Article

Not peer-reviewed version

Modulation of Staphylococcus aureus Biofilm Formation by Subinhibitory Concentrations of Biogenic Silver Nanoparticles and Simvastatin

Ana Carolina Furian Da Silva , Sindy Magri Roque , Marta Cristina Teixeira Duarte , Gerson Nakazato , Nelson Durán , Karina Cogo-Müller *

Posted Date: 25 October 2023

doi: 10.20944/preprints202310.1572.v1

Keywords: Staphylococcus areus; Statins; Biofilm; Antimicrobials; Silver nanoparticles

Article

Modulation of *Staphylococcus aureus* Biofilm Formation by Subinhibitory Concentrations of Biogenic Silver Nanoparticles and Simvastatin

Ana Carolina Furian da Silva [1], Sindy Magri Roque [1], Marta Cristina Teixeira Duarte [2], Gerson Nakazato [3], Nelson Durán [4] and Karina Cogo Müller [1,*]

[1] Laboratory of Pharmacology of Antimicrobials and Microbiology, School of Pharmaceutical Sciences, Universidade Estadual de Campinas (UNICAMP), Campinas, 13083-871, São Paulo, Brazil
[2] Laboratory of Microbiology, Pluridisciplinary Center for Chemical, Biological and Agricultural Research, Universidade Estadual de Campinas (UNICAMP), Campinas, 13148-218, São Paulo, Brazil
[3] Department of Microbiology, Center of Biological Sciences, University of Londrina (UEL), Londrina, 86057-970, Paraná, Brazil
[4] Laboratory of Urogenital Carcinogenesis and Immunotherapy, Universidade Estadual de Campinas (UNICAMP), Campinas, 13083-970, São Paulo, Brazil
* Correspondence: karinacm@unicamp.br

Abstract: *Staphylococcus aureus* is a causative agent of nosocomial infections and its antibiotic-resistant strains are concerning. Solutions are being explored to improve the treatment of these infections, including repositioning drugs such as statins and using nanoparticles with antimicrobial properties. This study aimed to evaluate the antimicrobial effects of simvastatin (SIM) and biologically synthesized silver nanoparticles (bio-AgNPs) in isolate form and in combination by assays of minimum inhibitory concentration (MIC), an *in vitro* biofilm model, and the association of antimicrobials against clinical strains of *S. aureus*. Bio-AgNPs showed a 53.8 ± 1.23-nm mean diameter and standard deviation, a 0.23 polydispersity index, and a −25.66 ± 2.19-mV mean potential and standard deviation. Transmission electron microscopy confirmed the formation of nanoparticles and the presence of Ag0 and AgCl. *S. aureus* strains were sensitive to bio-AgNPs and SIM, showing 31.88-187.5 and 74.66-149.32 μM concentrations, respectively. Our association assay showed 2.0 fractional inhibitory concentration indices (i.e., indifferent for clinical strains) and 0.32 values for the standard ATCC 29213 strain (synergy). Our biofilm inhibition assays with isolated SIM and bio-AgNPs showed decreased biofilm formation from 4× to ⅛ MICs, showing no synergism in association. These findings evince that simvastatin and bio-AgNPs at subinhibitory concentrations can serve as antimicrobial agents against *S. aureus* biofilm.

Keywords: *Staphylococcus aureus*; statins; biofilm; antimicrobials; silver nanoparticle

Introduction

Staphylococcus aureus is one of the most important bacteria agents in nosocomial infections, whose antibiotic-resistant or non-susceptible strains (including to vancomycin, daptomycin, and ceftaroline) cause increasing concern[1,2] Methicillin-resistant *Staphylococcus aureus* (MRSA) stand out among the most prevalent resistant strains. WHO 2020 global data showed a 24.9% average proportion of infection cases with MRSA.[3] Staphylococcus aureus can cause a range of infections, including skin and soft tissue infections[1,4] (including those in catheters and prosthetic devices), implant-associated infections, bacteremia, endocarditis, osteomyelitis, and pneumonia.[1]

MecA is a crucial gene that provides MRSA with the inherent ability to grow in the presence of penicillin-like antibiotics. This gene is present in all MRSA strains and codes for penicillin-binding protein 2a (PBP2a).[5] PBPs are enzymes located on the cell membrane and serve essential functions in microbial growth, cell division, and cell structure.[6] Further compounding the problem is the fact

 doi:10.20944/preprints202310.1572.v1

that MRSA has a high capacity to form biofilms on biotic and abiotic surfaces.[7–9] These biological communities are believed to account for nearly 80% of all human infections. Furthermore, one of their most significant attributes is their high resistance to antibiotics, disinfectants, host immune defenses, and environmental stress.[10,11]

Solving these problems requires searching and developing new antibacterial compounds. Metallic nanoparticles offer a possible antimicrobial agent. Many studies have shown that silver nanoparticles (AgNP) have a synergistic effect with other clinically used antibiotics and even other antimicrobial substances.[12,13] These associations between AgNP and antimicrobial drugs can direct the latter to specific targets and boost their effect by decreasing aquisition of resistance to antimicrobial.[12,14]

The great advantage of synthesizing AgNP is the possibility of producing them from plant extracts or microorganisms (green synthesis). This biogenic sourcing process is usually easy to perform, economically viable, secure, and scalable.[15] The fungus *Fusarium oxysporum* is one of the microorganisms that can produce silver nanoparticles. The methodology to produce them consists of adding silver nitrate ($AgNO_3$) to a fungal extract, which, by the action of reducing enzymes such as nitrate reductase, will reduce the silver and synthesize silver nanoparticles, attested by the color change to the extract to an yellowish-brown.[16,17]

Repositioning drugs that show antimicrobial activity as a side effect configures an alternative against antibiotic-resistant strains.[18] Drug associations can also produce synergism as some drugs tested under a monotherapy regimen fail to show significant antibacterial activity but are effective when associated with antibiotics, considerably reducing their dose.[19–21]

Statins are one of the most prescribed drugs in the world, with up to 200 million people worldwide using them daily.[22] These drugs are important lipid-lowering agents (exerting their effect by inhibiting the enzyme 3-hydroxy-3methyl-glutaryl-Coenzyme-A reductase — HMG-CoA), decreasing the synthesis of cholesterol and the low-density lipoproteins circulating in the body. They have a good margin of safety with a low frequency of side effects.[23]

Studies have evaluated the effects of statins (called pleiotropic effects) in addition to lowering cholesterol. We can highlight, for example, their antioxidant,[24] anticarcinogenic,[25] anticoagulant,[26] anti-inflammatory,[27] and immunomodulating effects. [28]

An effect that has gained increasing prominence is their antimicrobial activity, especially that of simvastatin (SIM), shown to have antimicrobial activity against *S. aureus* in both planktonic growth and biofilm.[29,30] Simvastatin can also reduce the formation of multispecies biofilms and treat oral infections.[31,32]

The association between AgNP and SIM has already been shown to be synergistic against standard and resistant strains of *S. aureus*.[17] In isolation, studies show that both compounds act against *S. aureus* biofilm.[30,33–35] However, no study has evaluated a possible synergistic interaction between these drugs in the form of biofilm against MRSA. The biofilm formation of *S. aureus* in the clinical environment mainly affects the surfaces of implanted catheters and medical devices, requiring the replacement of these infected medical devices by new ones and the use of oral antibiotics as treatment, drastically increasing cure costs and time (which may span up to six months or more).[36,37] Thus, this study aimed to evaluate the pharmacological interaction between simvastatin and AgNP in an *in vitro* biofilm model, suggesting a future alternative as an adjuvant treatment to *S. aureus* bacterial biofilm.

