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The mesenchymal stromal cell secretome impairs methicillin-resistant *Staphylococcus aureus* biofilms via cysteine protease activity in the equine model

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Abstract

Mesenchymal stromal cells (MSCs) from various species, such as humans, mice, and horses, were recently found to effectively inhibit the growth of various bacteria associated with chronic infections, such as nonhealing cutaneous wounds, via secretion of antimicrobial peptides. These MSC antimicrobial properties have primarily been studied in the context of the planktonic phenotype, and thus, information on the effects on bacteria in biofilms is largely lacking. The objectives of this study were to evaluate the in vitro efficacy of the MSC secretome against various biofilm-forming wound pathogens, including the methicillin-resistant *Staphylococcus aureus* (*MRSA*), and to explore the mechanisms that affect bacterial biofilms. To this end, we used equine MSCs, because the horse represents a physiologically relevant model for human wound healing and offers a readily translatable model for MSC therapies in humans. Our salient findings were that the equine MSC secretome inhibits biofilm formation and mature biofilms of various bacteria, such as *Pseudomonas aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis*. Furthermore, we demonstrated that equine MSC secrete cysteine proteases that destabilize *MRSA* biofilms, thereby increasing the efficacy of antibiotics that were previously tolerated by the biofilms. In light of the rise of antibiotic-resistant bacterial strains as an increasing global health threat, our results provide the rationale for using the MSC secretome as a complementary treatment for bacterial skin infections in both humans and horses.

KEYWORDS

antimicrobial, biofilm, cutaneous wounds, mesenchymal stromal cells, *MRSA*, secretome

1 | INTRODUCTION

Mesenchymal stromal cells (MSCs) are adult multipotent progenitor cells that participate in the inflammatory, proliferative, and remodeling phases of tissue repair and therefore, are considered as promising therapeutic approach in regenerative medicine.[1,2] Although it was initially thought that MSC engraft in the injured tissue, more recent data showed that paracrine signaling primarily contributes to tissue

repair.[1–3] Previously, we demonstrated that MSC-secreted factors promote angiogenesis and increase the migration of dermal fibroblasts in the equine model.[3–5] Our laboratory uses the horse as a physiologically relevant model for the study of mechanisms involved in wound repair and to evaluate novel therapies for skin wounds.[6] The economic cost of treating skin wounds and related complications in humans and horses is high, and in both species, particular types of chronic wounds do not respond well to current therapies, leading to suffering and morbidity.[6]

Skin wounds are usually colonized with commensal bacteria that support a "healthy" wound milieu. However, wound infections with pathogenic bacteria lead to inflammatory host responses and delayed wound healing.[7] Recently, it was demonstrated that human MSCs secrete factors that kill bacteria by depolarizing the bacterial cell membrane.[8] We, and others demonstrated this antimicrobial effect with equine MSC as well, most notably via the secretion of antimicrobial peptides (AMPs).[9,10] Consequently, MSC-secreted factors represent a promising new approach to treat bacterially infected skin wounds in both humans and horses. This is of particular importance in light of the increasing global health threat of antibiotic-resistant bacteria. Indeed, bacteria have the intrinsic mechanism to mutate, and thus, adapt rapidly to environmental changes, including treatments with antibiotics (Abx). Overuse, wrong indication, and incorrect application of conventional Abx allow some bacteria to survive treatment and to become the origin of a highly resistant population.[11] For example, methicillin-resistant *Staphylococcus aureus* (*MRSA*) causes infections in different parts of the body, including the skin, which are difficult to treat because of its resistance to commonly used Abx.[12,13] By integration of the staphylococcal cassette chromosome *mec* (SCC*mec*), *S. aureus* becomes resistant against beta-lactam stable Abx.[14,15]

Bacteria exist predominantly in adherent, highly structured cellular communities, encased in a self-produced extracellular matrix. Biofilms have an extreme tolerance to environmental impacts, including antimicrobial agents, because the phenotype is protected by an extracellular matrix that mainly consists of polysaccharides, proteins, and extracellular DNA.[16,17] Furthermore, bacteria in biofilms take on a quiescent state and have an increased mutation rate, resulting in a high tolerance of bacteria against conventional Abx when present in biofilms.[18–20] Biofilms typically occur in cutaneous wounds and are often associated with therapy-resistant wound infections and wound chronicity.[7,21] Antimicrobial chemotherapy, even when aggressive, is often ineffective since the nonpathogenic bacterial populations is killed while pathogenic bacteria persists protected by the biofilm, and individual bacteria mutate, become antibiotic resistant, and recolonize the wound after completion of the therapy.[7,22] Therefore, new approaches are needed to effectively treat biofilms, especially those consisting of antibiotic-resistant bacteria.

Because the antimicrobial properties of MSC have primarily been studied in the context of the planktonic phenotype, the aim of the present study was to determine the efficacy of equine MSC-secreted factors on biofilms generated by a wide variety of relevant wound pathogens, including *MRSA*, in vitro. Our salient findings were that factors secreted by MSC could impair biofilm formation, as well as disrupt mature biofilms generated by these bacteria. Importantly, we found that these effects were mediated via a protease-dependent mechanism and that MSC-secreted factors allowed previously ineffective antibiotic treatments to become more effective at reducing bacterial survival.

Significance statement

This study demonstrated for the first time that mesenchymal stromal cells (MSCs) are effective against bacteria in biofilms, including the antibiotic-resistant methicillin-resistant *Staphylococcus aureus*, via secretion of active proteases that destabilize biofilms by protein degradation, resulting in increased antibiotic effectiveness. In light of the rise of antibiotic-resistant bacterial strains as an increasing global health threat, these results provide the rationale for using the MSC secretome as a complementary treatment for bacterial infections.

