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The antimicrobial properties of exogenous copper in human synovial fluid against *Staphylococcus aureus*

an in vitro study

From Western University, London, Canada

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Aims
The mechanism by which synovial fluid (SF) kills bacteria has not yet been elucidated, and a better understanding is needed. We sought to analyze the antimicrobial properties of exogenous copper in human SF against *Staphylococcus aureus*.

Methods
We performed in vitro growth and viability assays to determine the capability of *S. aureus* to survive in SF with the addition of 10 µM of copper. We determined the minimum bactericidal concentration of copper (MBC-Cu) and evaluated its sensitivity to killing, comparing wild type (WT) and CopAZB-deficient USA300 strains.

Results
UAMS-1 demonstrated a greater sensitivity to SF compared to USA300 WT at 12 hours ($p = 0.001$) and 24 hours ($p = 0.027$). UAMS-1 died in statistically significant quantities at 24 hours ($p = 0.017$), and USA300 WT survived at 24 hours. UAMS-1 was more susceptible to the addition of copper at four ($p = 0.001$), 12 ($p = 0.005$), and 24 hours ($p = 0.006$). We confirmed a high sensitivity to killing with the addition of exogenous copper on both strains at four ($p = 0.011$), 12 ($p = 0.011$), and 24 hours ($p = 0.011$). WT and CopAZB-deficient USA300 strains significantly died in SF, demonstrating a MBC-Cu of 50 µM against USA300 WT ($p = 0.011$).

Conclusion
SF has antimicrobial properties against *S. aureus*, and UAMS-1 was more sensitive than USA300 WT. Adding 10 µM of copper was highly toxic, confirming its bactericidal effect. We found CopAZB proteins to be involved in copper effluxion by demonstrating the high sensitivity of mutant strains to lower copper concentrations. Thus, we propose CopAZB proteins as potential targets and use exogenous copper as a treatment alternative against *S. aureus*.

Article focus
- The importance of exogenous copper and CopAZB proteins as possible antimicrobial targets against *Staphylococcus aureus.*
- Low copper concentrations present an alternative for treating *S. aureus* infections.

Key messages
- 10 µM of exogenous copper effectively killed both *S. aureus* strains.

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- Human synovial fluid demonstrates heterogenous bactericidal activity against different *S. aureus* strains.

Strengths and limitations

- This was an in vitro study analyzing different *S. aureus* strains in human synovial fluid.
- More evidence is necessary to define the efficacy, safety, and toxicity level of copper.

Introduction

Osteoarthritis (OA) is the most frequent pathology that affects joint cartilage and synovial fluid (SF), resulting in increased wear, inflammation, pain, and decreased mobility.[1–4] Fortunately, total joint replacement (TJR) is a well-known and effective procedure that relieves pain and improves the quality of life in patients with end-stage OA.[5] The total number of TJRs performed each year is increasing worldwide, and there is an expected annual incidence of four million arthroplasties in the USA by 2030.[5,6] Consequently, the number of revision surgeries is also expected to increase, with periprosthetic joint infections (PJIs) as one of the most challenging causes of reintervention.[5-11] Several studies have reported an estimated infection rate of 1% to 2% after total hip arthroplasty (THA) and total knee arthroplasty (TKA).[7,12-15]

S. aureus is a Gram-positive, round-shaped commensal bacterium that forms pairs, tetrads, and irregular "grape-like" structures, and is part of the body's normal microbiota.[16,17] It can also be an opportunistic pathogen, being the most isolated microorganism in all three major osteoarticular infections, including osteomyelitis,[18] native joint septic arthritis,[19] and PJI.[20,21] In almost all series and for all types of PJI, *S. aureus* is the most common causative organism, accounting for 18% to 73% of cases.[22-26] One of the principal characteristics of *S. aureus* is its capacity to develop antibiotic resistance, making treatment even more difficult.[27] Due to increasing multi-resistant microorganisms and methicillin-resistant *S. aureus* (MRSA) prevalence, the eradication rate of PJI continues to decrease, severely affecting patients, surgeons, and healthcare systems.[28] Parvizi et al[29,30] reported a 50% prevalence of MRSA in the USA. Although different pre- and postoperative measures, such as skin decolonization, routine antimicrobial prophylaxis, increased hand hygiene compliance, and patient isolation, contributed to reducing MRSA infections, it is still a significant cause of morbidity and mortality;[31,32] therefore, high surveillance and novel treatment options are needed.[16,33-36]

The host's immune system includes an innate response to pathogens in different body fluids, including SF. Current literature suggests that SF has defence factors to prevent infections apart from the cell-mediated immune response, and might participate in the joint's natural defence. Gruber et al[37] showed SF bactericidal activity against *S. aureus*. Microbes require iron, zinc, manganese, and copper as essential micronutrients to survive. Although iron is abundant, free iron in living organisms is reduced by sequestration into proteins such as transferrin and lactoferrin.[38,39] Watson et al[40] demonstrated that SF from patients with OA has minimum free iron concentration and bactericidal effect against *S. aureus*. As a result, the iron restriction would contribute to SF's bactericidal activity, and the complement system would kill bacteria.

Copper is the third most abundant essential transition metal in humans, and it is required by several cellular enzymes involved in redox reactions.[41,42] The normal range for total copper in blood is 85 to 180 µg/dl (13.3 to 28.3 µmol/l = 13.3 to 28.3 µM), and serum-free copper reference values have been routinely identified between 0 to 10 µg/dl (0 to 1.6 µmol/l = 0 to 1.6 µM). Excess copper can harm cells, potentially producing toxic reactive oxygen species.[43,44] Usually, more than 90% of the copper in the blood is bound to ceruloplasmin. The concentration of protein-free copper is estimated to be less than 5%, leaving the remaining amount bound to transcuperin, albumin, and amino acids. Intracellular copper is usually bound to chaperones because free copper is highly deleterious to cells.[45]

There is evidence of increased systemic and local copper availability during infections.[46] This suggests that the host environment uses copper's toxicity to fight microbes. Free copper is toxic for most bacteria, and they go to great lengths to avoid its accumulation in the intracellular/cytoplasmic compartment.[47,48] However, a better understanding of its antimicrobial properties due to its bactericidal activity might help to develop new treatment strategies for *S. aureus* infections. Unfortunately, due to increasing resistant organisms, the high prevalence of antibiotic resistance raises concerns and limits antibiotic options, decreasing our ability to eradicate infection.[49,50] Consequently, new alternatives are needed to keep pathogenic microorganisms at bay. In this sense, copper surfaces in hygiene-sensitive areas have been developed.[51] At this point, additional studies are required to determine copper's potential role in treating PJIs and the most cost-effective presentation to deal with this devastating complication.

Thus, adding exogenous copper to human SF is expected to produce a high killing of the different *S. aureus* strains in our in vitro study. This study will provide a better understanding of the pathogenesis of *S. aureus* in human SF and try to identify specific synovial proteins that might influence bacterial survival. Additionally, it is anticipated that this work will help to develop novel strategies such as new antimicrobials and therapies, which could, in turn, lead to significant cost savings and reduce patient morbidity and mortality associated with this challenging complication.

Methods

Synovial fluid collection and preparation

SF samples were collected from patients undergoing elective primary TKA or THA to treat symptomatic osteoarthritis. All patients with inflammatory arthropathy (i.e. rheumatoid arthritis, gout) or fibromyalgia and cases with multiple severe comorbidities were excluded.

The same surgical team (FDD, EV) performed all procedures in the operating theatre under sterile conditions. When TKA was performed, a tourniquet was applied around the thigh before starting the intervention to minimize blood contamination. After the skin incision and deep dissection, the joint capsule was exposed, and SF was aspirated using a sterile 18-gauge needle and 20 cc syringe.