Materials and methods

Substances and Experimental Groups:

Bio-AgNP (3 mM) and SIM (EMS, Pharmaceutical Industry, São Paulo, Brazil) suspensions were used for our antimicrobial activity assays. The bio-AgNP suspension was diluted in sterile water and the SIM one, in DMSO (Dimethylsulfoxide). The highest tested SIM concentration (597.2 μM) with 2.5% DMSO, an atoxic concentration to bacterial cultures.

 doi:10.20944/preprints202310.1572.v1

Bio-AgNP Synthesis

The bio-AgNPs were synthesized according to the method in Durán et al. [16] (Patent, 2006, PI 0605681-4A2; http://www.inpi.gov.br). The bio-AgNPs were prepared by a silver nitrate reduction catalyzed by a cell-free enzyme preparation of *F. oxysporum* (strain 551) from the culture collection of the Molecular Genetics Laboratory (Universidade de São Paulo, ESALQ, Brazil).[16,38] The fungus was cultured in an agar medium containing 0.5% yeast extract (BD, Franklin Lakes, NJ, USA), 2% malt extract (BD), and 2% agar (BD) and incubated at 28°C for seven days. After the fungus grew, the produced biomass was added to sterile distilled water (0.1 g/mL) under agitation at 150 rpm (Agitator Tecnal, SP, Brazil) for 72 h. After vacuum filtration, 0.01 mol/L of silver nitrate ($AgNO_3$, Nuclear, SP, Brazil) was added to the supernatant (Filtrate fungal - FF) and protected from light.

Bio-AgNP Characterization

The hydrodynamic radius, zeta potential, and polydispersion index (PDI) of the bio-AgNPs were determined by dynamic light scattering using ZetaSizer NanoZS (Malvern Panalytical®, Malvern UK). Transmission electron microscopy (TEM) was performed using JEM-1400Plus (*Jeol®, Akishima*, Japan) to confirm AgNP morphology and size. Particle size was determined using dynamic light scattering (DLS).

Bio-AgNP and Simvastatin Antimicrobial Activity

The antimicrobial activity of bio-AgNPs alone or in combination with SIM was investigated by the microdillution method and determination of minimum inhibitory concentration (MIC) in *S. aureus* strains, an antimicrobial combination, and biofilm inhibition assays.

Bacteria and growing conditions

The following microorganism strains were used: methicillin-resistant *S. aureus* (MRSA) strains isolated from sputum samples (HC 3817719, 10106876, and 9120358); methicillin-susceptible *S. aureus* (MSSA) strains from blood cultures (HC 12092392, 985444, 909, 1734, 1744, and 1641); and standard *S. aureus* strains (ATCC 43300, 33591, 29213, and ATCC 6538). The strains were kindly provided by Professor Carlos Emilio Levy of the School of Medical Sciences, Department of Clinical Pathology, University of Campinas, Brazil. The cultures were stored in a tryptic soy broth medium (TSB-Difco Co., Detroit, MI, USA) with 20% glycerol (Sigma-Aldrich, San Luis, Missouri, USA) at −80°C and the bacteria were cultured in a tryptic soy agar medium (TSA, Difco Co., Detroit, MI, USA) and incubated in aerobiosis.

Minimum inhibitory concentration (MIC) assay

MIC was determined according to Clinical and Laboratory Standards Institute recommendations.[39] Serial dilutions (2×) of simvastatin (previously diluted in DMSO) and Bio-AgNP were prepared in 100 µL of Mueller Hinton broth medium (MHB, Difco Co., Detroit, MI, USA) using 96-well plates. Total concentrations ranged from 597.2 to 0.28 and from 750 to 0.36 µM for SIM and AgNP, respectively. The bacteria were reactivated and cultured in TSA for 24 h at 35°C. From the grown cultures, a microbial suspension was prepared and diluted until it reached 0.1 absorbance (turbidity equivalent to 0.5 in the McFarland scale) under a 660-nm wavelength, resulting in a concentration of 1×10^8 CFU/mL. From the 1×10^8 CFU/mL microbial suspension, a suspension with 1×10^6 CFU/mL was prepared using a reservoir with 9.9 mL of MHB medium and 100 µL of inoculum. Finally, 100 µL of the dilution were added to each plate well (5×10^5 UFC/well final concentration). The plates were incubated for 24 h at 35°C in aerobiosis and visually analyzed for turbidity, whereas absorbance was assessed in a spectrophotometer (ƛ = 660 nm). Moreover, 30 µL of the resazurin (Inlab, Diadema, São Paulo, Brazil) stain (0.01% solution in water) were added to each well, which were incubated for two hours and visually read to confirm the obtained results.

 doi:10.20944/preprints202310.1572.v1

Antimicrobial combination assay

A microdilution association method was used to evaluate the possible interaction between bio-AgNPs and simvastatin.[30,40] In it, dilutions with different concentrations of each substance were associated and their antimicrobial activity evaluated. For this, 96-well plates containing MHB medium were used. The used concentrations were the same as in the MIC assays. A 100-µL aliquot of the bacterial suspension in each well was used with a final 5×10^5 CFU/well concentration. The plates were incubated for 24 h at 35°C in aerobiosis and visually analyzed for turbidity, whereas absorbance was assessed in a spectrophotometer (ƛ = 660 nm). Finally, 30 µL of the resazurin stain (0.01% solution in water) were added to each well, which were incubated for two hours and visually read. Results were analyzed by their fractional inhibitory concentration index (FICI) values, calculated as follows: $\Sigma = FICI_A + FICI_B = MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$, in which the A and B MICs are those of the isolated substances and that of AB and BA, those of A and B combined. FICI values <0.5 represent synergism; those between 0.5 and 1.0, an additive effect; those between 1.0 and 4.0, indifference; and those >4.0, antagonism.[41]

Biofilm formation inhibition assay

Before this assay, the capacity of all bacterial strains to form biofilm was evaluated. The HC 3817719 (MRSA), 10106876 (MRSA), 9120358 (MRSA), 1734 (MSSA), 909 (MSSA), and 12092392 (MSSA) strains were found to form more biofilm and were thus chosen for our biofilm inhibition assay. For it, 96-well concave-bottomed plates were used and concentrations from 4× to ⅛ MIC were chosen as our study simvastatin and Bio-AgNP range. A bacterial suspension was prepared and diluted until it reached 0.100 absorbance (660-nm wavelength). Then, 100 µL of the previously diluted suspension (50 µL of suspension in 10 ml of TSB medium supplemented with 1% glucose (Labsynth, Diadema, São Paulo, Brazil) and 100 µL of the culture medium were added to each well. After 24 h of incubation at 35°C, the plates were washed with deionized distilled water to remove dead or non-adherent cells. The wells were allowed to dry at room temperature and optical density measurements (540nm) were performed to quantify the biofilm formed in each well after the addition of 0.4% violet crystal solution and 100% ethanol.[42]

Statistical Analyses

Data distribution was verified by the Shapiro-Wilk test and homoscedasticity, by Levene's test. Data were compared with the control group by analysis of variance (ANOVA) and Tukey's post-test and analyzed and graphs were generated based on them using Bioestat 5.0 (Mamirauá, Belém, Brazil) and GraphPad Prism 8.0 (San Diego, USA). A 0.05% significance level was established.

Results

Bio-AgNP Characterization

We evaluated nanoparticle diameter by DLS. Bio-AgNPs showed a 53.8 ± 1.23-nm mean diameter and standard deviation, a 0.23 polydispersity index, and a −25.66 ± -2.19 mV mean potential and standard deviation. Thus, the nanoparticles showed dispersion and homogeneous distributions.