2 | MATERIALS AND METHODS

2.1 | Cells

Equine peripheral blood-derived MSCs were isolated and characterized, exactly as described previously.[3,4,23] Blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014-0038). Briefly, peripheral blood mononuclear cells were isolated using density gradient centrifugation on Percoll and were seeded at a density of 16×10^4cells/cm^2 in a T_{75} flask in culture medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Corning, Acton, Massachusetts), supplemented with 30% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, Georgia), 1% penicillin/streptomycin (P/S) (Invitrogen, Grand Island, NY), 2 mM L-glutamine (Invitrogen) and 10^{-11} M low dexamethasone (Sigma Aldrich, St Louis, Missouri). At 70% confluency, cells were trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and were further cultured in expansion medium, which is identical to the culture medium but without dexamethasone. The equine dermal fibroblast cell line NBL6 (ATCC, Manassas, Virginia) was cultured in DMEM supplemented with 10% FBS and 1% P/S.

2.2 | Collection of conditioned media and treatment with inhibitor

Conditioned media (CM) were collected from MSC from three different horses upon 70% confluency of the cells. To this end, 9×10^5 MSCs were seeded in a T_{75} flask in 10 mL of antibiotic-free medium, consisting of DMEM and 10% FBS. After 2 days of culture, medium was removed, cells were washed three times with phosphate-buffered saline (PBS), and cultured in 6 mL of DMEM. Twenty hours later,

supernatant was collected after centrifugation twice for 5 minutes at 300*g* and stored at −80°C until further use. A similar approach was used to collect CM from NBL6 cells (control CM).

To determine the potential role of proteases secreted by MSC, MSC CM was incubated with 10 μM E-64 protease inhibitor (Sigma Aldrich) for 30 minutes at room temperature (RT) on an orbital shaker at 200 rpm before use. E-64-treated DMEM was included as controls.

2.3 | Bacterial cultures

Field isolates of *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Acinetobacter baumannii*, and *Aerococcus viridans* were obtained through the Animal Health Diagnostic Center at Cornell University (courtesy, Dr Thachil). The *MRSA* strain *USA300* and *MRSA USA300 Δsigβ* mutants were a kind gift from Dr Whittaker, Cornell University. *S. aureus* 25923 (ATCC) and *MRSA* were maintained on Tryptic Soy (TS) agar (Sigma Aldrich), whereas *P. aeruginosa*, *S. epidermidis*, *A. baumanni* and *A. viridans* were maintained on Luria-Bertani (LB) agar (Life Technologies, Rockville, Maryland) at 4°C for up to 2 weeks. For each experiment, a single colony of the appropriate strain was incubated in 4 mL of either LB (Life Technologies) or TS broth (Hardy Diagnostics, Santa Maria, California), depending on the strain, on an orbital shaker at 200 rpm, overnight at 37°C in a warm room. Overnight cultures were then used for further experimentation, as described below.

2.4 | CM-planktonic bacteria cocultures

To test the antibacterial effect of MSC CM on planktonic bacteria, overnight cultures were first diluted 1:100 in LB or TS broth and further incubated, on an orbital shaker at 200 rpm, at 37°C in a warm room until they reached their exponential growth phase. The exponential growth phase was determined by absorbance reading of 1 mL culture at 600 nm using an Ultraspec 2100 pro spectrophotometer (Amersham, Pharmacia Biotech, Cambridge, UK). Hundred microliters of media with following conditions were added in triplicate wells of a 96-well plate: (a) MSC CM; (b) NBL6 CM; (c) DMEM (negative control); and (d) P/S (1% in DMEM (positive control). Next, 100 μL of LB or TS broth containing 1×10^3 bacteria were added to each well and plates were incubated, on an orbital shaker at 200 rpm, at 37°C in a warm room for 8 hours (*A. baumanni*, *A. viridans*, *MRSA*, and *S. aureus*) or 12 hours (*P. aeruginosa*, *S. epidermidis*). The absorbance of the cultures was read using a 96-well Multiskan EX plate reader (Thermo Fisher Scientific, Rochester, New York) at 600 nm, and bacterial growth is presented as the absorbance relative to the absorbance of the DMEM control that was set to a 100%.

2.5 | CM-biofilm cocultures

To test the antibacterial effect of MSC CM on bacteria in biofilms, the microtiter dish biofilm assay was used, as previously described.[9,24] Briefly, 50 μL of overnight bacteria cultures, 1:100 diluted in the appropriate broth, was added to wells of 96-well U-bottom microtiter plates. Susceptibility of bacteria to various Abx was first tested on biofilm formation as follows: 50 μg/mL gentamicin, 50 μg/mL ampicillin, or 1% P/S were applied in triplicate and biofilms were allowed to form for 24 hours at 37°C in warm room on a rocking shaker. To test the effect on bacteria in mature biofilms, biofilms formed for 24 hours at 37°C in a warm room on a rocking shaker before 50 μg/mL gentamicin, 50 μg/mL ampicillin, or 1% P/S were applied in triplicate and incubated for another 24 hours.

