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Copper as an Antimicrobial Agent against Opportunistic Pathogenic and Multidrug Resistant *Enterobacter* Bacteria

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Infections by *Enterobacter* species are common and are multidrug resistant. The use of bactericidal surface materials such as copper has lately gained attention as an effective antimicrobial agent due to its deadly effects on bacteria, yeast, and viruses. The aim of the current study was to assess the antibacterial activity of copper surfaces against *Enterobacter* species. The antibacterial activity of copper surfaces was tested by overlying 5×10^6 CFU/ml suspensions of representative Enterobacter strains and comparing bacterial survival counts on copper surfaces at room temperature. Iron, stainless steel, and polyvinylchloride (PVC) were used as controls. The mechanisms responsible for bacterial killing on copper surfaces were investigated by a mutagenicity assay of the *D-cycloserin* (cyclA gene), single cell gel electrophoresis, a staining technique, and inductively coupled plasma mass spectroscopy. Copper yielded a significant decrease in the viable bacterial counts at 2 h exposure and a highly significant decrease at 4 h. Loss of cell integrity and a significantly higher influx of copper into bacterial cells exposed to copper surfaces, as compared to those exposed to the controls, were documented. There was no increase in mutation rate and DNA damage indicating that copper contributes to bacterial killing by adversely affecting cellular structure without directly targeting the genomic DNA. These findings suggest that copper's antibacterial activity against *Enterobacter* species could be utilized in health care facilities and in food processing plants to reduce the bioburden, which would increase protection for susceptible members of the community.

Keywords: Enterobacter, opportunistic pathogen, multidrug resistant, copper, antibacterial activity

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Introduction

The *Enterobacter* species that are distributed in water, soil, sewage; dairy products, and vegetables are part of the commensal enteric flora and can be present in the normal human gut in small numbers. They may cause urinary tract infections, wound infections and intravenous line associated infections. They may also cause infection outbreaks in communities and healthcare-associated infections (Zhu *et al*., 2010). Plant pathogenic bacteria are responsible for major economical losses in agricultural industries worldwide, prompting massive research efforts to understand their ecology, pathology, and epidemiology. Studies of many agriculturally relevant pathogens, such as *Pseudomonas syringae*, *Xylella fastidiosa*, *Erwinia amylovora*, *Enterobacter*, *Burkholderia*, and *Xanthomonas oryzae*, reviewed by Kirzinger *et al.* (2011), have revealed an extensive specialization toward plant systems, such as type III secretion systems, plant hormone analogs, and enzymes that target plant-specific cell wall components. Despite this, we often neglect the fact that the interactions of phytopathogenic bacteria are not confined to plants, and may include other organisms in the environment. Recent studies have begun to show that many plant pathogens have the capacity to colonize other hosts outside of the plant kingdom, including insects, animals, and humans (Kirzinger *et al*., 2011).

The ubiquitous presence of street foods in most Asian countries is one of the fascinating aspects of urban social life. However, street foods have been reported to be contaminated by pathogens originating from plants during processing or by cross contamination of food materials and have been implicated in food-borne epidemics, particularly in developing countries (Kubheka *et al*., 2001; Azana and Ortega, 2004).

Outbreaks of infections caused by *Enterobacter* species have been reported in neonatal intensive-care units (NICU), surgical wards, and burn units, where they originate and spread through cross-transmission, contaminated intravenous fluids or total parenteral nutrition solutions, and other contaminated pharmaceutical products or medical equipment. Moreover, the most alarming evidence exists that many of these strains exhibit multidrug resistance, making clinically relevant antimicrobials ineffective (Falkiner, 1992; Ono and Yamamoto, 1992; Acolet *et al*., 1994; Al Ansari *et al*., 1994; Sanders and Sanders, 1997; Ronveaux *et al*., 1999; Xu *et al*., 2010). An increasing involvement of *Enterobacter* in these outbreaks, particularly in hospitals, has raised the need to identify methods to put this pathogenic microorganism at bay.