Figure 1 represents the nanoparticles we evaluated by transmission electron microscopy (TEM). We found spherical and homogeneous nanoparticles dispersed in the suspension.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 October 2023 doi:10.20944/preprints202310.1572.v1

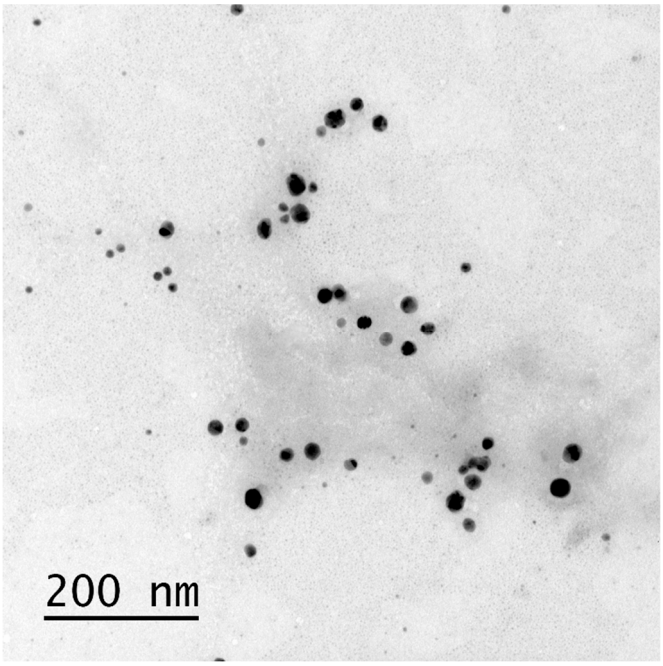

Figure 1. Characterization by transmission electron microscopy (TEM) of bio-AgNP suspensions synthesized by F. oxysporum and deposited in a copper sample port coated with parlodium film at 200nm.

Minimum inhibitory concentration (MIC) and antimicrobial combination (FICI) assays

SIM showed inhibitory activity at low concentrations in all nine clinical strains. For most strains, MICs values ranged from 74.66 to 149.32 μM (Table 1). Bio-AgNPs showed inhibitory activity at concentrations above SIM, from 31.88 to 187.5 μM (Table 1). Both DMSO and water (used as solvents) failed to interfere with antimicrobial activity.

In association, bio-AgNPs and simvastatin showed equal MIC values to the isolated compounds, resulting in a 2.0 FICI indifferent association (Table 1).

Table 1. Minimum inhibitory concentration (MIC), antimicrobial association, and fractionated inhibitory concentration (FICI) for S. aureus strains. Concentrations expressed in μM.

Strains	MIC		Association MIC	FICI index
	SIM (μM)	Bio-AgNP (μM)	SIM + Bio-AgNP	
S. aureus ATCC 29213	74.66	31.88	74.66 + 31.88	0.32
S. aureus ATCC 43300	149.32	63.75	149.32 + 63.75	2.0
S. aureus ATCC 33591	149.32	31.88	149.32 + 31.88	2.0
S. aureus ATCC 6538	74.66	31.88	74.66 + 31.88	2.0
S. aureus HC 3817719 (MRSA)	74.66	187.5	74.66 + 187.5	2.0
S. aureus HC 10106876 (MRSA)	74.66	187.5	74.66 + 187.5	2.0
S. aureus HC 9120358 (MRSA)	74.66	187.5	74.66 + 187.5	2.0
S. aureus HC 12092392 (MSSA)	74.66	93.75	74.66 + 93.7	2.0
S. aureus HC 985444 (MSSA)	74.66	93.75	74.66 + 93.75	2.0
S. aureus 909 (MSSA)	149.32	187.5	149.32 + 187.5	2.0
S. aureus 1734 (MSSA)	74.66	93.75	74.66 + 93.75	2.0
S. aureus 1744 (MSSA)	74.66	187.5	74.66 + 187.5	2.0
S. aureus 1641 (MSSA)	74.66	187.5	74.66 + 187.5	2.0

Biofilm formation inhibition assay

This assay tested the ability of SIM and bio-AgNPs (alone and in association) to inhibit biofilm formation at concentrations ranging from 4× to ⅛MIC, i.e., from 9.31 to 597.2 and from 11.7 to 750 μM for simvastatin and AgNP, respectively. Figures 2–4 show the optical density readings of biofilm

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 October 2023 doi:10.20944/preprints202310.1572.v1

formation in 96-well plates stained with violet crystal after 24 h of formation for the ATCC 29213, MSSA, and MRSA strains, respectively. In general, we found that SIM and bio-AgNPs inhibited biofilm formation from 4× to ⅛ MIC concentrations for almost all strains ($p<0.05$, ANOVA, Tukey) in comparison with the control group. Results for isolated AgNP evince that the MRSA HC 3817719 strain showed a slight increase in bacterial growth in relation to control, with statistical differences at the ¼ and ⅛ MIC concentrations. We observed the same profile for the HC 9120358 strain at the ⅛ MIC concentration, although statistically indifferent.

The association of bio-AgNP and SIM also inhibited the biofilm with a profile similar to that for isolated substances, showing inhibition at concentrations from 4× to ⅛ MIC. When in association, the 2×MIC concentration for the MSSA HC 909 strain showed a higher growth profile than the control, but without statistical differences. For the MRSA HC 3817719 strain, the ⅛ MIC concentration showed a statistical difference ($p>0.05$, ANOVA, Tukey).

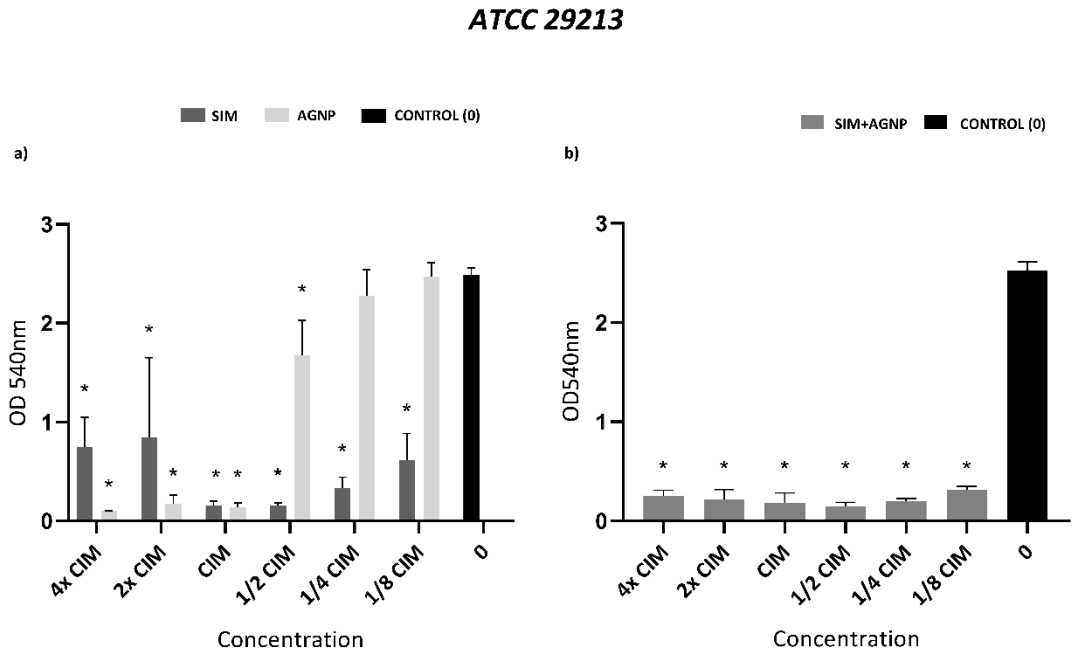

Figure 2. Graph a) represents biofilm inhibition for SIM and bio-AgNP and graph b) represents biofilm inhibition by association at concentrations from 4× to ⅛ MIC for the standard S. aureus strain after 24 h. The expressed values refer to the mean and standard deviation of the absorbance readings (OD 540 nm). * indicates statistical differences from the control. (ANOVA, 2 criteria, Tukey). Column "0" indicates the growth of the standard ATCC 29213 strain.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 October 2023 doi:10.20944/preprints202310.1572.v1