To test the effect of MSC CM on biofilm formation, 50 μL of MSC CM, NBL6 CM, DMEM (negative control), and 1% P/S (positive control) were added in triplicate and biofilms formed for 24 hours at 37°C in a warm room on a rocking shaker. To investigate the effect of beta-lactam stable Abx on *MRSA* biofilms, 50 μg/mL oxacillin was included. To test the effect of MSC CM on mature biofilms, 96-well U-bottom plates were prepared as described above, but biofilms were allowed to form for 24 hours at 37°C in a warm room on a rocking shaker, followed by the addition of 50 μL of MSC CM, NBL6 CM, DMEM (negative control), and 1% P/S (positive control) in triplicate. Treated mature biofilms were incubated for another 24 hours. Crystal violet staining was performed by removing medium, washing the wells with distilled water, adding 125 μL of 0.1% crystal violet for 5 minutes, washing the wells carefully twice with distilled water, and allowing the plates to air dry for at least 2 hours. Dye was solubilized using 125 μL of 30% glacial acetic acid, transferred to new 96-well flat bottom plates, and absorbance was measured at 550 nm on an Infinite 200 pro plate reader (Tecan, Morrisville, North Carolina). To determine colony forming units (CFU)/mL, 10 μL of each condition was removed and serially diluted 1:10 in LB broth or TS broth, respectively. Ten microliters of appropriate dilutions were drop-plated on LB agar plates or TS agar plates, respectively. Bacterial colonies were counted by visual inspection after overnight incubation at 37°C.

2.6 | Protease array

To screen for proteases in equine MSC and NBL6 CM, a Human Proteome Profiler Protease Array was used, according to manufacturer's instructions (R&D Systems, Minneapolis, Minnesota). We have used human profiler arrays successfully to detect other types of proteins (eg, angiogenesis-related proteins, cytokines, etc.) in CM from equine cells.[3,9] Positive signals were visualized using the ChemiDoc MP Imaging system (Bio-Rad, Hercules, California), normalized to the background, and quantified by measuring the sum of pixels intensities within the spot boundary pixel area using image analysis software (Image Laboratory 4.1; Bio-Rad).

2.7 | Confocal microscopy

To evaluate the composition of biofilms, confocal microscopy was used. To this end, overnight bacteria cultures were diluted 1:100 in

appropriate broth, and 400 μL was added to a well of a four-well chambered cover glass (Thermo Fisher Scientific). After 24 hours incubation at 37°C in a warm room on a rocking shaker, supernatants were removed, and 200 μL of MSC CM, DMEM (negative control), or Abx (positive control) were added. After another 24 hours of incubation, supernatants were removed again and biofilms stained as follows: protein content of the bacterial extracellular matrix was visualized using 1.4 μM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) diluted in Sypro Ruby Protein Staining (Thermo Fisher Scientific), as previously described.[25] Volume of live bacteria in the biofilms was assessed using a staining protocol consisting of 1.4 μM DAPI and 15 μM propidium iodide in PBS.[26] Images were acquired using a confocal laser scanning microscope (Zeiss, Oberkochen, Germany) with a camera controlled by ZEN imaging software. Images were analyzed using the Fiji ImageJ software (https://imagej.net/Fiji).

To test for the efficacy of P/S on biofilms that were pretreated with MSC CM, DMEM (negative control), or Abx (positive control), experiments were performed as described above, but in this case, 200 μL of 1% P/S was added to the biofilms for 4 hours prior to performing the staining and analyzing the images.

2.8 | Western blot analysis

Samples (MSC CM and NBL6 CM) were centrifugated at 4000*g* for 30 minutes to a 10× concentration using 3-kDa Centriprep Centrifugal Filters (Merck Millipore Ltd, Tullagreen, Ireland). Samples were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on a 15% gel, and transferred to Immobilin polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) using a transblot turbo system (Bio-Rad). Membranes were blocked in 5% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS), and incubated with 0.25 μg/mL human cathepsin B antibody (R&D Systems) or 1 μg/mL human cathepsin V antibody in TBS with 5% BSA overnight at 4°C on a rotating platform. Blots were washed three times for 10 minutes with TBS-Tween, followed by incubation with a 1:20 000 dilution of horseradish peroxidase (HRP)-conjugated donkey anti-goat (Jackson ImmunoResearch Labs) for 1 hour at RT. All blots were washed three times for 10 minutes with TBS-Tween, and then visualized by chemiluminescence using Clarity Western ECL (Bio-Rad). Membranes were probed in parallel with a human fibronectin antibody (Sigma Aldrich), diluted 1:5000, as a