After collection, all samples were centrifuged for ten minutes at 4,700 g within 12 hours of the procedure to remove any possible red blood cell and lymphocyte contamination. The cell-free SF supernatant was then aspirated and

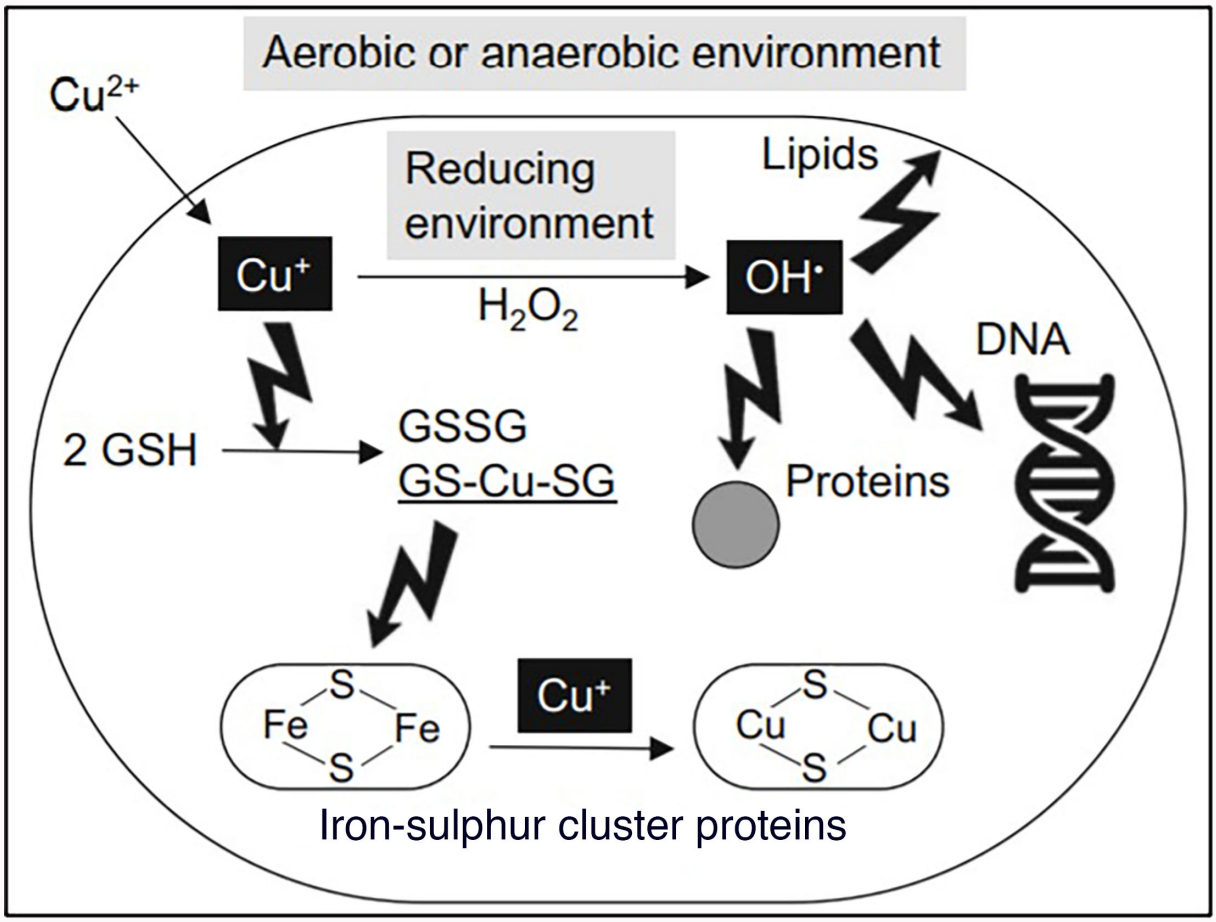

Fig. 1

Mechanisms of copper toxicity.[52,53] Copper violates the bacterial wall. The reducing environment of the cytoplasm reduces copper (Cu++ to Cu+), which can participate in Fenton-type reactions, produce highly reactive hydroxyl radicals, and affect lipids, proteins, and DNA. Cu+ can also lead to thiol depletion in the glutathione (GSH) pool, proteins, and free amino acids. Under anaerobic conditions, GSH copper complexes (GS-Cu-SG) can act as copper donors for metalloenzymes. The most common mechanism of copper toxicity is the displacement of iron from iron-sulphur cluster proteins.

transferred to cryotubes under sterile conditions. The samples with evident blood contamination after centrifugation were excluded from the analysis. All eligible samples were frozen and stored in a -80°C freezer until further analysis. All SF samples were labelled and separated by donor with a unique identification number without recording any patient's identifiable information.

At the time of experimentation, randomly selected samples were taken out from the -80°C freezer and put in the incubator at 37°C for five to ten minutes to thaw. After each experiment and analysis, the SF samples were destroyed. According to the surgeon's criteria, all patients were followed with routine appointments to detect any unexpected complications. No follow-up assessment was necessary for the present analysis.

S. aureus strains and routine culture

Different *S. aureus* strains previously found to be sensitive and resistant, UAMS-1 and USA300 wild type (WT), respectively, were used throughout this study. Bacteria were routinely cultured at 37°C in liquid tryptic soy broth (TSB) (Difco, UK) with shaking overnight at 200 rpm or on solid tryptic soy agar (1.5% w/v) (TSA) plates. When necessary, *S. aureus* strains were cultured in the presence of 3 µg/ml erythromycin to allow for selection of resistance markers.

It is essential to mention that all analyses and comparisons between strains were ideally made with the same human SF sample to avoid a possible bias in the results. When this was not possible, a different SF sample was used following the same methodology. A total of 55 different human SF samples were used in this study.

At the same time, a minimum of eight different tubes (n = 8) containing each *S. aureus* strain (UAMS-1 and USA300 WT) were used in every assay to reach appropriate statistical results. The only exception was the low-pH environment viability assay, where only four tubes for each strain were included in the analysis.

S. aureus survival in synovial fluid in vitro

A total of 11 human SF samples from different living donors were removed from the freezer, thawed, and diluted in sterile saline solution immediately before in vitro growth assays. To analyze the effect of SF against *S. aureus*, in vitro growth assays were performed with different *S. aureus* strains. We decided to use two of the most prevalent strains associated with human infections, including one sensitive and one resistant strain,

Table I. *Staphylococcus aureus* survival in saline solution in vitro.

Time, hrs	Mean UAMS-1, CFU/ml (SD) (n = 8)	Mean USA300 WT, CFU/ml (SD) (n = 8)	p-value
0	7.66 × 10e5 (9.03 × 10e5)	1.36 × 10e6 (1.01 × 10e6)	0.140
2	1.01 × 10e6 (1.74 × 10e6)	1.32 × 10e6 (1.55 × 10e6)	0.920
4	6.1 × 10e5 (6.4 × 10e5)	1.37 × 10e6 (1.21 × 10e6)	0.090
12	2.12 × 10e5 (1.16 × 10e5)	1.45 × 10e6 (4.7 × 10e5)	0.001
24	4.12 × 10e5 (1.11 × 10e6)	5.6 × 10e5 (4.5 × 10e5)	0.290

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; WT, wild type.

Table II. *Staphylococcus aureus* survival in synovial fluid in vitro.

Time, hrs	Mean UAMS-1, CFU/ml (n = 8) (SD)	USA300 WT, CFU/ml (n = 8) (SD)	p-value*
0	9.35 × 10e5 (5.72 × 10e5)	1.66 × 10e6 (9.09 × 10e5)	0.082
2	1.74 × 10e6 (1.1 × 10e6)	9.38 × 10e5 (5.33 × 10e5)	0.114
4	1.1 × 10e6 (7.8 × 10e5)	5.74 × 10e5 (7.23 × 10e5)	0.206
12	5.9 × 10e5 (1.66 × 10e5)	2.03 × 10e6 (8.98 × 10e5)	0.001
24	1.29 × 10e5 (2.05 × 10e5)	3.67 × 10e6 (3.96 × 10e6)	0.030

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; WT, wild type.

such as the osteomyelitis isolate UAMS-1 and USA300 WT, respectively.

Overnight cultures of each *S. aureus* strain were grown in 5 ml of TSB and then normalized to an optical density (OD_{600}) of 1.0 in sterile saline solution. Tubes containing either 20% SF (UAMS-1) or 50% SF (USA300 WT) in sterile saline solution were inoculated with 10 µl of the *S. aureus* strain suspension at OD_{600} = 1.0 to start each tube at an initial OD_{600} 0.01, equivalent to ~ 2 to 4 × 10^6 CFU/ml. The samples were incubated at 37°C for two, four, 12, and 24 hours, after which the samples were serially diluted, and 10 µl were droplet-plated on TSA plates. After overnight incubation at 37°C, the colonies were counted to determine the final number of colony-forming units per ml (CFU/ml) for each sample.

The effect of copper on *S. aureus* viability in synovial fluid

Due to the well-known toxicity of copper (Figure 1) and the presence of ceruloplasmin in SF, viability assays were performed to determine the capability of different *S. aureus* strains to survive in SF with the addition of exogenous copper. As done previously, 12 random SF samples were thawed immediately before dilution in sterile saline solution to determine *S. aureus* survival or death. SF samples were diluted in saline to varying concentrations from 20% to 50% along with no synovial fluid controls, and 1 µl of 10 mM copper sulphate ($CuS0_4$) was added to achieve a working concentration of 10 µM copper in each sample. Similarly, tubes containing paired samples of SF or saline alone were included as controls without adding copper. All samples were plated on TSA immediately after inoculation to assess the starting CFU/ml. Samples were again plated on TSA after four, 12, and 24 hours of incubation at 37°C to determine the resultant number of CFU/ml.