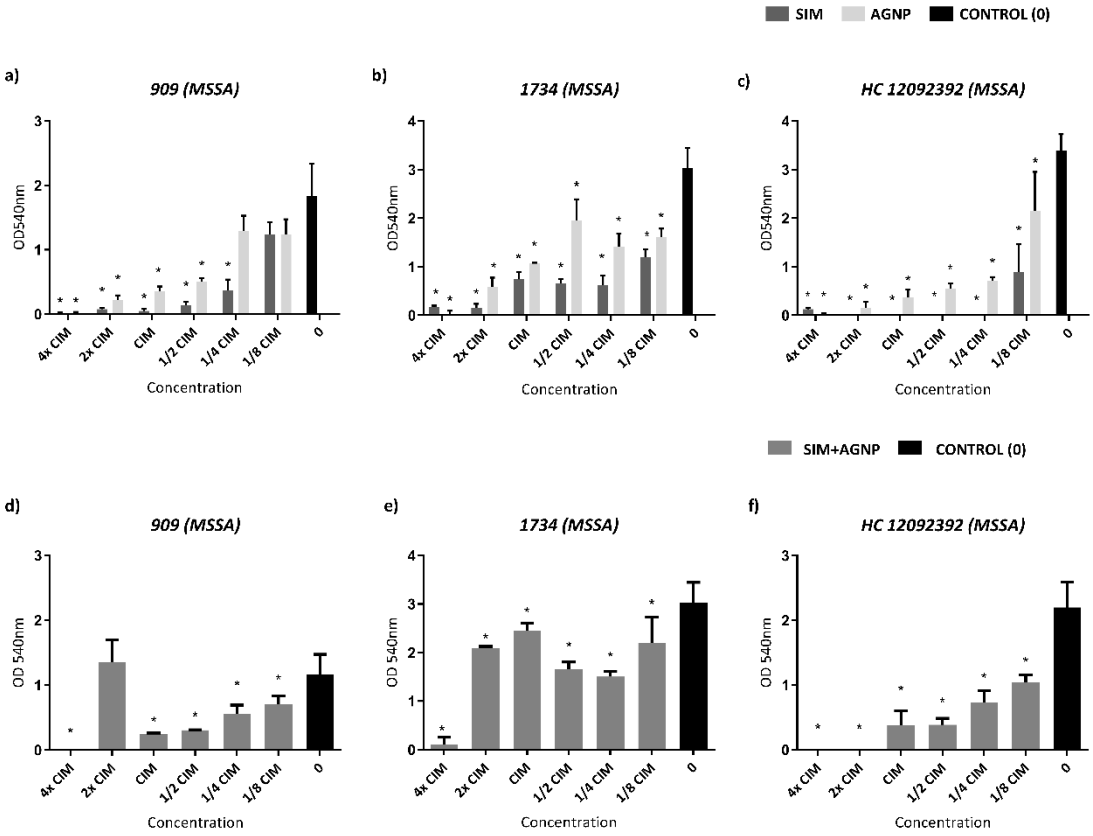

Figure 3. Graphs a), b), and c) represent biofilm inhibition for SIM and bio-AgNP at concentrations from 4× to ⅛MIC for methicillin-sensitive S. aureus strains (MSSA) after 24 h. Graphs d), e), and f) represent biofilm inhibition by association for MSSA after 24 hours. Values expressed are the mean and standard deviation of the absorbance readings (OD 540 nm). * indicates statistical differences from the control. (ANOVA, 2 criteria, Tukey). Column "0" indicates the growth of the standard ATCC 29213 strain.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 October 2023 doi:10.20944/preprints202310.1572.v1

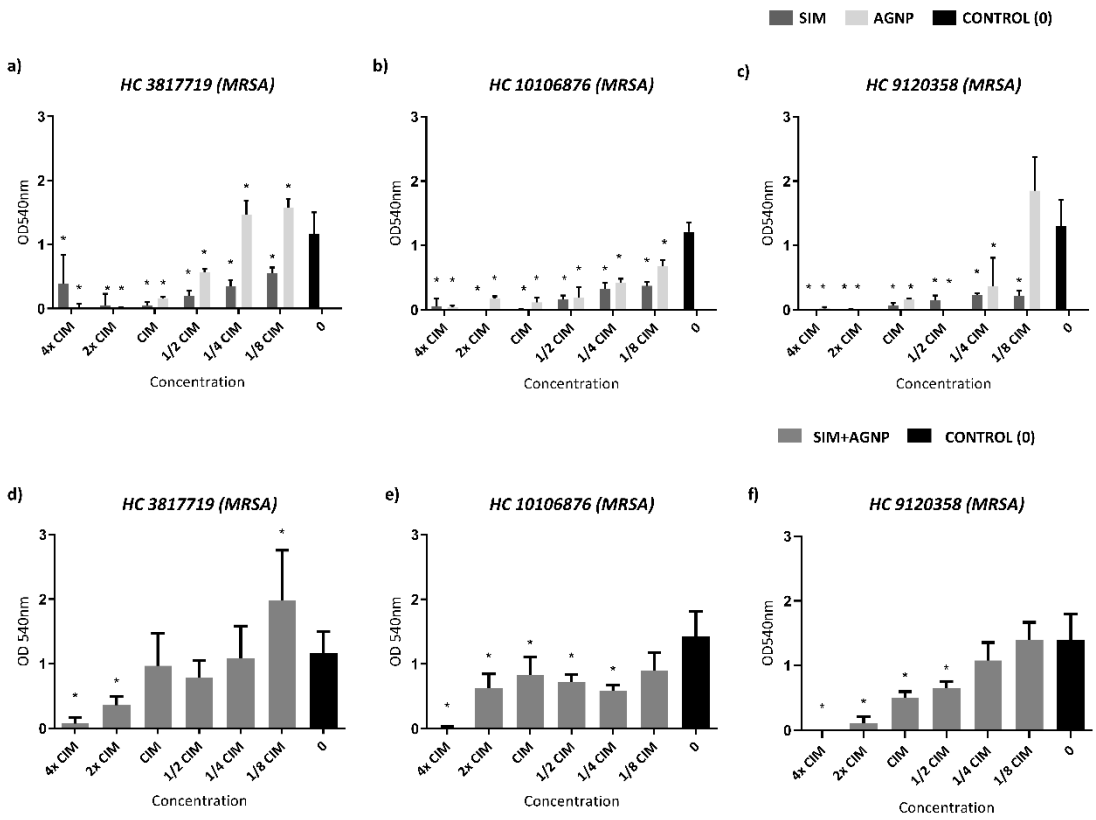

Figure 4. Graphs a), b), and c) represent biofilm inhibition for SIM and bio-AgNP at concentrations from 4× to ⅛MIC for methicillin-resistant S. aureus strains (MRSA) after 24 h. Graphs d), e), and f) represent biofilm inhibition by association for MRSA after 24 hours. Values expressed are the mean and standard deviation of the absorbance readings (OD 540 nm). * indicates statistical differences from the control. (ANOVA, 2 criteria, Tukey). Column "0" indicates the growth of the standard ATCC 29213 strain.

Discussion

Staphylococcus aureus is a gram positive bacterium often found in nosocomial infections that may resist antibiotics and complicate treatment.[1,2] Drug repositioning may configure a promising approach to treating infections, especially in the face of growing microbial resistance.[18] Statins (especially simvastatin) have been studied for their antimicrobial potential, especially against *S. aureus*.[30] Moreover, the use of silver nanoparticles as a topical agent in wounds or medical materials has been investigated to control on *S. aureus* biofilm.[31,32] This study produced and characterized bio-AgNPs, testing them in isolation and in association with simvastatin against clinical strains of *S. aureus*. Both substances showed antimicrobial activity at subinhibitory concentrations, reducing *S. aureus* biofilm formation. However, when associated, they showed synergism only for the standard *S. aureus* strain, with no change in activity against clinical MRSA and MSSA strains.