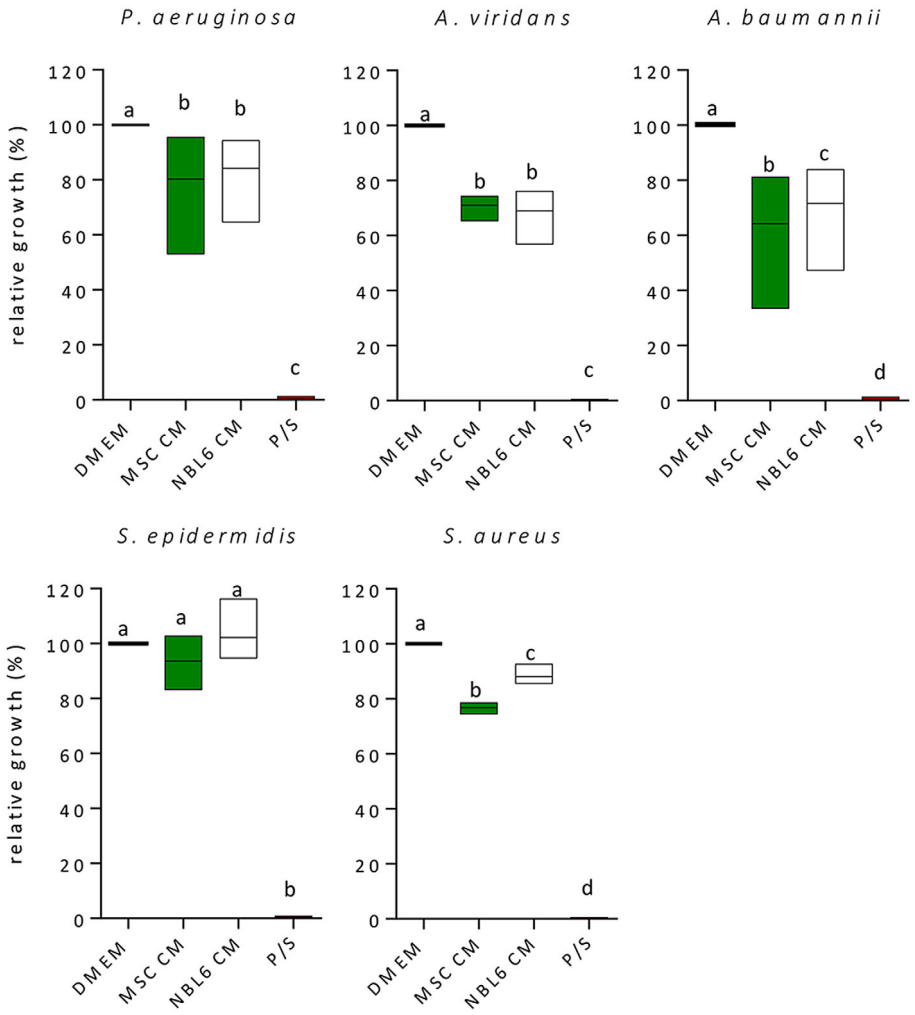


FIGURE 1 The conditioned medium (CM) of equine mesenchymal stromal cell (MSC) inhibits the growth of various wound-related planktonic bacteria. The relative growth of the planktonic form of *Pseudomonas aeruginosa*, *Aerococcus viridans*, *Acinetobacter baumannii*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* grown for 8 or 12 hours in Dulbecco's modified Eagle medium (DMEM), MSC CM, dermal fibroblast (NBL6) CM, and 1% penicillin/streptomycin (P/S). Different letters indicate statistically significant ($P < .05$) differences; n = 3

loading control. Gels were imaged on a BioRad ChemiDoc MP system (Bio-Rad).

2.9 | Statistical analysis

Two-way analysis of variance followed by a Tukey's multiple comparison test was performed to determine statistically significant differences at $P < .05$. GraphPad Software was used for the analysis (GraphPad Software, La Jolla, CA). Data given are the mean of three replicates with SDs.

3 | RESULTS

3.1 | MSC secretome inhibits the growth of various planktonic bacteria that commonly colonize cutaneous wounds

Our group previously showed that factors secreted by equine MSC can inhibit the growth of *Escherichia coli* and *S. aureus*, representative Gram-negative (–) and Gram-positive (+) bacteria, respectively.[9] Because cutaneous wounds can also be colonized by other bacteria, we assessed the antimicrobial potential of MSC against field isolates of *P. aeruginosa* and *A. baumannii* (Gram–), and *A. viridans* and *S. epidermidis* (Gram+).[27–29]

To this end, bacteria were cultured in the presence of MSC-secreted factors, delivered as CM. Bacterial growth was evaluated by measuring the absorbance of bacterial cultures at 600 nm, as previously described,[9] and expressed relatively to DMEM (negative control) which was set to 100%. In the presence of MSC CM, a significant inhibition of bacterial growth was observed for all bacteria tested (*P. aeruginosa*, *A. viridans*, *A. baumannii*, and *S. aureus*), except *S. epidermidis* (Figure 1). Because skin fibroblasts are known to have antimicrobial properties as well,[30,31] we repeated these experiments with CM collected from the equine dermal fibroblast cell line NBL6 and found that NBL6 CM affected bacterial growth similarly as MSC CM, with exception of *S. aureus*, where MSC CM was significantly more effective at inhibiting bacterial growth compared with NBL6 CM (Figure 1). As expected, growth of all bacterial strains was inhibited in the presence of P/S (positive control).

3.2 | MSC secretome prevents biofilm formation and diminishes mature biofilms of various bacteria

Encouraged by the finding that factors secreted by equine MSC can inhibit the growth of these various planktonic bacteria, we next sought to determine the effect of the MSC secretome on these bacterial strains when present in biofilms. Indeed, bacteria colonize the skin predominantly in this adherent phenotype that causes a variety of therapy-resistant infections.[29]

First, we investigated the effect of MSC CM on biofilm formation, using a well-established in vitro biofilm assay that we used