Copper is an essential element that works as a cofactor of aerobic metabolism; however, in low amounts or in excess,

Table 1. Copper antimicrobial activity against *Enterobacter* species tested using paper disc method

Bcc species and strains	Inhibition area diameter (mm)[a]
E. cloacae (LMG2683)	15.5±0.3
E. cancerogenus (LMG2693)	17.2±0.5
E. agglomerans (LMG2557)	15.3±0.09
E. helveticus (LMG23732)	15.4±1.0
E. turicensis (LMG23730)	10.3±0.16
E. nimipressuralis (LMG10245)	9.5±0.9
E. pyrinus (LMG22970)	13.3±2.0
E. amnigenus (LMG2784)	14.1±0.5
E. oryzae (LMG24251^T)	16.4±0.6
E. moris (LMG25706^T)	14.9±0.7
E. radicineitans (DSM16656^T)	14.7±0.5

[a] Values are means standard deviation of three assays with three paper disc/plate

it can have deleterious effects (Rensing and Grass, 2003). The tendency of copper to alternate between cuprous Cu (I) and cupric (II) ion oxidation states, and the inability of the cell to differentiate between copper and trace metals such as zinc and nickel are considered to be the basis of copper toxicity (Espirito Santo *et al.*, 2011). The use of copper by human civilization dates back to the 6th and 5th millennia (Dollwet and Sorenson, 1985); however, it was not until 2008 that its ability to inactivate microbes upon contact was identified, as reviewed by Grass *et al.* (2011), and rigorously tested by the US Environmental Protection Agency (EPA), which lead to the registration of copper alloys by the EPA as antimicrobial agents. The use of copper surfaces as an antimicrobial agent is supported by the EPA and has attracted people's attention. Additionally, the use of copper vessels to provide potable water has been adopted as a low-cost alternative for protection from bacterial infections in developing countries.

Keeping these antecedents in mind, the purpose of our study was to determine the copper antimicrobial effects on *Enterobacter* species, to assess which *Enterobacter* species is the most highly susceptible to copper, and the factors that contribute to bacterial killing on copper surfaces.

Materials and Methods

Bacterial strains and culture conditions

The antibacterial activity of copper contact killing was evaluated using suspensions of representative *Enterobacter* strains acquired from Prof. J Swing at the Belgian Co-ordinated Collection of Microorganisms, LMG Bacteria Collection, University of Ghent, Belgium and from locally identified sources listed in Table 1. All *Enterobacter* strains were stored in 20–30% glycerol (Shanglin Industries, China) at -80°C and were cultured for 24–48 h in Brain Heart Infusion (BHI) agar at 37°C. Single colonies were inoculated in 20 ml BHI broth and incubated at 37°C overnight. On the following day, cultures were diluted 1:100 with 10 ml fresh BHI in 50 ml flasks to an optical density (OD) of approximately 3.0, at 600 nm, which was determined using a spectrophotometer (Perkin Elmer Lambda35 UV/VIS).

Copper metallic surfaces and copper powder

Copper coupons (1.5 cm × 1.5 cm × 0.5 mm) with composition Cu (99.96), Ni (0.005), Zn (0.001), Se (0.002), Fe (0.001), Bi (0.002), Ag (0.012), P (0.019), As (<0.001), Sb (<0.001), Pb (<0.001), Sn (<0.001), Te(<0.001), (Teachn Industrial Technology Development Co., Limited, Hunan, China) and copper powder (Cu 99.9%, P<0.001, Fe<0.005, Sb<0.002, Sn<0.003%) Sangon Biotech Shanghai, were tested to evaluate copper's antibacterial activity against antibiotic-resistant *Enterobacter* bacteria. Iron sheets (1.5 cm × 1.5 cm × 0.5 mm) (Hangzhou Sukalp Trading Co., Ltd), stainless steel sheets (the metal predominantly used in health care environments), and polyvinylchloride (PVC) obtained locally were used as controls.

Viable bacterial counts on copper and control surfaces

The viable bacterial counts were measured as published by Mehtar *et al.* (2008) with some modifications. 20 µl bacterial suspensions (5×10^7 CFU/ml), prepared in phosphate buffer saline (PBS), were overlaid on sterile copper, PVC, iron, and stainless steel surfaces and kept in Petri dishes maintained in a closed plastic box at room temperature. The viable bacterial counts were measured at 0, 2, 4, 6, and 8 h to determine the effect of copper surfaces on bacterial growth at various time intervals. After incubation to different time points, coupons were placed in 10 ml of PBS with 2 mm glass beads (PBSG), centrifuged for 30 sec at 300×g, and then serially diluted to perform the bacterial counts. The assays were performed in duplicate with three replications.

Antibacterial activity of copper powder

The antibacterial activity of copper powder was determined using the paper disc assay as described by Fit *et al.* (2009). Briefly, 1 ml of overnight bacterial culture was diluted to 0.5 OD on the McFarland scale and pipetted onto the surface of a solidified Muellar Hinton (MH) agar plate and spread by rotating the plate slowly by hand. Sterile paper discs (6 mm) were placed at MH agar plates. The copper powder (0.25 mg/disc) was placed on paper discs (4 discs/plate) permeated with distilled water to determine the inhibition zone caused by the copper powder of each paper disc. Paper discs loaded with sodium chloride (NaCl 0.25 mg/disc) were used as a control. The plates were incubated for 24–48 h at 37±1°C and the results were measured by determining the mean of inhibition area diameters.

