfaces (>90% copper), but the pathogen survived for over an hour. In another study, *S. enterica* and *Campylobacter jejuni* showed a reduction of approximately 4 log in 4 h when exposed to metallic (electrolytic purity) copper sheets.[31]

In copper exposure studies, one of the most important factors associated with microbial death time, is the medium selected to suspend the microorganisms under testing. In general, shorter bacterial death times are observed when bacteria are suspended in PBS, a non-nutritive solution. On the contrary, longer death times (>1 h) are reached when bacteria are suspended in culture media. For instance, Espírito Santo et al.[30] added EDTA and bathocuproine disulfonate to the suspension media, increasing bacterial death time. Those results indicated that the presence of copper chelating substances reduce the antimicrobial activity of copper surfaces. In our study, we observed longer bacterial death times when organic matter from poultry carcasses were added to copper surfaces before being exposed to bacteria, thus reducing the antimicrobial activity of copper alloys. We decided to use poultry carcass rinse water over the copper surface to mimic conditions in a hypothetical poultry processing plant. Although the reason for higher survival rates in the presence of organic matter is not clear, it is thought that compounds such as protein or carbohydrates might interact with copper, acting like copper chelating complexes which prevent or delay the activity of copper over cell membranes.[20,32]

Interestingly, *L. monocytogenes* strain L452 showed a higher tolerance to copper antimicrobial activity; which is related to the higher MIC-Cu of this bacterium in comparison to *S.* Enteritidis S410 and *E. cloacae* E11.[30] Also, when *L. monocytogenes* was exposed to treated copper surfaces, a significantly higher T(4D) was observed compared to *S.* Enteritidis T(4D). These differences may be related to the presence of specific *Listeria* mechanisms related to copper homeostasis, such as: transporters, systems to extra- and intracellular sequestration, enzymatic detoxification and cell wall. Which seem to be more efficient in Gram positive than in Gram negative bacteria.[33]

The results of the antimicrobial activity of copper over the three microorganisms in the study suggest a potential use of copper surfaces for the control of foodborne pathogens in the poultry industry. However, some potential drawbacks need to be considered. For example, the transference of copper from surfaces to food needs to be addressed. Faúndez et al.[31] reported copper transference values under 2.5 mg/100 g for poultry meat after 50 min of exposure when testing a 99.999% copper alloy (electrolytic copper). Transference values were similar for poultry meat, liver, almond, and seafood.[31,34] The alloy used in this study had a lower percentage of copper, thus we might expect lower transference levels. Since values shown

by Faúndez et al.[31] are close to the upper limit of the acceptable daily intake of copper for adults, more studies are required to determine the copper transference of different copper alloys that display antimicrobial activity.

Under situations where organic matter was present, we identified lower antimicrobial activity. This aspect must be considered when using copper surfaces to control bacterial pathogens since the presence of organic matter in the poultry processing plant is common, thus reducing the effectiveness of this antibacterial alternative. The use of copper surfaces, as any other antimicrobial alternative for the industry, may be one of many tools to help reduce the presence of pathogens in processing plants. Copper surfaces may help to prevent biofilm formation; however, this strategy is not one that will eliminate pathogens from the environment. The use of good manufacturing practices and food safety management systems will remain the basis for improving food safety in processing plants.

In conclusion, the antibacterial activity of copper surfaces for bacterial pathogens was not affected by the presence of a representative of the poultry microbiota. Conversely, when bacteria were exposed to surfaces containing organic material, survival times were significantly longer. The results of this study support new trends that promote the use of copper as contact surfaces for foods. Additionally, our conclusions consider aspects that better simulate conditions of diverse food pathogens in distinct food matrices. We believe that the use of high percentage copper alloys as contact surfaces could help to reduce the presence of pathogens in the poultry and food industry. Additional studies that consider conditions such temperature, the presence of other bacteria, and those conducted in real settings are necessary.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from ENLACE ENL018/16 and FONDECYT (No. 1171575). Authors thank Ms. Estela Blanco for editing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2018.06.008.

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