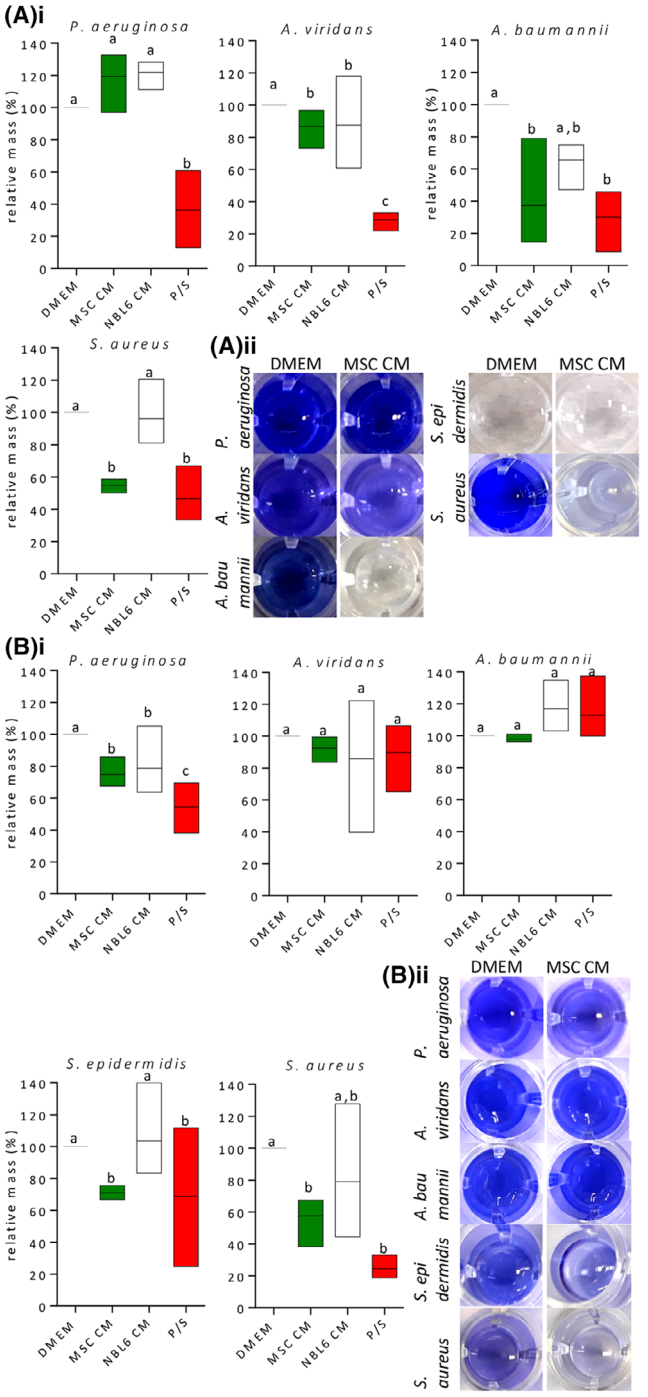
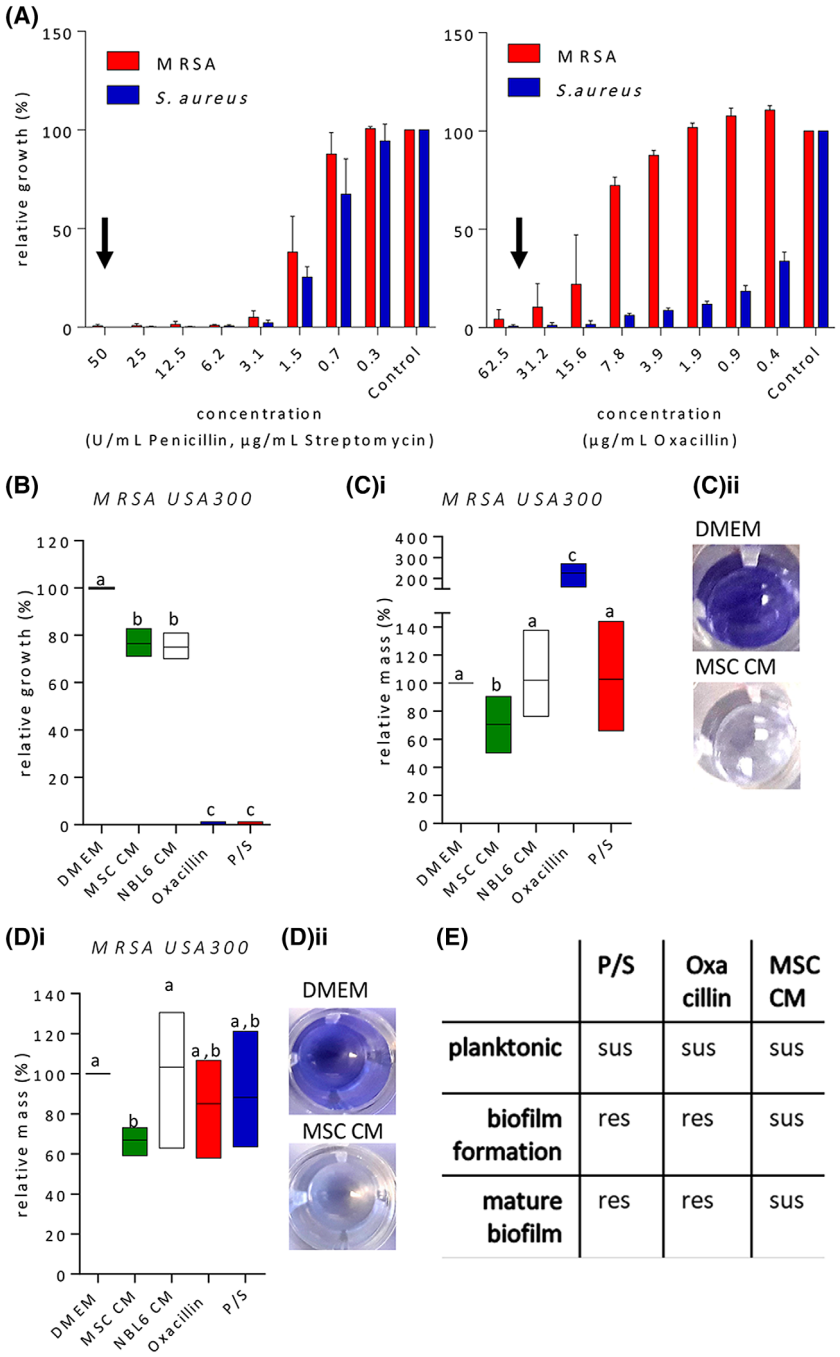