The MBC is the lowest amount of an antimicrobial agent required to cause a 3-logarithmic microbial death (99.9% killing) in the size of the standard inoculum. To identify the MBC-Cu, we performed viability assays using a twofold dilution series of copper in SF and saline. On the other hand, different copper export proteins have been identified in bacteria,[54–56] and Solioz et al[54] identified P1B-type ATPases in *S. aureus*. All *S. aureus* strains have a conserved operon encoding a P1B-1-type ATPase copper efflux transporter (copA) and a copper chaperone protein (copZ)[57] encoded as part of the core genome. Some HA-MRSA strains also have an additional copper exporting P1B-3 type ATPase, designated copB, encoded on a plasmid that is either free or integrated into the genome.[58]

Different mutant microorganisms with CopA deficiency have been demonstrated to have inadequate copper efflux and increased copper sensitivity.[59–61] To elucidate the mechanism of bacterial killing, we used the USA300 WT strain due to its inherent high resistance and the availability of mutants deficient for CopAZB protein, which collaborates with bacterial survival by pouring copper out of its cytoplasm. In this context, we tried to analyze the effects of deficient CopAZB protein on sensitivity to SF killing and determine the MBC-Cu.

The WT and CopAZB-deficient USA300 strains were streaked onto TSA plates and grown overnight at 37°C. Isolated colonies were inoculated into individual glass test tubes containing 5 ml TSB and grown overnight with shaking. The next day, bacteria were diluted in sterile saline solution to obtain an OD_{600} of 1.0. Next, these bacterial suspensions were used to inoculate SF and sterile saline solution with 1 in 100 dilutions (final OD_{600} of 0.01) with and without the addition of copper at a range of concentrations. Exogenous copper sulphate was added to the SF and saline in a twofold dilution series ranging from 100 µM down to 1.5 µM along with a no copper control. A total of 11 different human SF samples were used for this analysis.

All samples were plated on TSA immediately after inoculation to assess the starting CFU/ml. After a 24-hour incubation at 37°C, samples were again plated on TSA to count the resultant number of CFU/ml. The MBC-Cu was defined as the lowest concentration of copper, resulting in significant bacterial killing following overnight incubation at 37°C.

Analysis of the acidic environment

Low-pH environments (< 6.5) can reduce the survival of gram-positive pathogens.[62] Indeed, weak acids would have antimicrobial activity because the undissociated form of weak acids passes easily through the cell membrane.[62] Because of

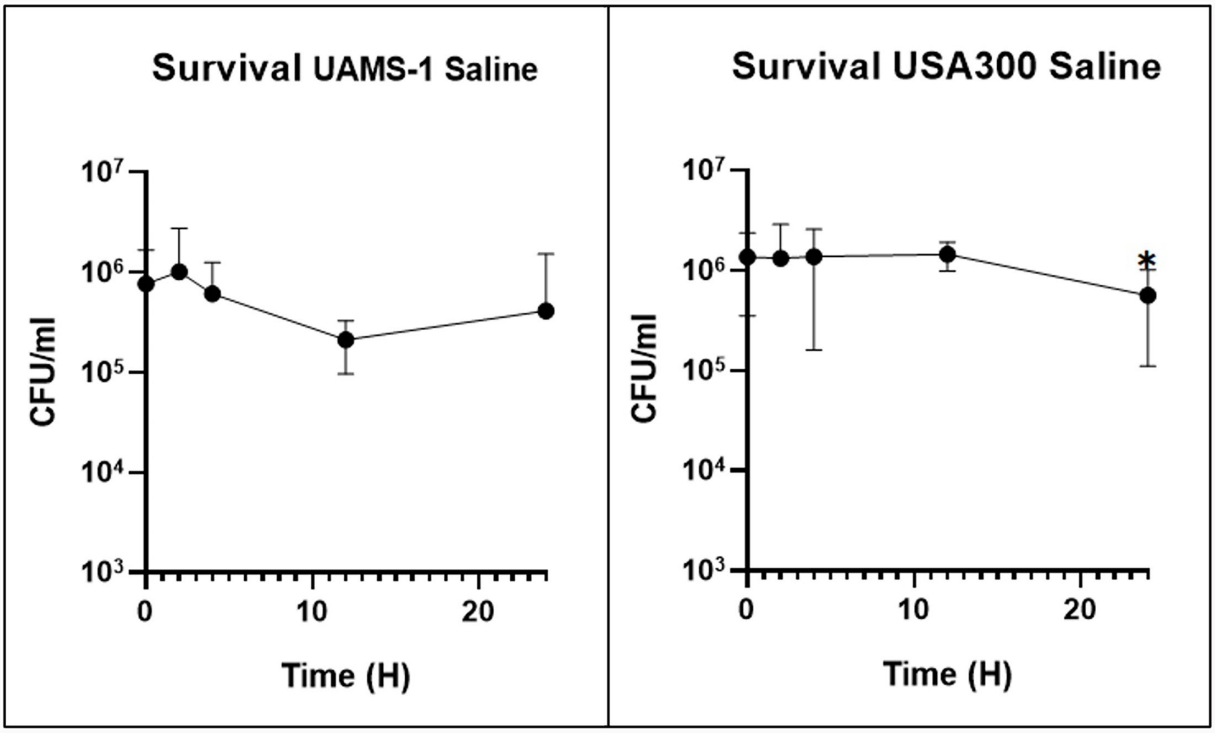

Fig. 2
Staphylococcus aureus survival in saline solution in vitro. Variables were considered statistically significant at *p < 0.05. CFU, colony-forming units.

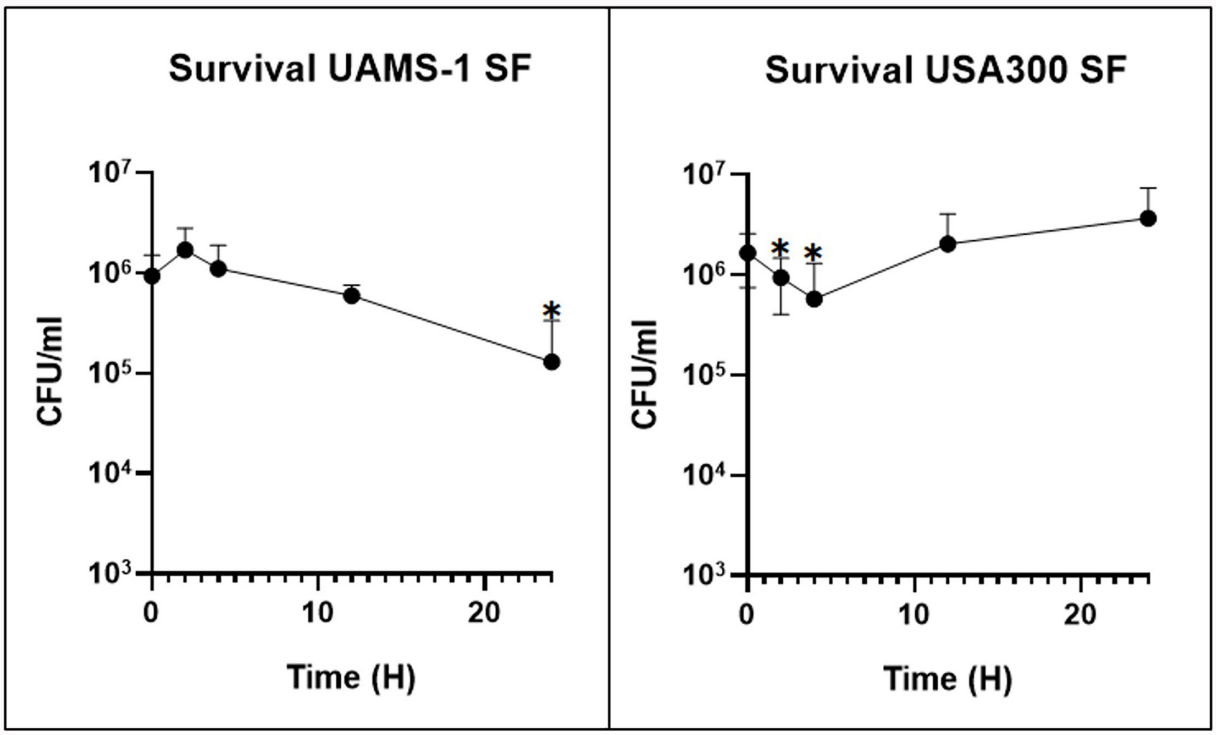

Fig. 3
Staphylococcus aureus survival in synovial fluid in vitro. Variables were considered statistically significant at *p < 0.05. CFU, colony-forming units; SF, synovial fluid.

the acidic characteristics of local environmental pH with the addition of exogenous copper, we then sought to analyze whether the SF low-pH environment would influence bacterial killing.

As done previously, four random SF samples were thawed immediately before dilution in sterile saline solution to determine *S. aureus* survival or death. SF samples were diluted in saline to varying concentrations from 20% to 50%

Table III. The effect of copper on *Staphylococcus aureus* viability in saline solution.