We prepared our silver nanoparticles by biologically obtaining metallic nanoparticles from fungi,[16] which have greater advantages over chemical and physical methods. Fungi are easy to grow, can occupy large surface areas, and facilitate nanoparticle synthesis due to their easy biomass manipulation.[43,44] Fungal mycelia exhibit a superior ability to withstand high-flow pressure, agitation, and other challenging conditions encountered in bioreactions when compared to other microbes and plants.[45] Furthermore, they possess the remarkable capability of cost-effective large-scale synthesis, requiring only a minimal quantity of biomass.[46] Moreover, they neither harm the environment nor require toxic chemicals or radiation.[15] The obtained bio-AgNPs had adequate size (below 100 nm) and good polydispersity and zeta potential indices, as observed in previous studies using biogenic production.[16] The diameter we observed in our transmission electron microscopy

images showed smaller particles according to our distribution histogram (5–30 nm). This stems from the fact that the DLS measures hydrodynamic radii, i.e., it considers particles and the stabilization layers around them — the protein layer from the synthesis of the fungal filtrate.[16] Bio-AgNPs showed a −25.66 ± 2.19-mV zeta potential, corroborating Raj et al. [47] A negative zeta potential indicates the predominance of particles with surface electric charges, contributing to the repulsion between nanoparticles, reducing aggregation, and increasing their stability.[48]

Both bio-AgNPs and simvastatin have shown antimicrobial activity against opportunistic pathogens and oral cavity bacteria. [22,30,49] Assays of SIM and bio-AgNP minimum inhibitory concentration showed good antimicrobial activity, inhibiting the bacterial growth of all *S. aureus* strains in concentrations from 74.66 to 37.27 μM (SIM) and from 187.5 to 93.75 μM (bio-AgNP). Our research group previously found similar MIC values for SIM [30]. For the bio-AgNPs tested against *S. aureus*, we found MIC values in the same range as in this study.[17,30,50] When tested against clinical strains of *Pseudomonas aeruginosa,* bio-AgNPs showed an inhibitory concentration comparable to the one in this study (62.5 μM).[51]

According to FICI, association trials showed no synergistic effect against the clinical strains of *S. aureus*, unlike that for the ATCC 29213 strain, which resulted in a synergistic effect. Figueiredo et al. found the same synergistic result against a standard (ATCC 25923) and an MRSA strain (MRSA N315).[17] Thus, for standard *S. aureus* strains, the association of bio-AgNP and SIM can promote a synergistic effect — rare for clinical strains isolated from patients. Therefore, the association of these substances for clinical use may fail to offer additional benefits when compared to isolated products.

Other studies have found synergism between antibiotics and silver nanoparticles. Habash et al., found that AgNP coated with 10-and 20-nm citrate boosted the effect of tobramycin in both planktonic cultures and *P. aeruginosa* strain biofilm.[52] AgNP averaging 45 nm in size (from the aqueous extract of *Zea mays* leaf residues) showed synergistic activity against *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *S. aureus*, and *Salmonella typhimurium* when associated with antibiotics such as kanamycin and rifampicin B.[13] Based on these findings, our research group conducted studies associating simvastatin and silver nanoparticles. We then found synergism in standard strains but not in clinically isolated strains, as described in this study.

When testing the ability of isolated substances to inhibit biofilm formation, in general, concentrations below the MIC, i.e., ranging from 4× to ⅛MIC for SIM and bio-AgNP, showed no inhibition. For HC 3817719 (a resistant strain), we obtained a biofilm growth increase greater than the control in ⅛ and ¼ MIC concentrations (isolated bio-AgNPs) and in the ⅛ concentration (association). Other studies have shown increased biofilm in subinhibitory bio-AgNP concentrations, when compared to other bacterial genera.[51,53] The increase can be attributed to AgNPs inducing the generation of reactive oxygen species (ROS), resulting in oxidative stress[54]. ROS can cause detrimental alterations to cellular components and harm proteins, DNA, and lipids[55]. The heightened oxidative stress likely applied selective pressure during the initial stages of biofilm development, augmenting its capacity for formation.[53] Substance association showed no superior effect for biofilm inhibition than isolated substances. Using a standard strain of *S. aureus*, we observed that, despite its synergy, the association of SIM and bio-AgNP failed to increase its antibiofilm effect when compared to isolated substances. In the biofilm of different species of *Arpergillus*, inhibition by associating bio-AgNP and simvastatin at concentrations from 2× to 8× MIC also resembled isolated substances.[56] This study found a synergistic effect between bio-AgNP and SIM when tested in fungi in its association assay. So, despite the synergism between substances in association assays, when tested on biofilm, the inhibition profile of the association remained similar to that of isolated substances.[56]

AgNPs-based medical devices have been greatly explored, such as different-type catheters (glass, plastic, polyurethane), for their effectiveness in antibacterial and antibiofilm applications.[57] Plastic and polyurethane catheters implanted with AgNPs significantly reduced infection rates and prevented multispecies biofilm formation. [58–60] A study on hemodialysis catheters found that using polyurethane catheters for vascular access and blood filtration causes infections that often lead to patients' death, but photochemically depositing AgNPs at the infection site inhibited bacterial

 doi:10.20944/preprints202310.1572.v1

growth. The authors showed that the developed surface coating can produce safe, cost-effective catheters with low infection rates.[61]

The toxicity of silver ions is questionable, but some studies have shown that incorporating them into nanoparticles decreases their cytotoxicity. Using cell culture assays, bio-AgNP showed no cytotoxicity against immune cells (T, B, and NK cells) from three to 72 h of exposure.[62,63] In contact with mouse (Balb/c) skin fibroblasts, exposure to bio-AgNP preserved cellular structures, a result seen on TEM images of IC20 nontoxic concentrations (91.77 μg/mL).[64,65]

Thus, based on our data, we conclude that the association of SIM and bio-AgNP performs no better than isolated substances in clinical strains of *S. aureus.* Clinically, the association may be interesting to treat or prevent biofilm formation/infections from more than one bacterial species since their sensitivity to each substance may differ, thus increasing their spectrum of action. For this, further studies with other bacterial species should be conducted to evaluate the possible interaction between these substances and the relevance of their associated clinical use.

Conclusion

Our findings evince that SIM and bio-AgNP have antimicrobial activity at subinhibitory concentrations and may be used in hospitals (impregnated in materials and to treat wound and burn infections). Although not synergistic, these compounds maintained antimicrobial activity when associated in suspension and biofilm. Further studies are needed to evaluate the clinical relevance and mechanisms of antimicrobial action of the association of these substances.

Authors' Contributions: Conceptualization K.C.M. and N.D.; methodology K.C.M., N.D., A.C.F.S. and S.M.R; software K.C.M., A.C.F.S., S.M.R; M.C.T.D., validation A.C.F.S. and S.M.R; investigation K.C.M., A.C.F.S. and S.M.R.; resources K.C.M., N.D. and M.C.T.D.; data curation K.C.M., A.C.F.S. and S.M.R; writing—original draft preparation, K.C.M., A.C.F.S. and S.M.R; writing—review and editing K.C.M., N.D., M.C.T.D., A.C.F.S., S.M.R., G.N; visualization, K.C.M., N.D., M.C.T.D., A.C.F.S., S.M.R., G.N; supervision K.C.M.; project administration, K.C.M and A.C.F.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study received a grant and a Research Grant from the São Paulo Research Foundation (Process no.: 2018/21769-4 and 2018/20593-0).

Ethical Approval and Consent to Participate: Not applicable.

Consent to Publication: Not applicable

Data Availability Statement: Data is contained within the article.

Acknowledgements: We would like to thank Professor Carlos Emilio Levy for providing us with bacterial samples and FAPESP (2018/21769-4 and 2018/20593-0) for its financial support and Espaço da Escrita – Pró-Reitoria de Pesquisa – UNICAMP – for the language services provided.

Conflict of Interests: The authors declare no conflict of interest.