FIGURE 2 The conditioned medium (CM) of equine mesenchymal stromal cell (MSC) inhibits biofilm formation and reduces biofilms of various wound-related bacteria. Relative biofilm mass of *Pseudomonas aeruginosa*, *Aerococcus viridans*, *Acinetobacter baumannii*, and *Staphylococcus aureus* was determined either during biofilm formation, A, or after 24 hours of growth when biofilms where established, B, when incubated for 24 hours with Dulbecco's modified Eagle medium (DMEM), MSC CM, dermal fibroblast (NBL6) CM, and 1% penicillin/streptomycin (P/S). Absorbance of bacterial cultures was measured at 550 nm and expressed relatively to DMEM (negative control) which was set to 100%. Different letters indicate statistically significant ($P < .05$) differences; n = 3 (i). Representative images of crystal violet uptake by biofilms of the various bacteria grown in (i) DMEM or (ii) MSC CM (ii)

previously.[9] Because biofilms are known to often tolerate Abx, three different Abx protocols, namely, 50 μg/mL gentamicin, 50 μg/mL ampicillin, and a 1% P/S combination, were tested to determine the most effective Abx control. As we observed an overall highest inhibitory effect when biofilms were treated with P/S (Figure S1), we decided to include this Abx as control in our experiments. Biofilm formation in the presence of DMEM was observed within 24 hours for all bacterial strains, except *S. epidermidis*, and thus, this bacterial strain could not be evaluated in this experiment (Figure 2A(ii)). We found that MSC CM significantly impaired biofilm formation of all bacterial strains tested (*A. viridans*, *A. baumannii*, and *S. aureus*), except *P. aeruginosa* (Figure 2A). Moreover, MSC CM was as effective as the positive control P/S for *A. baumannii* and *S. aureus* (Figure 2A(i)). Similar to MSC CM, NBL6 CM did not affect biofilm formation of *P. aeruginosa* and impairing biofilm formation of *A. viridans*, but in contrast to MSC CM, NBL6 CM was ineffective at impairing biofilm formation of *A. baumannii* and *S. aureus* (Figure 2A(i)).

Second, we investigated the effect of MSC CM on biofilms that are already established. To this end, we repeated the experiments using mature biofilms, where bacteria formed biofilms for 24 hours before they were exposed to the different treatments. When comparing the results between intervention during biofilm formation (Figure 2A) and intervention when the biofilm is already established (Figure 2B), several interesting observations were made. Where *S. epidermidis* was unable to form biofilms



(E)

	P/S	Oxacillin	MSC CM
planktonic	sus	sus	sus
biofilm formation	res	res	sus
mature biofilm	res	res	sus

FIGURE 3 The conditioned medium (CM) of equine mesenchymal stromal cell (MSC) inhibits the growth of methicillin-resistant *Staphylococcus aureus* (*MRSA*) in planktonic and biofilm form. A, Relative bacterial growth of *S. aureus* and *MRSA* isolate *USA300* grown in different concentrations of penicillin/streptomycin (P/S) and oxacillin. Arrow indicates concentrations used for all experiments. B, The relative growth of the planktonic form of *MRSA USA300* grown for 8 hours in Dulbecco's modified Eagle medium (DMEM), MSC CM, dermal fibroblast (NBL6) CM, 50 μg/mL oxacillin, and 1% P/S. Different letters indicate statistically significant ($P < .05$) differences; n = 3. Relative biofilm mass of *MRSA USA300* was determined either during biofilm formation , C, or after 24 hours of growth when biofilms where established, D, when incubated for 24 hours with Dulbecco's modified Eagle medium (DMEM), MSC CM, dermal fibroblast (NBL6) CM, 50 μg/mL Oxacillin, and 1% Penicillin/Streptomycin (P/S). Absorbance of bacterial cultures was measured at 550 nm and expressed relatively to DMEM (negative control) which was set to 100%. Different letters indicate statistically significant ($P < .05$) differences; n = 3
(i). Representative images of crystal violet uptake by *MRSA* biofilms grown in DMEM or MSC CM (ii). E, Overview of susceptibility of *MRSA USA300* to P/S, oxacillin, and MSC CM. sus, susceptible; res, resistant

in the presence of DMEM (Figure 2A(ii)), this bacterial strain did form biofilms in its traditional growth medium, LB broth, and thus could be included for further analysis (Figure 2B). We found that MSC CM could significantly inhibit the biofilm mass of mature biofilms made by *S. aureus* and *S. epidermidis*. However, although the MSC CM was capable to significantly impair biofilm formation by *A. viridans* and *A. baumannii*, it no longer was effective once the biofilm was established; and vice versa, although the MSC CM was unable to impair biofilm formation by *P. aeruginosa*, it was effective in significantly reducing its biofilm mass in mature biofilms (Figure 2A,B). Overall, the inhibiting effects of the treatments (including the positive P/S control) were less pronounced on mature biofilms when compared with the new biofilms (Figure 2A,B).

These findings were corroborated when using CFU/mL as another read-out in addition to crystal violet absorbance readings, with the exception of *A. baumannii* and *S. aureus*, where MSC CM treatment showed a similar trend of biofilm mass inhibition but did no longer reached significance (Figure S2A,B).

Collectively, these data show that the MSC secretome negatively affects biofilms, either during formation or after the mature biofilm is established or both, of various bacteria that colonize cutaneous wounds. Moreover, the MSC secretome maintained its activity under conditions where P/S, very effective against bacteria in their planktonic phenotype, was unable to reduce mature biofilms.