Mutagenicity assay

Uptake of *D-cycloserin*, an inhibitor of bacterial cell wall biosynthesis, into the cell wall leads to toxicity and finally bacteriostasis, but not to cell death. Mutation in the *cyc*A gene makes cells resistant to this antimicrobial agent. The use of *D-cycloserin* against *E. coli* leads to the inactivation of cycA gene, allowing the detection of *D-cycloserin* resistant clones and this strategy can be utilized to measure the number of mutant cells (Fehér *et al.*, 2006). Mutagenesis of *Enterobacter* cells growing on copper surfaces was tested as described by Esprito Santo *et al.* (2011). Cells were applied for 10 sec (an exposure period shorter than that required for

massive onset of lethal damage) to the surface of the metal coupons, removed as described above, and spread on solidified minimal medium with glycerol as the sole carbon source for determination of total CFU and on minimal medium containing glycerol and 20 µl/ml *D-cycloserine* to select for *cycA* mutants. Colonies were counted after 24 h of incubation. They were considered to have originated from mutations in the *cycA* gene. The percentage of *cycA* mutants was calculated by dividing the number of CFU of *cycA* mutants by the total number of CFU. As controls, cells were exposed for the same period of time to stainless steel or stainless steel with 0.25% (w/v) formaldehyde.

Single Cell Gel electrophoresis (SCGE)

Comet Assay or SCGE was performed as described by Singh *et al.* (1988) with some changes. *E. cancerogenus* suspensions with 5×10^7 CFU/ml were streaked on dry copper surfaces using sterile cotton swabs for 10 sec and 30 sec while stainless steel were used as control. Cells were removed with 10 ml PBSG containing 20 µM EDTA, treated with lysozyme (20 mg/ml), and incubated at 37°C for 20 min. Cells were then mixed with 0.8% agarose and applied to glass slides precoated with 1.5% agarose. After complete gelling and solidification of agarose cell suspensions, slides were immersed in lysis buffer [2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 10, 10% Triton X-100, and 1% dimethyl sulfoxide (DMSO) to prevent oxidation during lysis] and were carefully agitated at 25 rpm at 4°C for 5 min. After washing with deionized water, slides were treated with denaturation buffer (300 mM NaOH and 1 mM EDTA, pH>13), incubated briefly with excess Tris-borate-EDTA (TBE) buffer, and subjected to electrophoresis at 25 mV at 15 mA for 3 min. Slides were removed, washed with ice-cold deionized water, immersed into absolute ethanol, and air dried overnight. Finally, slides were stained with ethidium bromide (Sangon Biotech China) in TBE and incubated for 1 min in the dark. Fluorescence was then observed (excitation wavelength [λ_{Ex}] of ~490 nm, emission wavelength [λ_{Em}] of ~520 nm) with an inverted confocal fluorescence microscope (Olympus DP50 BX 51).

ICP-MS analysis

The influx of copper into 12 Enterobacter species was analyzed using inductively coupled plasma mass spectroscopy (ICP-MS) (Agilent ICP-MS model 7500a) as described by Esprito Santo *et al.* (2011). *Enterobacter* cells were exposed to copper and PVC surfaces for 4 h as described for the viability assays. Surface challenged cells on moist copper surfaces, PVC, iron, and stainless steel were removed and washed with ice-cold PBSG buffer containing 20 µM EDTA, and viable bacterial counts were determined by plating as described above. In parallel, samples were mineralized with concentrated 70% (v/v) nitric acid for 2 h at 70°C and diluted to a final concentration of 5% (v/v) nitric acid. Germanium as $Ge(NO_3)_3$ was added at a final concentration of 50 ppb as an internal standard. Samples were loaded using an autosampler and then analyzed. Each sample contained three replicates.

Testing cell disintegration using general staining methods

To examine cellular changes, *E. cancerogenus* suspensions were exposed to dry metallic copper surfaces and stainless steel as described above. Cells were resuspended with 100 µl PBS buffer on the coupons, transferred onto a glass slide and air dried. The cells were stained with safranin O, and the glass slides were examined under oil immersion or at 40X using light microscopy (Axioskop 2 Plus *Zeiss* fluorescence microscope).