Abbreviations

MRSA	methicillin-resistant *Staphylococcus aureus*
Bio-AgNP	Biologically synthesized silver nanoparticles
AgNP	Silver nanoparticles
EDS	Energy Dispersive X-ray Spectroscopy
TEM	Transmission Electron Microscopy
SIM	Simvastatin
FICI	Fractional Inhibitory Concentration Index
WHO	World Health Organization
PBP2a	Penicillin-binding protein 2a
MRSA	Methicillin-resistant *Staphylococcus aureus*
HMG-CoA	3-hydroxy-3-methylglutaryl-CoenzymeA reductase
LDL	Low density lipoprotein
DMSO	Dimethylsulfoxide

DLS	Dynamic light scattering
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth
MIC	Minimum Inhibitory Concentration
CLSI	Clinical and Laboratory Standards Institute
MHB	Mueller Hinton Broth
OD	Optical density
TEM	Transmission Electron Microscopy
JCPDS	Joint Committee on Powder Diffraction Standards
XRD	X-ray Diffraction Spectroscopy
ATCC	American Type Culture Collection

References

1. Hassoun, A.; Linden, P.K.; Friedman, B. Incidence, Prevalence, and Management of MRSA Bacteremia across Patient Populations—a Review of Recent Developments in MRSA Management and Treatment. *Crit Care* **2017**, *21*, 211, doi:10.1186/s13054-017-1801-3.
2. Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D.L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y.; et al. Discovery, Research, and Development of New Antibiotics: The WHO Priority List of Antibiotic-Resistant Bacteria and Tuberculosis. *Lancet Infect Dis* **2018**, *18*, 318–327, doi:10.1016/S1473-3099(17)30753-3.
3. OMS, W.H.O. *Global Report on Infection Prevention and Control*; 2022; ISBN 9789240051164.
4. Lee, A.S.; de Lencastre, H.; Garau, J.; Kluytmans, J.; Malhotra-Kumar, S.; Peschel, A.; Harbarth, S. Methicillin-Resistant Staphylococcus Aureus. *Nat Rev Dis Primers* **2018**, *4*, 18033, doi:10.1038/nrdp.2018.33.
5. Fishovitz, J.; Hermoso, J.A.; Chang, M.; Mobashery, S. Penicillin-Binding Protein 2a of Methicillin-Resistant Staphylococcus Aureus</i>. *IUBMB Life* **2014**, *66*, 572–577, doi:10.1002/iub.1289.
6. Kong, K.-F.; Schneper, L.; Mathee, K. Beta-Lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. *APMIS* **2010**, *118*, 1–36, doi:10.1111/j.1600-0463.2009.02563.x.
7. Abd El-Hamid, M.I.; Y. El-Naenaeey, E.; M kandeel, T.; Hegazy, W.A.H.; Mosbah, R.A.; Nassar, M.S.; Bakhrebah, M.A.; Abdulaal, W.H.; Alhakamy, N.A.; Bendary, M.M. Promising Antibiofilm Agents: Recent Breakthrough against Biofilm Producing Methicillin-Resistant Staphylococcus Aureus. *Antibiotics* **2020**, *9*, 667, doi:10.3390/antibiotics9100667.
8. Cascioferro, S.; Carbone, D.; Parrino, B.; Pecoraro, C.; Giovannetti, E.; Cirrincione, G.; Diana, P. Therapeutic Strategies To Counteract Antibiotic Resistance in MRSA Biofilm-Associated Infections. *ChemMedChem* **2021**, *16*, 65–80, doi:10.1002/cmdc.202000677.
9. Silva, V.; Almeida, L.; Gaio, V.; Cerca, N.; Manageiro, V.; Caniça, M.; Capelo, J.L.; Igrejas, G.; Poeta, P. Biofilm Formation of Multidrug-Resistant MRSA Strains Isolated from Different Types of Human Infections. *Pathogens* **2021**, *10*, 970, doi:10.3390/pathogens10080970.
10. Lebeaux, D.; Chauhan, A.; Rendueles, O.; Beloin, C. From in Vitro to in Vivo Models of Bacterial Biofilm-Related Infections. *Pathogens* **2013**, *2*, 288–356, doi:10.3390/pathogens2020288.
11. Penesyan, A.; Gillings, M.; Paulsen, I. Antibiotic Discovery: Combatting Bacterial Resistance in Cells and in Biofilm Communities. *Molecules* **2015**, *20*, 5286–5298, doi:10.3390/molecules20045286.
12. Fayaz, A.M.; Balaji, K.; Girilal, M.; Yadav, R.; Kalaichelvan, P.T.; Venketesan, R. Biogenic Synthesis of Silver Nanoparticles and Their Synergistic Effect with Antibiotics: A Study against Gram-Positive and Gram-Negative Bacteria. *Nanomedicine* **2010**, *6*, 103–109, doi:10.1016/j.nano.2009.04.006.
13. Patra, J.K.; Baek, K.-H. Antibacterial Activity and Synergistic Antibacterial Potential of Biosynthesized Silver Nanoparticles against Foodborne Pathogenic Bacteria along with Its Anticandidal and Antioxidant Effects. *Front Microbiol* **2017**, *08*, doi:10.3389/fmicb.2017.00167.
14. Gimenez, I.F.; Anazetti, M.C.; Melo, P.S.; Haun, M.; De Azevedo, M.M.M.; Durán, N.; Alves, O.L. Cytotoxicity on V79 and HL60 Cell Lines by Thiolated-<I>B</I>-Cyclodextrin-Au/Violacein Nanoparticles. *J Biomed Nanotechnol* **2005**, *1*, 352–358, doi:10.1166/jbn.2005.041.
15. Siddiqi, K.S.; Husen, A.; Rao, R.A.K. A Review on Biosynthesis of Silver Nanoparticles and Their Biocidal Properties. *J Nanobiotechnology* **2018**, *16*, 14, doi:10.1186/s12951-018-0334-5.
16. Durán, N.; Marcato, P.D.; Alves, O.L.; De Souza, G.I.; Esposito, E. Mechanistic Aspects of Biosynthesis of Silver Nanoparticles by Several Fusarium Oxysporum Strains. *J Nanobiotechnology* **2005**, *3*, 8, doi:10.1186/1477-3155-3-8.
17. Figueiredo, E.; Ribeiro, J.; Nishio, E.; Scandorieiro, S.; Costa, A.; Cardozo, V.; de Oliveira, A.; Durán, N.; Panagio, L.; Kobayashi, R.; et al. New Approach For Simvastatin As An Antibacterial: Synergistic Effect With Bio-Synthesized Silver Nanoparticles Against Multidrug-Resistant Bacteria</P>. *Int J Nanomedicine* **2019**, *Volume 14*, 7975–7985, doi:10.2147/IJN.S211756.