3.3 | MSC secretome inhibits the growth of *MRSA* both in planktonic and biofilm form

Our current data, combined with our previously work,[9] clearly show that the MSC secretome is effective against *S. aureus*, both in

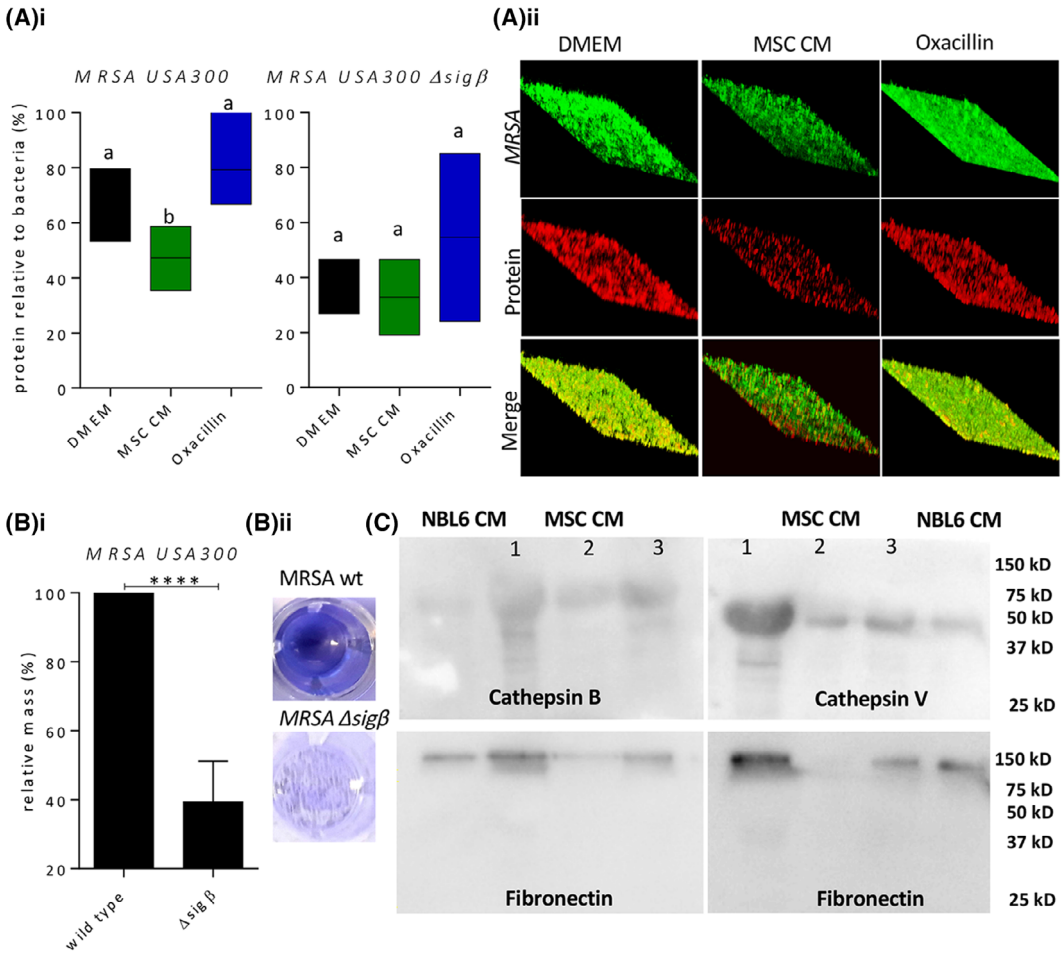


FIGURE 4 The conditioned medium (CM) of equine mesenchymal stromal cell (MSC) reduces protein content in methicillin-resistant *Staphylococcus aureus* (*MRSA*) *USA300* biofilms. A, Protein volume relative to bacterial volume in mature *MRSA USA300* and *MRSA USA300 Δsigβ* biofilms incubated in Dulbecco's modified Eagle medium (DMEM), MSC CM, dermal fibroblast (NBL6) CM, and 50 μg/mL oxacillin. Biofilms were stained with 4′,6-diamidino-2-phenylindole (to visualize bacteria) and Sypro Ruby Protein Staining (to visualize proteins), and images were acquired using a confocal laser scanning microscope. Different letters indicate statistically significant ($P < .05$) differences; n = 3 (i). Representative confocal images. Green panel shows bacteria, red panel shows proteins, merge panel shows composition of bacteria and proteins (ii). B, Relative biofilm mass of *MRSA USA300* and *MRSA USA300 Δsigβ* after 24 hours of growth (when biofilms where established) when incubated for 24 hours with Dulbecco's modified Eagle medium (DMEM). ****$P < .001$; n = 3 (i). Representative images of crystal violet uptake by *MRSA* and *MRSA USA300 Δsigβ* biofilms grown in DMEM (ii). C, Western blotting to evaluate the presence of cathepsins B and V in the CM of equine MSC obtained from three different horses and NBL6. Fibronectin was included as loading control

planktonic and biofilm form. This prompted us to evaluate the efficacy of the MSC secretome against the clinically relevant *MRSA* isolate *USA300*[32] as well. As already mentioned in the introduction, *MRSA* causes therapy-resistant infections due to the development of resistance against beta-lactam stable Abx, for example, oxacillin. Therefore, we performed a concentration kinetic experiment with P/S, used as a positive control for the other bacteria, and oxacillin, to evaluate the inhibitory effect of these two Abx on the growth of planktonic *S. aureus* and *MRSA USA300*. We found that *S. aureus* and *MRSA USA300* were equally susceptible to P/S, starting at ~1.5 μg/mL (Figure 3A). However, *S. aureus* was susceptible to oxacillin at concentrations as low as 0.5 μg/mL, whereas *MRSA USA300* only became susceptible to this Abx at a concentration of ~8 μg/mL, indicating that this strain is indeed resistant to oxacillin (Figure 3A). We then evaluated the effect of MSC CM on *MRSA USA300* and included both Abx as positive controls at a concentration of 50 μg/mL, which effectively inhibits the growth of this bacterial strain (arrow, Figure 3A).