Analysis of copper contents in meat

In order to investigate the potential acquisition of copper by food exposed to copper surfaces, copper contents were measured in meat as described by Faundez *et al.* (2004). Briefly, 1×1 cm meat pieces (about 5 g each) were exposed

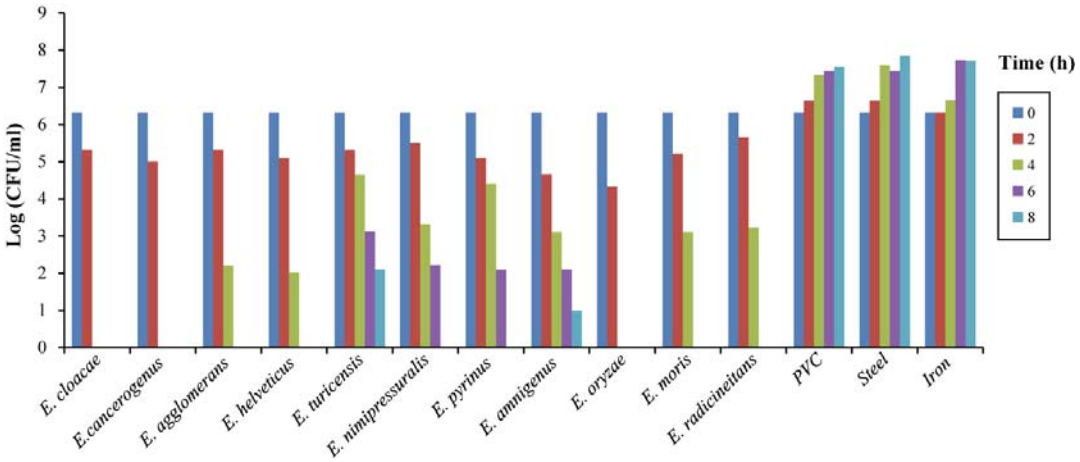

Fig. 1. Survival of *Enterobacter* suspensions on Copper, PVC and stainless steel surfaces. 5×10^7 CFU/ml *Enterobacter* cell suspensions were overlaid on copper, PVC, or stainless steel surfaces. Sampling was performed at 0, 2, 4, 8, and 10 h. The plots indicate the comparison between the logarithms of the mean number of surviving bacteria (CFU/ml) on copper and control surfaces. Fig. 1 shows the significant differences among *Enterobacter* species exposed to copper, PVC and stainless steel surfaces after 2 h, and these differences were highly significant after 4, and 8 h ($P<0.05$).

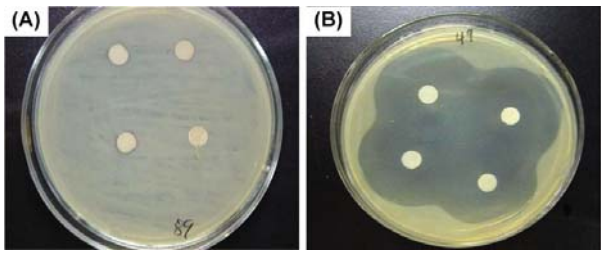

Fig. 2. ***Enterobacter*** **sensitivity to copper powder vs control (NaCl).** (A) No inhibition zone obtained for the control (NaCl). (B) Inhibition zone obtained for copper powder.

to copper surfaces for 0, 30, 60, and 90 min at room temperature and copper contents in the meat pieces were detected using ICP/MS. Similarly, to check the potential effect of bacterial load on the amount of copper detected in the exposed meat, the same methodology was performed in a subset of 3 samples of meat contaminated with *Enterobacter* (1×10^5 CFU/ml).

Statistical analyses

The data were subjected to analysis of variance (ANOVA), and the mean values were compared using the least significant differences test. The level of significant difference was set at $p<0.05$.

Results

Antibacterial efficiency of metallic copper surfaces against *Enterobacter*

Enterobacter suspensions (approximately 1×10^7 CFU/ml) overlaid on copper coupons for 0, 2, 4, 6, and 8 h had decreasing numbers of viable bacteriaover time on the copper surfaces, as compared to iron, PVC, or stainless steel. The species *E. cloacae*, *E. cancerogenus*, *E. agglomerans*, *E. helveticus*, *E. pyrinus*, *E. amnigenus*, *E. oryzae*, *E. moris*, *E. radicineitans*, and *E. sacchari* showed significantly lower counts on copper surfaces at 2–4 h compared with those obtained on PVC, iron or stainless steel surfaces, at room temperature. These differences were significantly greater at 6–8 h ($P>0.05$) (Fig. 1). At 8 h, the antibacterial activity of copper was at maximum, while no lethal effects of PVC or stainless steel were noted at that time (Fig. 1).