18. Foletto, V.S.; da Rosa, T.F.; Serafin, M.B.; Bottega, A.; Hörner, R. Repositioning of Non-Antibiotic Drugs as an Alternative to Microbial Resistance: A Systematic Review. *Int J Antimicrob Agents* **2021**, *58*, 106380, doi:10.1016/j.ijantimicag.2021.106380.
19. Annamanedi, M.; Varma, G.Y.N.; Anuradha, K.; Kalle, A.M. Celecoxib Enhances the Efficacy of Low-Dose Antibiotic Treatment against Polymicrobial Sepsis in Mice and Clinical Isolates of ESKAPE Pathogens. *Front Microbiol* **2017**, *8*, doi:10.3389/fmicb.2017.00805.
20. de S. Machado, C.; da Rosa, T.F.; Serafin, M.B.; Bottega, A.; Coelho, S.S.; Foletto, V.S.; Rampelotto, R.F.; Lorenzoni, V.V.; de L. Marion, S.; Hörner, R. In Vitro Evaluation of the Antibacterial Activity of Amitriptyline and Its Synergistic Effect with Ciprofloxacin, Sulfamethoxazole–Trimethoprim, and Colistin as an Alternative in Drug Repositioning. *Medicinal Chemistry Research* **2020**, *29*, 166–177, doi:10.1007/s00044-019-02470-x.
21. Sankar, S.; Thangamalai, R.; Padmanaban, S.; Kannan, P.; R, S.M.; S, A.C. In-Vitro Synergistic Antibacterial Effect of Atorvastatin and Ampicillin against Resistant Staphylococcus Spp and E.Coli Isolated from Bovine Mastitis. **2019**, doi:10.1101/695817.
22. Ko, H.H.T.; Lareu, R.R.; Dix, B.R.; Hughes, J.D. Statins: Antimicrobial Resistance Breakers or Makers? *PeerJ* **2017**, *5*, e3952, doi:10.7717/peerj.3952.
23. Pose, E.; Trebicka, J.; Mookerjee, R.P.; Angeli, P.; Ginès, P. Statins: Old Drugs as New Therapy for Liver Diseases? *J Hepatol* **2019**, *70*, 194–202, doi:10.1016/j.jhep.2018.07.019.
24. Stoll, L.L.; McCormick, M.L.; Denning, G.M.; Weintraub, N.L. Antioxidant Effects of Statins. *Drugs of Today* **2004**, *40*, 975, doi:10.1358/dot.2004.40.12.872573.
25. Tsubaki, M.; Fujiwara, D.; Takeda, T.; Kino, T.; Tomonari, Y.; Itoh, T.; Imano, M.; Satou, T.; Sakaguchi, K.; Nishida, S. The Sensitivity of Head and Neck Carcinoma Cells to Statins Is Related to the Expression of Their Ras Expression Status, and Statin-Induced Apoptosis Is Mediated via Suppression of the Ras/ERK and Ras/MTOR Pathways. *Clin Exp Pharmacol Physiol* **2017**, *44*, 222–234, doi:10.1111/1440-1681.12690.
26. Hsu, C.; Brahmandam, A.; Brownson, K.E.; Huynh, N.; Reynolds, J.; Lee, A.I.; Fares, W.H.; Ochoa Chaar, C.I. Statin Therapy Associated with Improved Thrombus Resolution in Patients with Deep Vein Thrombosis. *J Vasc Surg Venous Lymphat Disord* **2019**, *7*, 169-175.e4, doi:10.1016/j.jvsv.2018.10.020.
27. Li, G.; Zhao, J.; Li, B.; Zhang, X.; Ma, J.; Ma, X.; Liu, J. The Anti-Inflammatory Effects of Statins on Patients with Rheumatoid Arthritis: A Systemic Review and Meta-Analysis of 15 Randomized Controlled Trials. *Autoimmun Rev* **2018**, *17*, 215–225, doi:10.1016/j.autrev.2017.10.013.
28. Sheridan, A.; Wheeler-Jones, C.P.D.; Gage, M.C. The Immunomodulatory Effects of Statins on Macrophages. *Immuno* **2022**, *2*, 317–343, doi:10.3390/immuno2020021.
29. Jerwood, S.; Cohen, J. Unexpected Antimicrobial Effect of Statins. *Journal of Antimicrobial Chemotherapy* **2007**, *61*, 362–364, doi:10.1093/jac/dkm496.
30. Graziano, T.S.; Cuzzullin, M.C.; Franco, G.C.; Schwartz-Filho, H.O.; de Andrade, E.D.; Groppo, F.C.; Cogo-Müller, K. Statins and Antimicrobial Effects: Simvastatin as a Potential Drug against Staphylococcus Aureus Biofilm. *PLoS One* **2015**, *10*, e0128098, doi:10.1371/journal.pone.0128098.
31. de Carvalho, R.D.P.; Casarin, R.C.V.; de Lima, P.O.; Cogo-Müller, K. Statins with Potential to Control Periodontitis: From Biological Mechanisms to Clinical Studies. *J Oral Biosci* **2021**, *63*, 232–244, doi:10.1016/j.job.2021.06.002.
32. Parolina de Carvalho, R.D.; de Andrade Moreno, J.; Roque, S.M.; Chan, D.C.H.; Torrez, W.B.; Stipp, R.N.; Bueno-Silva, B.; de Lima, P.O.; Cogo-Müller, K. Statins and Oral Biofilm: Simvastatin as a Promising Drug to Control Periodontal Dysbiosis. *Oral Dis* **2022**, doi:10.1111/odi.14446.
33. Sun, T.; Huang, J.; Zhang, W.; Zheng, X.; Wang, H.; Liu, J.; Leng, H.; Yuan, W.; Song, C. Simvastatin-Hydroxyapatite Coatings Prevent Biofilm Formation and Improve Bone Formation in Implant-Associated Infections. *Bioact Mater* **2023**, *21*, 44–56, doi:10.1016/j.bioactmat.2022.07.028.
34. Ansari, M.; Khan, H.; Khan, A.; Cameotra, S.; Alzohairy, M. Anti-Biofilm Efficacy of Silver Nanoparticles against MRSA and MRSE Isolated from Wounds in a Tertiary Care Hospital. *Indian J Med Microbiol* **2015**, *33*, 101–109, doi:10.4103/0255-0857.148402.
35. Tarusha, L.; Paoletti, S.; Travan, A.; Marsich, E. Alginate Membranes Loaded with Hyaluronic Acid and Silver Nanoparticles to Foster Tissue Healing and to Control Bacterial Contamination of Non-Healing Wounds. *J Mater Sci Mater Med* **2018**, *29*, 22, doi:10.1007/s10856-018-6027-7.
36. Lebeaux, D.; Ghigo, J.-M.; Beloin, C. Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. *Microbiology and Molecular Biology Reviews* **2014**, *78*, 510–543, doi:10.1128/MMBR.00013-14.
37. Cluzet, V.C.; Gerber, J.S.; Nachamkin, I.; Metlay, J.P.; Zaoutis, T.E.; Davis, M.F.; Julian, K.G.; Royer, D.; Linkin, D.R.; Coffin, S.E.; et al. Duration of Colonization and Determinants of Earlier Clearance of Colonization With Methicillin-Resistant Staphylococcus Aureus. *Clinical Infectious Diseases* **2015**, *60*, 1489–1496, doi:10.1093/cid/civ075.