Time, hrs	Mean UAMS-1, CGU/ml (n = 8) (SD)	Mean USA300 WT, CFU/ml (n = 8) (SD)	p-value*
0	6.83 × 10e6 (5.50 × 10e5)	6.4 × 10e6 (2.7 × 10e6)	0.920
0 + copper	6.1 × 10e6 (7.31 × 10e5)	6.34 × 10e6 (2.24 × 10e6)	0.670
4	3 × 10e6 (1.26 × 10e6)	5 × 10e6 (1.84 × 10e6)	0.060
4 + copper	1.83 × 10e6 (1.66 × 10e6)	3.25 × 10e6 (2.14 × 10e6)	0.140
12	3.6 × 10e5 (1.8 × 10e5)	4.28 × 10e6 (2.1 × 10e6)	< 0.001
12 + copper	6 × 10e4 (6.4 × 10e4)	3.52 × 10e5 (3 × 10e5)	0.003
24	3.8 × 10e4 (1.5 × 10e4)	1.25 × 10e6 (9.9 × 10e5)	< 0.001
24 + copper	1.8 × 10e31.800 (1.6 × 10e3)	1.6 × 10e4 (2.2 × 10e4)	< 0.001

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; WT, wild type.

Table IV. The effect of copper on *Staphylococcus aureus* viability in synovial fluid.

Time, hrs	Mean UAMS-1, CFU/ml (n = 8) (SD)	USA300 WT, CFU/ml (n = 8) (SD)	p-value*
0	6.5 × 10e6 (1.6 × 10e6)	7 × 10e6 (2.7 × 10e6)	0.670
0 + copper	5.6 × 10e6 (1.4 × 10e6)	7.25 × 10e6 (2 × 10e6)	0.240
4	5.1 × 10e6 (2.2 × 10e6)	5.1 × 10e6 (2.1 × 10e6)	0.870
4 + copper	9.6 × 10e4 (2.2 × 10e5)	3.84 × 10e6 (2.9 × 10e6)	0.001
12	4.7 × 10e6 (1.9 × 10e6)	5.8 × 10e6 (1.7 × 10e6)	0.170
12 + copper	4.3 × 10e4 (1.2 × 10e5)	1.76 × 10e5 (1.8 × 10e5)	0.005
24	4.4 × 10e6 (1.7 × 10e6)	6.9 × 10e6 (1.2 × 10e6)	0.015
24 + copper	1.7 × 10e3 (4.8 × 10e3)	2.5 × 10e5 (7.1 × 10e5)	0.006

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; WT, wild type.

along with no synovial fluid controls, and 1 µl of 10 mM copper sulphate (CuS04) was added to achieve a working concentration of 10 µM copper in each sample. Similarly, tubes containing paired samples of SF or saline alone were included as controls without adding copper. Finally, additional samples of SF or saline were included as an experimental analysis with the addition of hydrochloric acid (HCl) solution to determine if the acidic environment influenced the killing of the bacteria. Overnight cultures of each *S. aureus* strain were grown in 5 ml of TSB and then normalized to an optical density (OD_{600}) of 1.0 in sterile saline solution. All samples were plated on TSA immediately after inoculation to assess the starting CFU/ml. Samples were again plated on TSA after four, 12, and 24 hours of incubation at 37°C to determine the resultant number of CFU/ml.

SDS-PAGE and protein identification in synovial fluid

The amount of copper available in the host environment depends on binding proteins such as ceruloplasmin. Ceruloplasmin is a serum ferroxidase responsible for more than 90% of copper transportation,[63] and its weight ranges between 120 and 132 kDa.[40,64] In this sense, ceruloplasmin may be present in SF and may be responsible for the binding of copper.

To determine whether ceruloplasmin is responsible for the availability of copper in SF, we tried to identify the presence of ceruloplasmin in SF. A total of 18 random SF samples were thawed immediately before dilution in sterile saline solution to an initial concentration of 10% (vol/vol) in saline. Then, 10 µl of the previous diluted SF samples from different donors were mixed 1:1 with Laemmli protein buffer and boiled for ten minutes.

After cooling all samples, 7 µl of the ladder control solution was loaded. Then, 10 µl of nine SF samples were loaded, and a 12% SDS-PAGE was run in Tris-glycine buffer at 150 V and 500 mA for 90 minutes. The remaining nine samples were loaded and run through a second 12% SDS-PAGE in Tris-glycine buffer at 150 V and 500 mA but for over 90 minutes. Upon completion, the gel was removed from the dock and stained overnight with InstantBlue Coomassie Protein Stain (Abcam, UK). After staining the gel, we used an automated spot picker to identify the individual protein bands from the gel.

Statistical analysis

Continuous variables were expressed as means and SDs or medians and IQRs depending on whether they had a normal distribution. Data were compared using the independent-samples *t*-test, where data were normally distributed, and the Mann-Whitney U test otherwise. Variables were considered statistically significant at $p < 0.05$. All analyses were performed using IBM SPSS Statistics v15.0 (SPSS, USA).

Results

Human SF has a heterogenous bactericidal response against different *S. aureus* strains. In sterile saline solution and without SF, both control strains died at the final endpoint. We only found a higher sensitivity to killing for UAMS-1 after 12 hours ($p = 0.001$) compared to USA300 WT (Table I).

Despite an initial growth after the first two hours ($p = 0.330$), UAMS-1 died after four ($p = 0.060$), 12 ($p = 0.330$), and 24 hours ($p = 0.090$) without being statistically significant. By contrast, USA300 WT survived for the first 12 hours but showed significant bacterial death after 24 hours ($p = 0.040$) (Figure 2).

In contrast, we observed a different bactericidal effect of SF against each strain. UAMS-1 was highly sensitive and significantly died after 12 ($p = 0.001$) and 24 hours ($p = 0.030$,

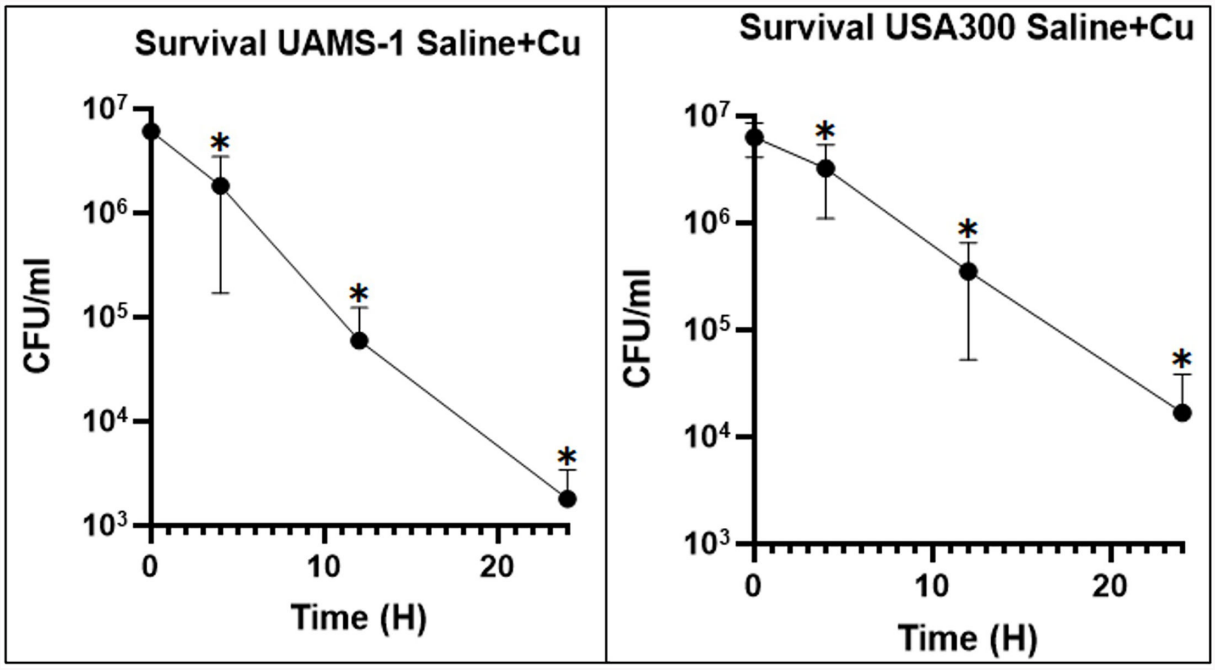

Fig. 4
The effect of copper (Cu) on *Staphylococcus aureus* viability in saline. The addition of copper resulted in significant killing of both strains after four, 12, and 24 hours. Variables were considered statistically significant at *p < 0.05. CFU, colony-forming units.