The viable bacterial counts of *E. nimipressuralis* and *E. turicensis* were reduced at each time interval compared to controls, but these differences became statistically significant ($P<0.05$) at 6–8 h (Fig. 1) and full inhibition was achieved at 10 h (data not shown). The results indicated that *Enterobacter* strains, from different environmental sources, were significantly susceptible to copper, with some significant differences among the species.

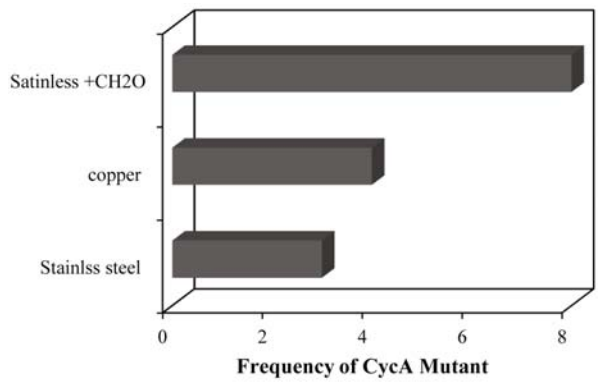

Fig. 3. Treatments using dry metallic copper, stainless steel, and stainless steel with formaldehyde to assess the frequencies of *cycA* mutants.

Antibacterial efficiency of metallic copper powder against *Enterobacter*

To investigate if pure copper powder possesses antibacterial effects, all *Enterobacter* strains were tested with copper powder using the paper disc assays. By measuring the mean of the inhibition zone in diameters (mm) of these tested *Enterobacter* species, we noted inhibitory effects of copper powder up to 17.2 mm for *E. cancerogenus* (Fig. 2B) and described in Table 1, while no inhibitory effect was recorded in the control (Fig. 2A). *E. oryzae*, *E. cloacae*, *E. agglomerans*, and *E. helveticus* were also more sensitive to the activity of copper, with an inhibitory effect of 16.4, 15.5, 15.3, and 15.4 mm, respectively (Table 1). The mean of the inhibition area diameters for other strains in this experimental panel, including *E. turicensis*, *E. nimipressuralis*, *E. pyrinus*, *E. amnigenus*, *E. moris*, *E. radicineitans*, and *E. sacchari* were 10.3, 9.5, 13.3, 14.1, 14.9, 14.7, and 14.9, respectively. These values were significantly higher compared to those where NaCl was used as control but less than *for E. cancerogenus*, *E. agglomerans*, *E. helveticus*, and *E. cloacae* ($P>0.05$).

These results agree with the data from the copper surface contact assay and demonstrate that copper powder has a strong antibacterial effect against Enterobacter species.

Copper surface mediated DNA damage

To determine the fate of *Enterobacter* bacterial DNA on dry metallic copper exposure we employed the *D-cycloserine* mutagenicity selection to examine the potential mutagenicity of copper on *Enterobacter*. We observed that exposures to dry copper surfaces did not increase the mutation rate in *Enterobacter* (Fig. 3). No significant differences were noted

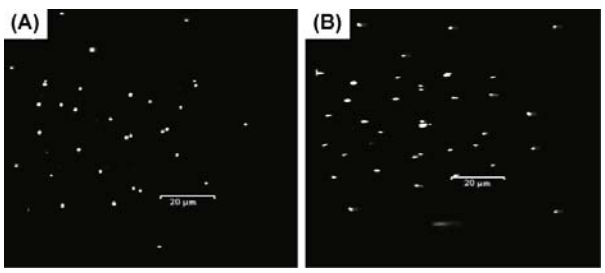

Fig. 4. Exposure of *Enterobacter* to copper surfaces causes DNA damage. (A) Exposure of *Enterobacter* to stainless steel control surfaces. (B) Exposure of Enterobacter to dry metallic copper surfaces. Bar=20 µm

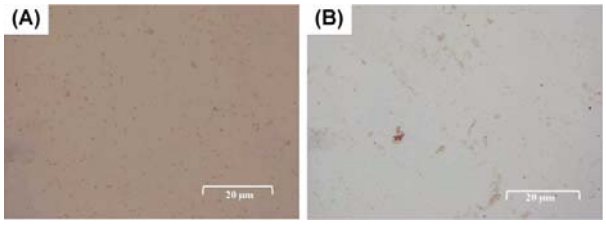

Fig. 5. Exposure of *Enterobacter* to dry metallic copper surfaces results in cellular disintegration. (A) Cells of *Enterobacter* exposed to stainless steel control surfaces for 10 sec. (B) Cells of *Enterobacter* exposed to dry metallic copper surfaces for 10 sec. Bar=20 μm

in the number of resistant mutants after *Enterobacter* cells were surface challenged to copper, while a significant increase in the number of mutants was observed when cells on control surfaces were additionally treated with formaldehyde, a known mutagen. This indicated that dry copper surfaces are not genotoxic.