38. Kumar, S.A.; Ansary, A.A.; Ahmad, A.; Khan, M.I. Extracellular Biosynthesis of CdSe Quantum Dots by the Fungus, <I>Fusarium Oxysporum</I> *J Biomed Nanotechnol* **2007**, *3*, 190–194, doi:10.1166/jbn.2007.027.
39. CLSI Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. *Clinical and Laboratory Standards Institute* **2018**, *11th ed*.
40. Odds, F.C. Synergy, Antagonism, and What the Chequerboard Puts between Them. *Journal of Antimicrobial Chemotherapy* **2003**, *52*, 1–1, doi:10.1093/jac/dkg301.
41. Doern, C.D. When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing. *J Clin Microbiol* **2014**, *52*, 4124–4128, doi:10.1128/JCM.01121-14.
42. Wu, W.-S.; Chen, C.-C.; Chuang, Y.-C.; Su, B.-A.; Chiu, Y.-H.; Hsu, H.-J.; Ko, W.-C.; Tang, H.-J. Efficacy of Combination Oral Antimicrobial Agents against Biofilm-Embedded Methicillin-Resistant Staphylococcus Aureus. *J Microbiol Immunol Infect* **2013**, *46*, 89–95, doi:10.1016/j.jmii.2012.03.009.
43. Honary, S.; Barabadi, H.; Gharaei-Fathabad, E.; Naghibi, F. Green Synthesis of Silver Nanoparticles Induced by the Fungus Penicillium Citrinum. *Tropical Journal of Pharmaceutical Research* **2013**, *12*, doi:10.4314/tjpr.v12i1.2.
44. Birla, S.S.; Tiwari, V.V.; Gade, A.K.; Ingle, A.P.; Yadav, A.P.; Rai, M.K. Fabrication of Silver Nanoparticles by *Phoma Glomerata* and Its Combined Effect against *Escherichia Coli* , *Pseudomonas Aeruginosa* and *Staphylococcus Aureus*. *Lett Appl Microbiol* **2009**, *48*, 173–179, doi:10.1111/j.1472-765X.2008.02510.x.
45. Soni, N.; Prakash, S. Synthesis of Gold Nanoparticles by the Fungus Aspergillus Niger and Its Efficacy against Mosquito Larvae. *Reports in Parasitology* **2012**, 1, doi:10.2147/rip.s29033.
46. Vala, A.K.; Shah, S.; Patel, R.N. Biogenesis of Silver Nanoparticles by Marine- Derived Fungus Aspergillus Flavus from Bhavnagar Coast, Gulf of Khambhat, India. *Journal of Marine Biology & Oceanography* **2014**, *03*, doi:10.4172/2324-8661.1000122.
47. Raj, S.; Chand Mali, S.; Trivedi, R. Green Synthesis and Characterization of Silver Nanoparticles Using Enicostemma Axillare (Lam.) Leaf Extract. *Biochem Biophys Res Commun* **2018**, *503*, 2814–2819, doi:10.1016/j.bbrc.2018.08.045.
48. Baharara, J.; Ramezani, T.; Hosseini, N.; Mousavi, M. Silver Nanoparticles Synthesized Coating with Zataria Multiflora Leaves Extract Induced Apoptosis in HeLa Cells Through P53 Activation. *Iran J Pharm Res* **2018**, *17*, 627–639.
49. Noronha, V.T.; Paula, A.J.; Durán, G.; Galembeck, A.; Cogo-Müller, K.; Franz-Montan, M.; Durán, N. Silver Nanoparticles in Dentistry. *Dental Materials* **2017**, *33*, 1110–1126, doi:10.1016/j.dental.2017.07.002.
50. Scandorieiro, S.; de Camargo, L.C.; Lancheros, C.A.C.; Yamada-Ogatta, S.F.; Nakamura, C. V.; de Oliveira, A.G.; Andrade, C.G.T.J.; Duran, N.; Nakazato, G.; Kobayashi, R.K.T. Synergistic and Additive Effect of Oregano Essential Oil and Biological Silver Nanoparticles against Multidrug-Resistant Bacterial Strains. *Front Microbiol* **2016**, *7*, doi:10.3389/fmicb.2016.00760.
51. Saeki, E.K.; Yamada, A.Y.; de Araujo, L.A.; Anversa, L.; Garcia, D. de O.; de Souza, R.L.B.; Martins, H.M.; Kobayashi, R.K.T.; Nakazato, G. Subinhibitory Concentrations of Biogenic Silver Nanoparticles Affect Motility and Biofilm Formation in Pseudomonas Aeruginosa. *Front Cell Infect Microbiol* **2021**, *11*, doi:10.3389/fcimb.2021.656984.
52. Habash, M.B.; Goodyear, M.C.; Park, A.J.; Surette, M.D.; Vis, E.C.; Harris, R.J.; Khursigara, C.M. Potentiation of Tobramycin by Silver Nanoparticles against Pseudomonas Aeruginosa Biofilms. *Antimicrob Agents Chemother* **2017**, *61*, doi:10.1128/AAC.00415-17.
53. Villa, F.; Remelli, W.; Forlani, F.; Gambino, M.; Landini, P.; Cappitelli, F. Effects of Chronic Sub-Lethal Oxidative Stress on Biofilm Formation by *Azotobacter Vinelandii*. *Biofouling* **2012**, *28*, 823–833, doi:10.1080/08927014.2012.715285.
54. Wang, L.; Hu, C.; Shao, L. The Antimicrobial Activity of Nanoparticles: Present Situation and Prospects for the Future. *Int J Nanomedicine* **2017**, *Volume 12*, 1227–1249, doi:10.2147/IJN.S121956.
55. Su, L.-J.; Zhang, J.-H.; Gomez, H.; Murugan, R.; Hong, X.; Xu, D.; Jiang, F.; Peng, Z.-Y. Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. *Oxid Med Cell Longev* **2019**, *2019*, 1–13, doi:10.1155/2019/5080843.
56. Bocate, K.P.; Reis, G.F.; de Souza, P.C.; Oliveira Junior, A.G.; Durán, N.; Nakazato, G.; Furlaneto, M.C.; de Almeida, R.S.; Panagio, L.A. Antifungal Activity of Silver Nanoparticles and Simvastatin against Toxigenic Species of Aspergillus. *Int J Food Microbiol* **2019**, *291*, 79–86, doi:10.1016/j.ijfoodmicro.2018.11.012.
57. Sahoo, J.; Sarkhel, S.; Mukherjee, N.; Jaiswal, A. Nanomaterial-Based Antimicrobial Coating for Biomedical Implants: New Age Solution for Biofilm-Associated Infections. *ACS Omega* **2022**, *7*, 45962–45980, doi:10.1021/acsomega.2c06211.
58. Agnihotri, S.; Mukherji, S.; Mukherji, S. Immobilized Silver Nanoparticles Enhance Contact Killing and Show Highest Efficacy: Elucidation of the Mechanism of Bactericidal Action of Silver. *Nanoscale* **2013**, *5*, 7328, doi:10.1039/c3nr00024a.

59. Roe, D.; Karandikar, B.; Bonn-Savage, N.; Gibbins, B.; Roullet, J.-B. Antimicrobial Surface Functionalization of Plastic Catheters by Silver Nanoparticles. *Journal of Antimicrobial Chemotherapy* **2008**, *61*, 869–876, doi:10.1093/jac/dkn034.
60. Seymour, C. Audit of Catheter-Associated UTI Using Silver Alloy-Coated Foley Catheters. *British Journal of Nursing* **2006**, *15*, 598–603, doi:10.12968/bjon.2006.15.11.21227.
61. Pollini, M.; Paladini, F.; Catalano, M.; Taurino, A.; Licciulli, A.; Maffezzoli, A.; Sannino, A. Antibacterial Coatings on Haemodialysis Catheters by Photochemical Deposition of Silver Nanoparticles. *J Mater Sci Mater Med* **2011**, *22*, 2005–2012, doi:10.1007/s10856-011-4380-x.
62. Mussin, J.; Robles-Botero, V.; Casañas-Pimentel, R.; Rojas, F.; Angiolella, L.; San Martín-Martínez, E.; Giusiano, G. Antimicrobial and Cytotoxic Activity of Green Synthesis Silver Nanoparticles Targeting Skin and Soft Tissue Infectious Agents. *Sci Rep* **2021**, *11*, 14566, doi:10.1038/s41598-021-94012-y.
63. Orta-García, S.T.; Plascencia-Villa, G.; Ochoa-Martínez, A.C.; Ruiz-Vera, T.; Pérez-Vázquez, F.J.; Velázquez-Salazar, J.J.; Yacamán, M.J.; Navarro-Contreras, H.R.; Pérez-Maldonado, I.N. Analysis of Cytotoxic Effects of Silver Nanoclusters on Human Peripheral Blood Mononuclear Cells ' *in Vitro* .' *Journal of Applied Toxicology* **2015**, *35*, 1189–1199, doi:10.1002/jat.3190.
64. Alves, M.F.; Paschoal, A.C.C.; Klimeck, T.D.F.; Kuligovski, C.; Marcon, B.H.; de Aguiar, A.M.; Murray, P.G. Biological Synthesis of Low Cytotoxicity Silver Nanoparticles (AgNPs) by the Fungus Chaetomium Thermophilum—Sustainable Nanotechnology. *Journal of Fungi* **2022**, *8*, 605, doi:10.3390/jof8060605.
65. Haidari, H.; Kopecki, Z.; Bright, R.; Cowin, A.J.; Garg, S.; Goswami, N.; Vasilev, K. Ultrasmall AgNP-Impregnated Biocompatible Hydrogel with Highly Effective Biofilm Elimination Properties. *ACS Appl Mater Interfaces* **2020**, *12*, 41011–41025, doi:10.1021/acsami.0c09414.