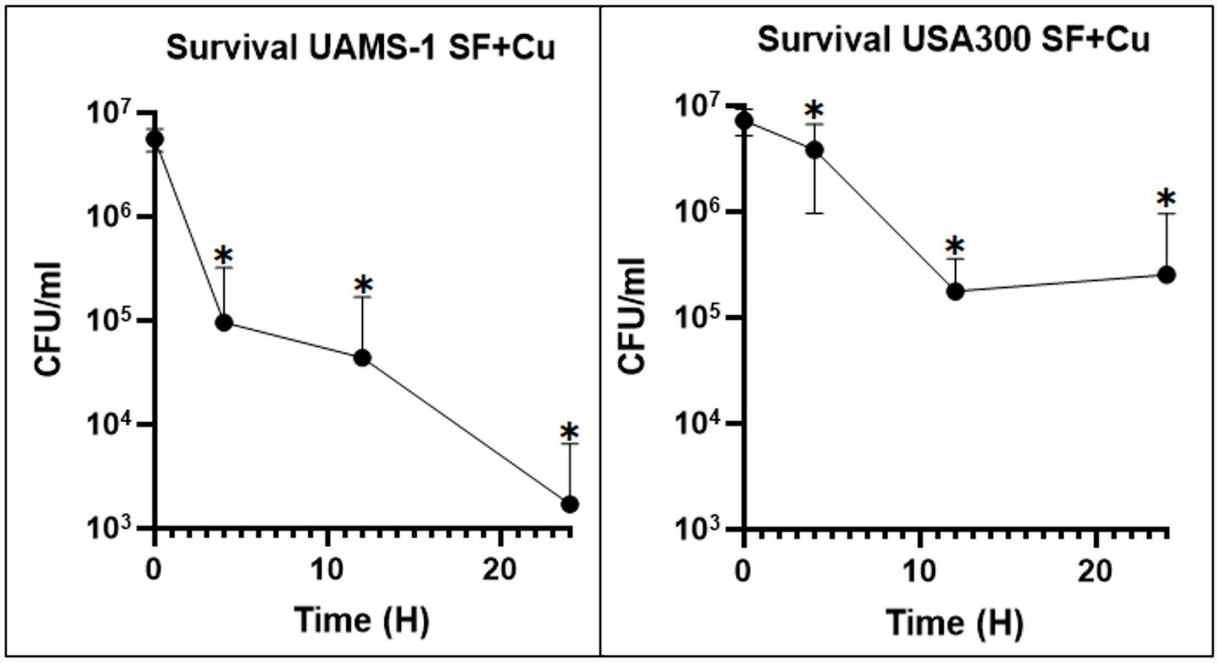

Fig. 5
The effect of copper on *Staphylococcus aureus* viability in synovial fluid (SF). The addition of copper (Cu) resulted in significant killing of both strains after four, 12, and 24 hours. Variables were considered statistically significant at *p < 0.05. CFU, colony-forming units.

both Mann-Whitney U test) when compared to USA300 WT (Table II).

Despite an initial growth after the first four hours (p = 0.670), UAMS-1 died after 12 hours (p = 0.090), achieving statistical significance at the 24-hour endpoint (p = 0.020). On the other hand, although USA300 WT showed a significant decrease of CFU/ml after two (p = 0.040) and four (p = 0.040) hours, it could finally survive and even grew after 12 (p = 0.530) and 24 hours (p = 0.670, all Mann-Whitney U test), suggesting an increased resistance to SF activity (Figure 3).

Although we confirmed the bactericidal activity of SF, the sensitivity varied significantly between both strains, with UAMS-1 being more sensitive than USA300 WT, affecting the overall survival of each strain.

Table V. Minimum bactericidal concentration of copper on *Staphylococcus aureus* USA300 wild type.

Time, hrs	Mean SF, CFU/ml (n = 8) (SD)	Mean SL, CFU/ml (n = 8) (SD)	p-value
0 + 100 µM copper	1.9 × 10e6 (7.9 × 10e5)	2.5 × 10e6 (7.8 × 10e5)	0.040
0 + 50 µM copper	1.6 × 10e6 (4.2 × 10e5)	2.4 × 10e6 (9.7 × 10e5)	0.010
0 + 25 µM copper	1.9 × 10e6 (1.1 × 10e6)	2.3 × 10e6 (7.2 × 10e5)	0.530
0 + 12.5 µM copper	1.5 × 10e6 (1.4 × 10e6)	2.1 × 10e6 (8.5 × 10e5)	0.030
0 + 6.25 µM copper	1.2 × 10e6 (7.1 × 10e5)	2.5 × 10e6 (1.5 × 10e6)	0.030
0 + 3.125 µM copper	1.1 × 10e6 (5.5 × 10e5)	1.9 × 10e6 (6.6 × 10e5)	0.020
0 + 1.5 µM copper	2.1 × 10e6 (7.9 × 10e5)	2.1 × 10e6 (6.5 × 10e5)	0.870
0 + 0 µM copper	2.1 × 10e6 (9.1 × 10e5)	2 × 10e6 (1.1 × 10e6)	0.920
24 + 100 µM copper	5.3 × 10e3 (8.4 × 10e3)	9.9 × 10e3 (8.3 × 10e3)	0.290
24 + 50 µM copper	7.9 × 10e3 (8.4 × 10e3)	7.7 × 10e3 (1.2 × 10e4)	0.920
24 + 25 µM copper	3.2 × 10e6 (4.3 × 10e6)	8.5 × 10e3 (8.9 × 10e3)	0.060
24 + 12.5 µM copper	3.7 × 10e6 (4.3 × 10e6)	1.2 × 10e4 (1.6 × 10e4)	0.008
24 + 6.25 µM copper	6.7 × 10e6 (4.3 × 10e6)	1.8 × 10e4 (2.1 × 10e4)	0.008
24 + 3.125 µM copper	1.2 × 10e6 (2.3 × 10e6)	8.6 × 10e3 (9.4 × 10e3)	0.010
24 + 1.5 µM copper	6.5 × 10e4 (1.2 × 10e5)	2 × 10e4 (2.5 × 10e4)	0.460
24 + 0 µM copper	2 × 10e6 (3.6 × 10e6)	7.3 × 10e5 (6.1 × 10e5)	0.400

CFU/ml, colony-forming units per ml; M, molar; SF, synovial fluid; SL, saline solution.

Table VI. Minimum bactericidal concentration of copper on *Staphylococcus aureus* USA300 CopAZB.

Time, hrs	Mean SF, CFU/ml (n = 8) (SD)	Mean SL, CFU/ml (n = 8) (SD)	p-value*
0 + 100 µM copper	1.9 × 10e6 (5.1 × 10e5)	2.1 × 10e6 (8.8 × 10e5)	0.150
0 + 50 µM copper	1.4 × 10e6 (6.9 × 10e5)	2.5 × 10e6 (8.7 × 10e5)	0.030
0 + 25 µM copper	2.1 × 10e6 (8.4 × 10e5)	2 × 10e6 (8.1 × 10e5)	0.960
0 + 12.5 µM copper	2 × 10e6 (6.5 × 10e5)	1.6 × 10e6 (5.5 × 10e5)	0.090
0 + 6.25 µM copper	1.3 × 10e6 (6 × 10e5)	1.7 × 10e6 (7.6 × 10e5)	0.600
0 + 3.125 µM copper	1.6 × 10e6 (7.4 × 10e5)	2.1 × 10e6 (9 × 10e5)	0.210
0 + 1.5 µM copper	1.8 × 10e6 (9.1 × 10e5)	2.1 × 10e6 (7 × 10e5)	0.560
0 + 0 µM copper	9.9 × 10e5 (8.5 × 10e5)	1.9 × 10e6 (6 × 10e5)	0.030
24 + 100 µM copper	6.7 × 10e4 (1.6 × 10e5)	0 (0)	< 0.001
24 + 50 µM copper	2.9 × 10e4 (3.1 × 10e4)	275 (778)	0.002
24 + 25 µM copper	1.1 × 10e5 (1.9 × 10e5)	13 (35)	< 0.001
24 + 12.5 µM copper	3.8 × 10e5 (6.6 × 10e5)	0 (0)	0.001
24 + 6.25 µM copper	3.8 × 10e5 (6.6 × 10e5)	313 (579)	0.005
24 + 3.125 µM copper	1.1 × 10e4 (1.6 × 10e4)	75 (139)	0.006
24 + 1.5 µM copper	4.6 × 10e4 (6.5 × 10e4)	8.8 × 10e3 (1.6 × 10e4)	0.050
24 + 0 µM copper	8.3 × 10e4 (1.8 × 10e5)	1.7 × 10e5 (2.7 × 10e5)	0.430

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; M, molar; SF, synovial fluid; SL, saline solution.

The addition of exogenous copper contributes to and increases the antimicrobial activity of SF against *S. aureus*. Due to the presence of ceruloplasmin as a copper-binding protein in SF and after demonstrating the bactericidal behaviour of SF against *S. aureus*, we then sought to analyze whether the addition of exogenous copper would contribute to bacterial killing.

Tables III and IV show the results of bacterial viability assays separated by *S. aureus* strains, while Figures 4 and 5 show the same data but as survival curves for each strain. Both control strains in saline solution and with exogenous copper significantly died after four hours. We observed a higher sensitivity to killing for UAMS-1 after 12 ($p = 0.003$) and 24 hours ($p < 0.001$) when compared to USA300 WT (Table III).