We also employed an alternative test for DNA damage. An SGCE assay was performed at 10 sec and 30 sec following exposure of *Enterobacter* cells to dry metallic copper surfaces. We visualized the comet tail or the DNA damage after gel electrophoresis in the cells exposed for 30 sec on dry metallic surfaces (Fig. 4B), and no tail or DNA fragmentation was observed in the cells exposed for 10 sec, similar to stainless steel surfaces (Fig. 4A). These results indicated that DNA is lethally damaged by the dry metallic copper surfaces at 30 sec exposure (which is more than enough time to kill bacteria on dry metallic surfaces or to induce acute toxicity) and that no lethal effects occur at 10 sec following exposure.

Cell disintegration by copper

We performed the general staining method after 30 sec exposure on dry metallic copper surfaces. It was observed that most of the cells exposed to dry metallic copper surfaces disintegrated into cellular debris (Fig. 5B). Cells exposed to iron or stainless steel surfaces remained unaffected (Fig. 5A). These results indicated that *Enterobacter* exposure to copper led to severe structural damage after 30 sec.

Accumulation of copper ions by bacterial cells

To measure the accumulation of copper contents by each

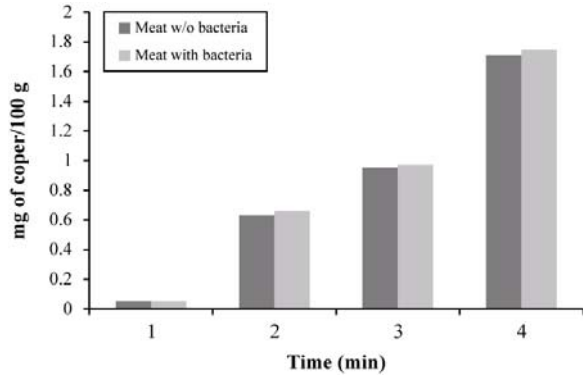

Fig. 7. The average of copper levels (mg/g) in 5 samples of meat without bacteria and with bacteria exposed on copper surfaces for 0, 30, 60, and 90 min.

Enterobacter species exposed to dry metallic copper surfaces, ICP-MS was performed. It was observed that copper accumulation in the *Enterobacter* cells exposed to moist copper surfaces was noticeably increased compared to PVC (Fig. 6). *E. cloacae*, *E. cancerogenus*, *E. agglomerans*, *E. helveticus*, *E. oryzae* accumulated significantly higher amounts of copper compared to PVC ($P>0.05$) (Fig. 6). *E. turicensis* and *E. nimipressuralis*, however, accumulated 7.3±0.2 μg/ml and 9.4 μg/ml copper, which was significantly elevated compared to PVC (Fig. 6), where it remained constant at 0.82±0.21 μg/ml ($P>0.05$), while it was lower than for *E. cloacae*, *E. cancerogenus*, *E. agglomerans*, *E. helveticus*, *E. pyrinus*, *E. amnigenus*, *E. oryzae*, *E. moris*, *E. radicineitans*, and *E. sacchari.* These results indicated that *Enterobacter* strains which accumulated more copper exhibited reduced survival time on copper surfaces compared to those that accumulated less copper. These data indicated that the influx of copper into *Enterobacter* bacterial cells could be one of the main factors for bacterial killing.

Acquisition of copper by beef

The possible acquisition of copper by food exposed to it was evaluated by placing pieces of meat on copper for varying periods of time. The copper contents increased in meat pieces after 30 min to 90 min from 0.05 to 1.71 mg copper/100g of meat (Fig. 7). Similarly, copper levels in meat pieces im-