Similarly, adding exogenous copper to SF significantly killed both strains after four, 12, and 24 hours (Figure 5).

As expected, UAMS-1 was more sensitive and significantly died after four ($p = 0.001$), 12 ($p = 0.005$), and 24 ($p = 0.006$, all Mann-Whitney U test) hours when compared to USA300 WT (Table IV).

These data again demonstrate that SF from 12 different donors has a higher bactericidal effect against UAMS-1 compared to USA300 WT, and the addition of exogenous copper significantly increased bacterial killing of both strains.

The deficiency of CopAZB protein enhances SF bacterial killing with lower copper concentrations. We found that the USA300 WT was significantly sensitive to killing with higher copper concentrations in SF after 24 hours. On the other hand, the control strain in saline solution was

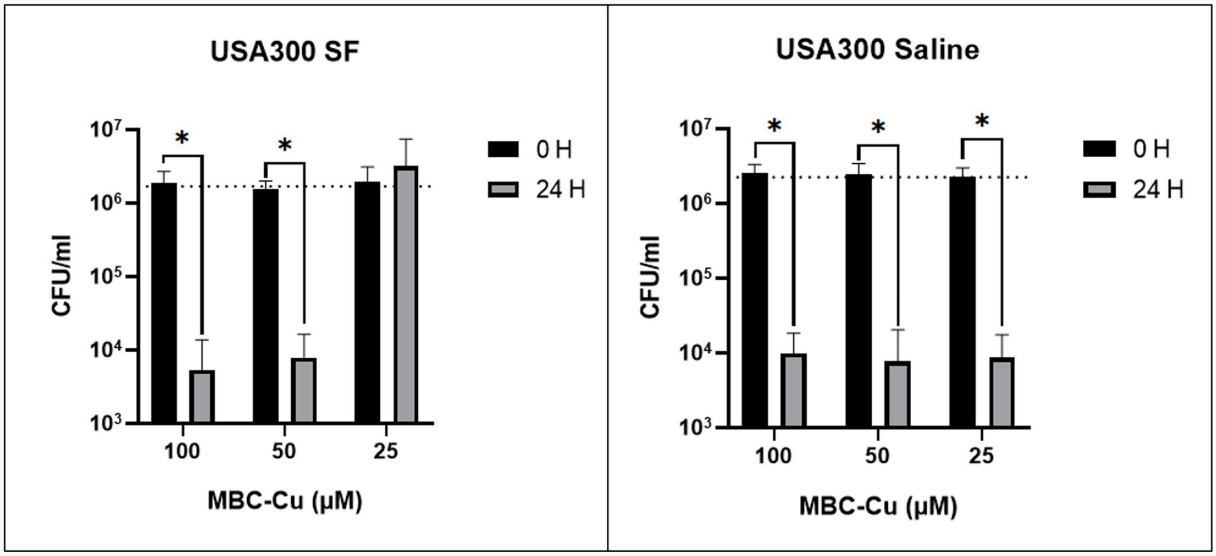

Fig. 6
Minimum bactericidal concentration of copper on *Staphylococcus aureus* USA300 wild type. Variables were considered statistically significant at *p < 0.05.

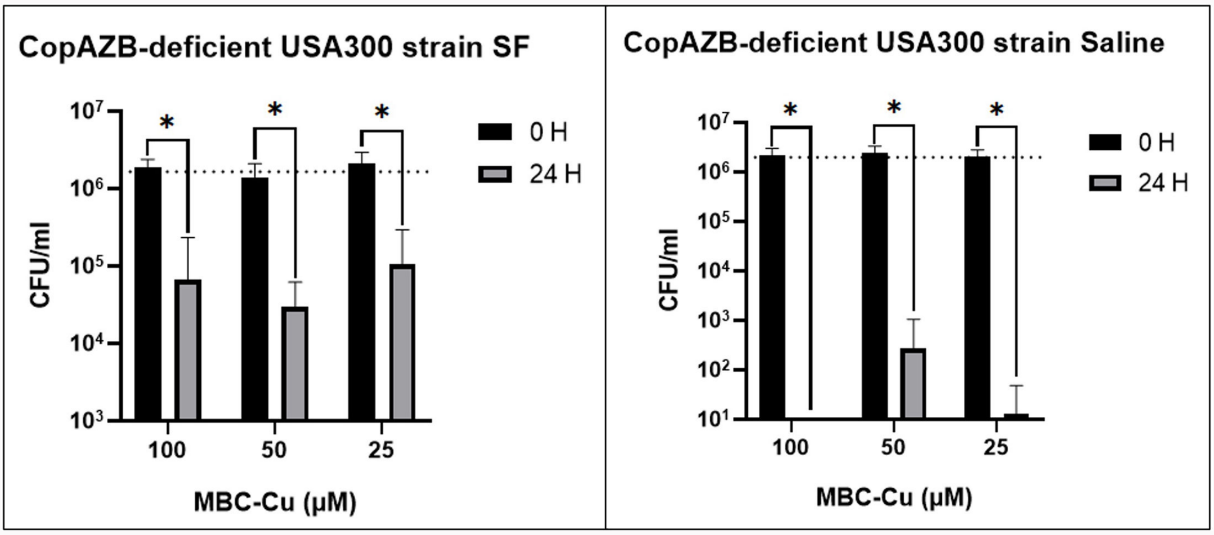

Fig. 7
Minimum bactericidal concentration of copper (MBC-Cu) on *Staphylococcus aureus* USA300 CopAZB. Variables were considered statistically significant at *p < 0.05. CFU,colony-forming units; SF, synovial fluid.

significantly sensitive to killing with any copper concentrations after 24 hours (Table V). Consequently, a 50 μM copper concentration was identified as the MBC-Cu (Figure 6).

Interestingly, we demonstrated that the USA300 CopAZB mutant strain was significantly sensitive to killing in SF with as little as 1.5 μM copper concentration or without adding it (0 μM) after 24 hours (Table VI). Moreover, the mutant strain significantly died even without copper (0 μM) at zero hours compared with the control group in saline solution (p = 0.030, Mann-Whitney U test) (Table VI).

These results highlight the importance of the CopAZB protein for bacterial survival, showing that the mutant strain cannot survive with small amounts of copper exposure. When analyzing the results in saline solution, we found a higher sensitivity to killing with any copper concentrations after 24 hours, being statistically significant (Figure 7 and Table VI). These data suggest that *S. aureus* requires CopAZB protein for resistance to killing when free or exogenous copper is available in SF; however, other proteins or defence mechanisms might play a role in resistance to the killing by SF.

Analysis of the acidic environment

The addition of exogenous copper is responsible for the increased antimicrobial activity of SF against *S. aureus*. As low-pH environments can reduce the survival of gram-positive pathogens, we then sought to analyze if the acidic environment could contribute to bacterial killing because of the addition of exogenous copper. For this purpose, additional samples of SF or saline were included with hydrochloric acid

Table VII. The effect of low-pH environment on *Staphylococcus aureus* viability in saline solution.

Time, hrs	Mean UAMS-1, CFU/ml (n = 4) (SD)	Mean USA300 WT, CFU/ml (n = 4) (SD)	p-value*
0 + copper	1.6 × 10e6 (1.4 × 10e6)	1.2 × 10e6 (1.6 × 10e6)	0.560
0 + HCl	1.5 × 10e6 (7.1 × 10e5)	2.1 × 10e6 (9.9 × 10e5)	0.240
0 -	1.8 × 10e6 (7.5 × 10e5)	2.3 × 10e6 (5.9 × 10e5)	0.380
24 + copper	1.3 × 10e3 (1.1 × 10e3)	1.4 × 10e4 (1.7 × 10e4)	1.000
24 + HCl	2 × 10e4 (1.1 × 10e4)	4.4 × 10e4 (1.8 × 10e4)	0.100
24 -	1.2 × 10e5 (1.7 × 10e5)	2.8 × 10e5 (3.3 × 10e5)	0.560

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; HCl, hydrochloric acid; WT, wild type.

Table VIII. The effect of low-pH environment on *Staphylococcus aureus* viability in synovial fluid.

Time, hrs	Mean UAMS-1, CFU/ml (n = 4) (SD)	Mean USA300 WT, CFU/ml (n = 4) (SD)	p-value*
0+ copper	2.8 × 10e6 (1.1 × 10e6)	1.65 × 10e6 (1.8 × 10e6)	0.380
0+ HCl	2.6 × 10e6 (1.6 × 10e6)	3.3 × 10e6 (7.9 × 10e5)	0.550
0 -	3.1 × 10e6 (7.1 × 10e5)	2.9 × 10e6 (1.8 × 10e5)	0.240
24+ copper	25 (29)	3.7 × 10e4 (2.3 × 10e4)	0.020
24+ HCl	1.4 × 10e6 (2.6 × 10e6)	5.3 × 10e6 (3.6 × 10e6)	0.240
24 -	1.1 × 10e6 (1.9 × 10e6)	5.8 × 10e6 (3.9 × 10e6)	0.240

*Mann-Whitney U test.
CFU/ml, colony-forming units per ml; HCl, hydrochloric acid; WT, wild type.