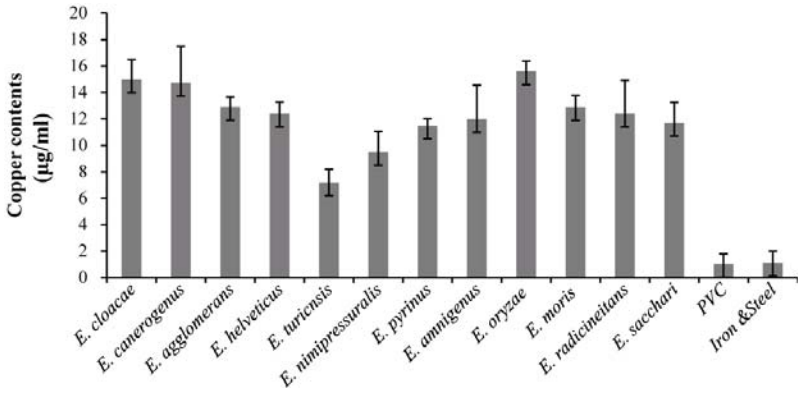

Fig. 6. The uptake of copper by *Enterobacter*. The differences in the copper levels accumulated by *Enterobacter* species during a 6 h exposure to copper surfaces compared to iron, stainless steel and PVC.

mersed in *Enterobacter* before exposure to copper surfaces were increased, with little difference compared to values obtained with uncontaminated meat (Fig. 7).

Taken all together, these findings suggest that copper has a strong antibacterial effect against *Enterobacter*. Copper accumulation within the cell, cell death, and DNA damage indicate that copper has lethal effects. Among the tested *Enterobacter* species, all were susceptible to copper within a small time range.

Discussion

The aim of the present study was to test new antibacterial alternatives for reducing the risk of *Enterobacter*, the emerging, highly infectious, and multidrug-resistant bacteria, in food processing and health care facilities, using copper as an antimicrobial agent. The results of this study indicate that the copper surface has strong antibacterial effects against *Enterobacter* bacteria. Copper surface tests were conducted at room temperature, a condition that imitates environmental surfaces.

Faundez *et al.* (2004) revealed that the inhibitory effect of copper was dependent on the time of exposure of *Salmonella enterica* and *Campylobacter jejuni* bacterial suspensions. In our study, we observed that the antibacterial activity of moist copper against *E. cloacae*, *E. cancerogenus*, *E. agglomerans*, *E. helveticus*, *E. oryzae*, *E. moris*, *E. radicineitans*, and *E. sacchari* was noticeable at 2 to 4 h but became more obvious at 8 h when no bacterial counts were obtained (Fig. 1). Similarly, the antibacterial activity of copper against *E. nimipressuralis* and *E. pyrinus* at 2, 4, and 6 h was significantly higher than iron, PVC, and stainless steel; however, this difference was more significant at 8 h (Fig. 1) when no bacterial counts were noted. *E. turicensis* and *E. amnigenus* were the exceptions, where some bacterial counts were recorded at 8 h (Fig. 1), however, the growth of these bacteria was completely inhibited at 10 h of exposure (data not shown). The differences in the time required for full antibacterial activity of copper against different bacterial species has been reviewed by Grass *et al.* (2011) as well as more recently reported by Ibrahim *et al.* (2011) for the *Burkholderia cepacia* complex, where they indicated that time is an important factor for bacterial killing. In our study, we observed that the time required for full antibacterial activity of copper for different *Enterobacter* species varies. The maximum time recorded for complete bacterial killing on dry copper surfaces was approximately 30 sec and on moist copper surfaces was 10 h in the case of *E. turicensis* and *E. nimipressuralis* (data not shown) and a minimum of 4 h for *E. cloacae* and *E. cancerogenus* (Fig. 1).

Our results indicate that time is a crucial factor for the survival of *Enterobacter* on copper surfaces, similar to other bacterial species reported by Grass *et al.* (2011). During antibacterial copper sheet assays, the appearance of the copper surfaces changed, where the metallic aspect of the new copper sheets turned a dark brown color and bacterial suspensions acquired a pale blue color that was indicative of the release of Cu^{+2} ions as shown by Faundez *et al.* (2004). Over time, this color became deeper. These data indicate that time is one of the vital factors contributing to bacterial killing and that *E. turicensis* and *E. amnigenus*, which required more time for complete inhibition, are more resistant than other species used in our experimental panel.

When testing the efficiency of the antibacterial effect of copper powder, it was evident that pure copper has significant antibacterial effects against Enterobacter species. *E. turicensis* and *E. nimipressuralis* were the only species whose sensitivity was low compared to all other strains tested in this study (Table 1). These findings support the results obtained from copper contact killing, demonstrating that copper has strong antibacterial effects against *E. turicensis* and *E. nimipressuralis* but less for other species tested in this study. Previously, various granulations of copper in combination with different salts, bismuth compounds, and bismuth salicylate were reported (Briand and Burford, 1999; Fit *et al.*, 2009) as antimicrobial agents against many gram negative pathogenic bacteria and when used in medicine preparation. Our results, finding significant antibacterial activity of copper powder against 12 Enterobacter species (Table 1), suggested further studies. The use of copper powder in combination with different salts, bismuth, and bismuth salicylate by medical experts could be investigated for their activity against Enterobacter species.