(HCl) to determine whether the acidic environment influences the killing of the bacteria.

As shown in Tables VII and VIII and Figures 8 and 9, adding copper was the critical factor for the increased bacterial killing in SF after 24 hours (p = 0.020, Mann-Whitney U test). Like the previous analysis, UAMS-1 showed a higher sensitivity to killing than USA300 WT.

SDS-PAGE and protein identification in synovial fluid

Two rounds of 12% SDS-PAGE were run using nine randomly diluted SF samples from different donors in each gel. The first SDS-PAGE was run for 90 minutes, and the nine SF samples ended the analysis successfully. The second SDS-PAGE was run for more than 90 minutes to identify proteins better. Still, unfortunately, one of the SF samples was deficient, and only eight samples ended the run correctly.

The gel demonstrated a heterogeneous amount of protein, around 122 kDa, compatible with the estimated size of ceruloplasmin in SF, as Watson et al[40] previously demonstrated (Figure 10). In this sense, ceruloplasmin would be responsible for the presence of copper in SF.

Discussion

The number of TJRs performed increases yearly; consequently, the number of revision surgeries is expected to grow as well.[5,6] PJI is known as one of the most challenging complications after TJR and has been reported as the leading cause of revision surgery,[7] with a prevalence between 1% and 2% among all arthroplasties. *S. aureus* is part of the human microbiota and is the most frequently isolated bacterial pathogen responsible for PJI after THA or TKA. It has a well-known ability to cause infections, plasticity to mutate, and develops highly resistant strains, resulting in a real threat to surgeons and the healthcare system. Although there is evidence of the bactericidal role of SF, the exact mechanism and the specific antimicrobial properties that might be implicated in killing *S. aureus* still need to be defined.[4] This study provides evidence that SF from hips and knees of osteoarthritic patients is bactericidal against *S. aureus*. Moreover, we have demonstrated that the addition of low copper concentrations exacerbates the bactericidal effect of SF and that *S. aureus* CopAZB protein-deficient strain, without the capacity to efflux copper ions from the cytoplasm, has increased sensitivity to SF and the lowest copper concentrations.

Regarding *S. aureus* survival analysis in SF, Watson et al[40] showed that SF from osteoarthritic patients has antimicrobial factors that restrict and kill *S. aureus*. Nonetheless, sensitivity variations exist, with the CA-MRSA strain LAC showing a high level of resistance. Similarly, we confirmed the heterogenous bactericidal activity of human SF against different *S. aureus* strains. UAMS-1 was highly sensitive and significantly died, whereas USA300 WT survived and even grew at the final endpoint, suggesting an increased resistance to SF activity.

It is challenging to confirm how SF can kill bacteria, partly because the precise SF composition of osteoarthritic joints is not widely known.[3] For example, as we already know, iron is an essential micronutrient required by most bacteria for crucial intracellular processes and their growth.[39,65–67] Nevertheless, free iron is almost non-existent in most host environments, as different iron-binding proteins like ferritin, transferrin, lactoferrin, and haemoglobin actively bind to iron and sequester most of it from the extracellular space, competing with bacteria.[65,68,69] On the other hand, bacteria, specifically *S. aureus,* can steal iron from the host iron-binding proteins by producing siderophores.[70,71] Watson et al[40] identified the presence of transferrin in SF of osteoarthritic knees. The study confirmed the capacity of SF to decrease iron availability for bacteria, resulting in a higher siderophore synthesis by *S. aureus*. They also demonstrated increased survival of *S. aureus* when adding free iron in SF, consistent

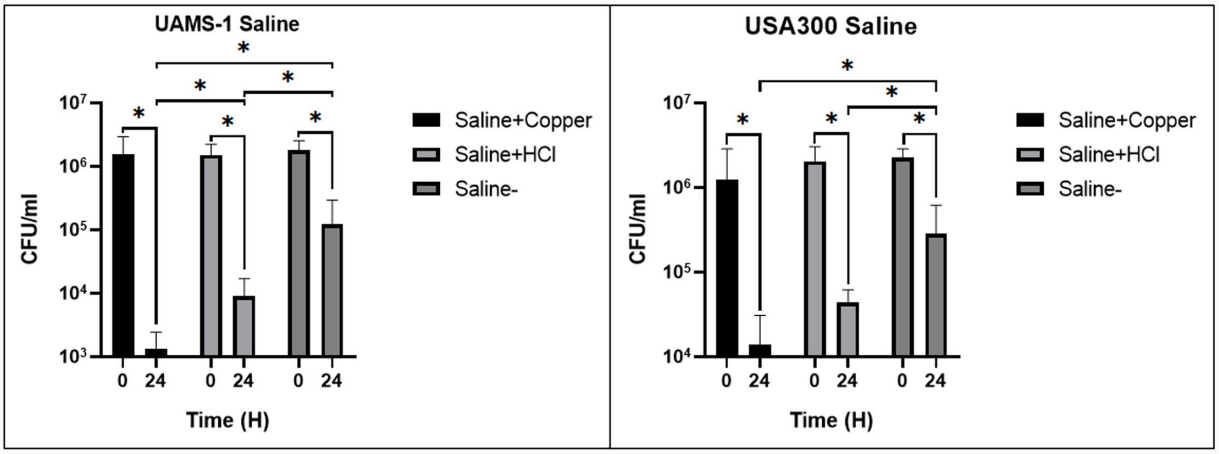

Fig. 8
The effect of low-pH environment on *Staphylococcus aureus* viability in saline solution. Variables were considered statistically significant at *p < 0.05. CFU, colony-forming units; HCl, hydrochloric acid.

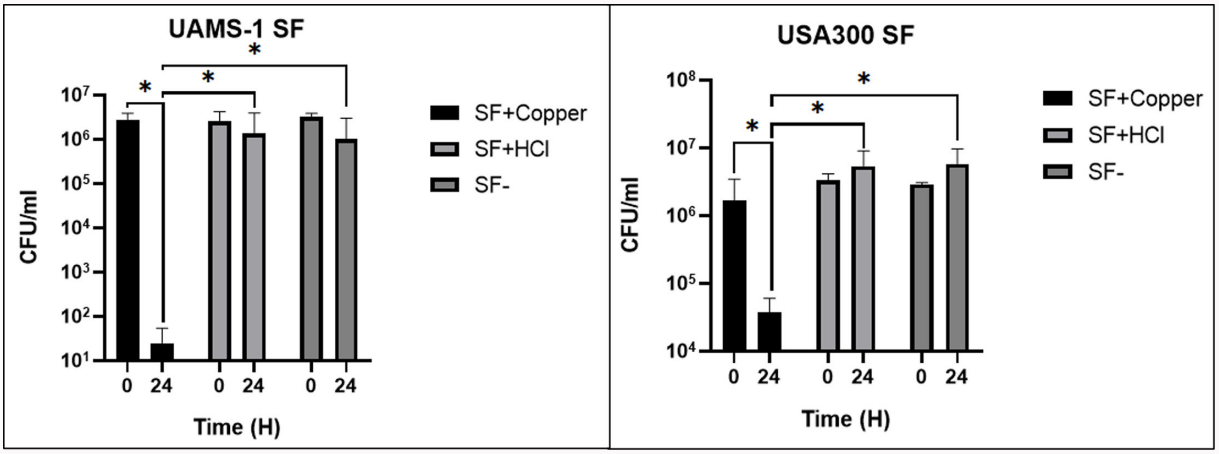

Fig. 9
The effect of low-pH environment on *Staphylococcus aureus* viability in synovial fluid (SF). Variables were considered statistically significant at *p < 0.05. HCl, hydrochloric acid.

with the concept that iron restriction decreases bacterial growth.

Many publications have already dealt with contact-killing kinetics upon bacteria exposure. Dry metallic copper surfaces seem more antimicrobial than moist ones, resulting in bacteria inactivation within a few minutes of exposure.[72,73] Touch surfaces frequently found in hospitals can be highly contaminated, and *S. aureus* can persist on such surfaces for months.[74] Systematic and efficient cleaning and proper hand hygiene decrease infections, but complete elimination seems impossible.[75] With the high prevalence of methicillin-reistant *S. aureus* (MRSA), nosocomial infections have become a primary concern for hospitals, and copper seems to be a promising alternative.[73] Thus, metallic copper surfaces would protect from microorganisms by reducing surface contamination.[51,76,77] Indeed, the antimicrobial properties of copper surfaces have been shown to reduce bacterial counts, indicating that copper surfaces are a reliable alternative to decrease the number and severity of hospital-acquired infections.[78–80]

During infections, microbes might have difficulty facing the bioavailability of copper and other essential and deleterious metals. In this sense, host environments take advantage of this challenging situation by using different strategies to starve pathogens of essential metals.[81,82] There is reduced availability of iron,[52,81,83] manganese, and zinc, which are also withheld at local sites of infection by high-affinity metal-binding proteins.[53,84] On the contrary, the exact opposite seems to be the case with copper concentrations. Instead of starvation, the host environment increases copper during infection to kill pathogens, with copper toxicity behaving as an antimicrobial agent.[85-88] In our study, we demonstrated that adding low copper concentrations increases the antimicrobial activity of SF against *S. aureus*. Although we showed a higher sensitivity to killing for UAMS-1 than for USA300 WT, interestingly, adding a working concentration of 10µM copper significantly killed both strains. This suggests that the addition of copper in SF, presumably via enhancing cytotoxic activity, increases the bactericidal activity of SF and, therefore, bacterial killing. Theoretically, copper that is free and also bound to ceruloplasmin might be insufficient to produce significant bacterial death. In this sense, the copper-additional environment may increase the bactericidal activity

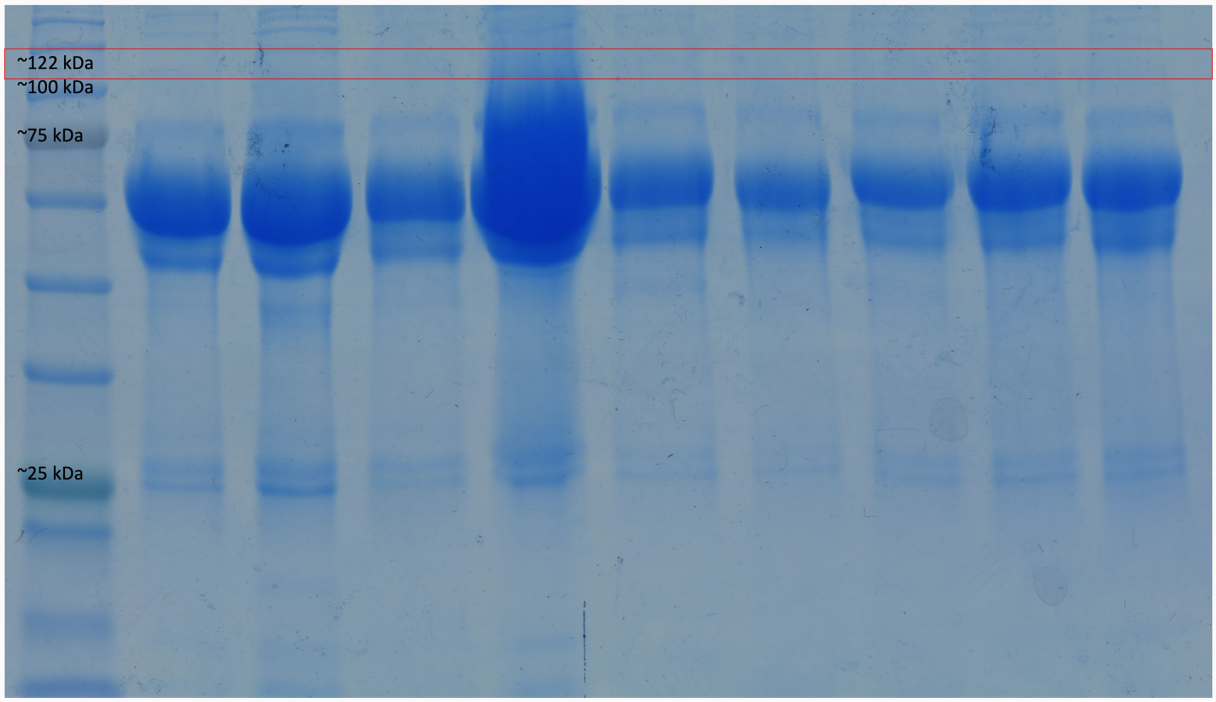

Fig. 10
Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identification in synovial fluid run for 90 minutes. Upon completion, the gel was removed from the dock and stained overnight with Instant Blue Coomassie Protein Stain. After staining the gel, we used an automated spot picker to identify the individual protein bands from the gel. The red box identifies the heterogeneous band in the different synovial fluid samples compatible with the estimated size of ceruloplasmin (122 kDa).

of SF, but other factors apart from the addition of copper would undoubtedly contribute to bacterial killing.

In our study, we only used a 10μM working concentration of copper, resulting in a significant killing of both strains. As we can see, the copper concentration used (10μM) was less than the normal range of total copper (13.3 to 28.3μM), but it was higher than the normal reference of free copper (0 to 1.6μM). As we know, a copper-binding protein such as ceruloplasmin is present in SF, and exogenous copper might bind to ceruloplasmin in SF. As a result, it is challenging to assess the real fraction of free copper and its theoretical toxic threshold. It would be vital to know the exact concentration of ceruloplasmin in SF, the possible free fraction of copper that might be available in SF, and how much of the added copper would be bonded to ceruloplasmin to realize the accurate working concentration of copper and avoid possible toxicity.

Different mutant microorganisms with CopA deficiency, such as *E. coli, Streptococcus pneumoniae*, and *Neisseria gonorrhoeae,* have been demonstrated to have inadequate copper efflux, intracellular accumulation, and increased copper sensitivity.[59–61] Our data on the differences in sensitivity between USA300 WT and CopAZB-deficient USA300 were significant. Regarding USA300 WT, we showed high sensitivity to higher copper concentrations in SF after 24hours, demonstrating a MBC-Cu of 50μM. On the other hand, USA300 CopAZB mutant strains were significantly sensitive to dying in SF with as little as 1,562 μM of copper after 24hours. We demonstrated that *S. aureus* needs CopAZB proteins to export the copper out of its cytoplasm to survive in SF. Indeed, we showed that CopAZB protein deficiency further enhanced sensitivity to SF and increased bacterial killing with low copper concentrations. Nevertheless, it is essential to mention that our analyses included only one microorganism, and the results should be interpreted as a study of a single microorganism in vitro.

As we know, SF may vary among humans, and its exact composition is yet to be defined. Ceruloplasmin is a serum ferroxidase responsible for more than 90% of copper transportation.[63] Also, it is known as an acute-phase reactant, and its concentration in plasma may increase during inflammation or infection.[89] Ceruloplasmin is a single polypeptide chain weighing 120 kDa to 132 kDa.[31,56] Our study demonstrated a heterogeneous amount of protein, around 122 kDa, compatible with the estimated size of ceruloplasmin in SF. It seems that ceruloplasmin concentration varies among different samples. Although it might be responsible for the presence of copper in SF, we could not confirm the exact concentration of ceruloplasmin, neither bonded copper nor free fraction.

Our study has potential limitations. Although we analyzed different *S. aureus* strains, this was an in vitro study in which the microorganisms had all the ideal conditions in which to grow. Additionally, despite including different *S. aureus* strains with various resistance levels, we did not include other prevalent bacteria like *S. epidermidis*, *E. coli*, *Streptococcus pneumoniae*, and *Neisseria gonorrhoea*. Our results should be interpreted as a study of a single microorganism in vitro. As we know, SF varies among humans, and its exact composition is unknown. We evidenced a heterogeneous amount of protein, around 122 kDa, compatible with ceruloplasmin, but we could not confirm the precise concentration of ceruloplasmin, bonded copper, or free fraction. It would be vital to know

the exact concentration of ceruloplasmin in SF, the possible free fraction of copper that might be available in SF, and how much of the added copper would be bonded to ceruloplasmin to realize the accurate working concentration of copper, its mechanism, and to avoid possible toxicity.

To conclude, this study highlights the vital importance of exogenous copper and the CopAZB proteins as possible antimicrobial tools against *S. aureus*; however, more evidence, including animal model studies, is required to further confirm our findings. Due to the prevalence of *S. aureus* PJIs, it is possible that treatment alternatives considering the use of exogenous copper or therapeutics targeting CopAZB protein would be effective in treating *S. aureus* infections. We demonstrated that 10μM exogenous copper effectively killed both *S. aureus* strains in different human SF samples. Therefore, our study supports using low copper concentrations as an alternative for treating *S. aureus* infections; however, more evidence is necessary to define the efficacy, safety, and toxicity level of copper in animal models before a possible application in humans. Finally, we showed that human SF from knees and hips of osteoarthritic patients demonstrates heterogenous bactericidal activity against different *S. aureus* strains, which require CopAZB protein and probably other related factors to efflux copper from the cytoplasm and resist SF destruction. Thus, further studies using exogenous copper and CopAZB protein are needed to investigate these possible therapeutic alternatives to decrease the incidence and improve treatment options for *S. aureus* infections.

Social media

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

Ethical review statement

This research was approved by the Western University Research Ethics Board and Lawson Research Institute. Informed consent was signed before patient enrolment. Informed consent was obtained from all individual participants included in the study.

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