Our results and that of others suggest that bacterial killing by copper proceeds by successive actions, namely membrane damage, copper influx into the cells, oxidative stress and DNA damage (Espirito Santo *et al.*, 2011). In this study, we noticed that a 10 sec exposure produced no DNA fragmentation or comet formation, similar to the control as shown in Fig. 4A, while on longer exposure, DNA fragmentation or tailed DNA was clearly visible as shown in Fig. 4B. Our SCGE results indicate that exposing *Enterobacter* to dry copper surfaces results in DNA damage that likely precedes cell death. These findings are consistent with those of Espirito-Santo *et al.* (2011), who demonstrated that exposing *E. coli* to dry metallic copper surfaces resulted in toxicity due to DNA damage following prolonged exposure. DNA mutation and DNA lesions are not the underlying cause for antimicrobial properties of dry metallic copper. We confirmed by the *D-cycloserin* mutation assay that exposute to a copper surface didn't increase the mutation rate in *Enterobacter*, indicating that DNA is not a major target during acute lethal metallic copper stress.

Copper accumulation in the bacterial cells is the key factor resulting in copper toxicity and subsequent cell death in E. coli (Espirito Santo *et al.*, 2011). In our study, the data obtained from ICP-MS demonstrates that the differences in the increased concentration of Cu contents (Fig. 6) and the differences in the time of complete killing of *Enterobacter* cells (Fig. 1) correlate. This correlation indicates that the more rapid antibacterial activity against *E. cloacae*, *E. cancerogenus*, *E. agglomerans*, and *E. helveticus* compared to *E. turicensis* was due to a higher intracellular accumulation of copper resulting in subsequent toxicity. It is clear that the contact killing of Enterobacter cells was due to the accumulation of copper, as free copper ions are likely to cause a selective change in the membrane (Ohsumi *et al.*, 1998), and the membrane is the direct target of copper exposure (Espirito Santo *et al.*, 2011). Contact killing also led to cellular changes

including cellular disintegration in *Enterobacter* cells (Fig. 5B), while no disintegration or cell debris was observed in cells exposed to stainless steel surfaces. This finding is consistent with previous studies that have demonstrated that exposure of bacterial cells to copper leads to cellular disruptions (Espirito Santo *et al*., 2011).

The uptake of copper into meat increased with the passage of time reaching 1.71 mg/100 g after 90 min exposure (Fig. 7) and similar results were obtained from meat exposed to copper surfaces after immersing the meat in bacteria. Faundez *et al*. (2004) reported that during food processing the normal time of exposure is not more than a few min, thus copper acquisition would remain very low. It should be stated that the use of copper is not intended to be the first line of defense against pathogenic bacteria; rather, the main application of copper would be in decreasing the bacterial load in food processing industries (Faundez *et al*., 2004).

Our study demonstrated that copper has antibacterial activity against *Enterobacter* species. Therefore, the levels of *Enterobacter*, which is an emerging, infectious, multidrug-resistant human pathogen, found in a variety of environmental niches, including the plant rhizosphere, soil, water, hospital patients, hospital equipment, and industrial contaminants, as reviewed by Tyler and Triplett (2009), could be reduced by using antimicrobial copper surfaces to prevent cross contamination. Copper is used in many effective antimicrobial agents, including fungicides, antifouling paints, antimicrobial medicines, oral hygiene products, hygienic medical devices, antiseptics, and in a host of other useful applications (Airey and Verran, 2007). Most importantly, it has been demonstrated that dry copper surfaces provide different antimicrobial properties than ionic copper, with potential implications for the challenge posed by multiple-drug-resistant germs (Espírito Santo *et al*., 2008). Experimental evidence demonstrated that copper-containing items significantly reduce the numbers of microorganisms in the clinical environment (Casey *et al*., 2010), and these are now being applied elsewhere for the wellbeing of susceptible members of the community and could be used against Enterobacter.

To date, studies have demonstrated the antimicrobial effect of copper on a range of disease-causing organisms, as reviewed by Grass *et al.* (2011); however, to the best of our knowledge, no previous information was available concerning the antibacterial activity of copper against *Enterobacter* species, which could be a risk through cross-contamination or contamination during food processing. Our data indicate that the use of copper could reduce the environmental bioburden of these bacteria, particularly in food processing industries as well as in the hospital environment. In addition, it would be of interest to investigate the effects that copper resistance genes have on the survival of these bacteria on copper surfaces.

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