In the presence of MSC CM, a significant inhibition of planktonic *MRSA USA300* growth was observed (Figure 3B). NBL6 CM similarly affected its growth, and as expected, both Abx completely inhibited *MRSA USA300* (Figure 3B). When evaluating biofilm formation, we found that MSC CM, but not NBL6 CM or P/S, was able to impair the formation of *MRSA USA300* biofilms (Figure 3C). Biofilm formation in the presence of oxacillin was significantly increased compared with the DMEM control (Figure 3C(i)). Enhanced biofilm formation in the presence of Abx, especially beta-lactam Abx such as oxacillin, has been reported previously for *MRSA*.[33,34] Lastly, even when *MRSA USA300* biofilms were already established, MSC CM was still capable of significantly inhibiting the biofilm mass, in contrast to NBL6 CM or any of the Abx (Figure 3D). Again, determining CFU/mL after treatments corroborated our findings, although the inhibitory trend of MSC CM on mature *MRSA* biofilms did no longer reach significance (Figure S2C,D).

Collectively, these results indicate that the secretome of MSC is effective against *MRSA USA300*, not only when growing planktonically but also when growing in biofilms. The latter is exciting, since under biofilm conditions, *MRSA USA300* tolerated high concentrations of P/S and oxacillin (Figure 3E).

3.4 | The MSC secretome reduces *MRSA* biofilms through cysteine protease activity

To further follow-up on the encouraging findings that the MSC secretome effectively impairs *MRSA* biofilms, we decided to investigate the underlying mechanism(s) in more detail. In general, biofilms

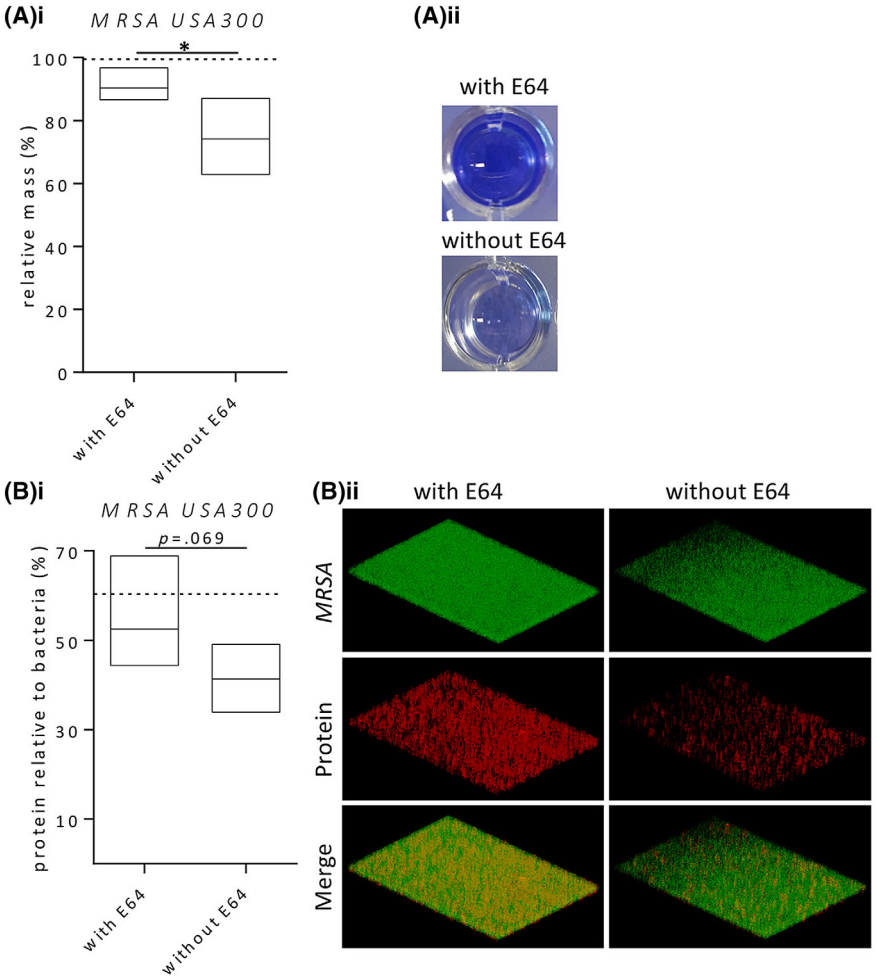


FIGURE 5 Cysteine proteases in the conditioned medium (CM) of equine mesenchymal stromal cell (MSC) partially reduces methicillin-resistant *Staphylococcus aureus* (*MRSA*) *USA300* biofilms. A, Relative biofilm mass of mature *MRSA USA300* was determined when incubated for 24 hours with MSC CM with or without the cysteine protease inhibitor E64. Absorbance of bacterial cultures was measured at 550 nm and expressed relatively to Dulbecco's modified Eagle medium (DMEM) (negative control) which was set to 100% (dotted line). *$P < .05$; n = 3 (i). Representative images of crystal violet uptake by *MRSA* biofilms grown in MSC CM with or without E64 (ii). B, Protein volume relative to bacterial volume in mature *MRSA USA300* biofilms incubated with MSC CM with or without the cysteine protease inhibitor E64, as analyzed by confocal microscopy and shown relatively to DMEM (negative control) (dotted line). n = 3; (i). Representative confocal images. Green panel shows bacteria, red panel shows proteins, merge panel shows composition of bacteria and proteins (ii)

consist of polysaccharides, proteins, extracellular DNA, and lipids. Although all components are important, *MRSA* biofilms primarily rely on proteins, and thus, protein degradation by proteases has been shown an efficient mechanism to dissolve mature *MRSA* biofilms.[35]

Therefore, we first evaluated whether the MSC CM affects the protein content in *MRSA* biofilms. To this end, mature *MRSA* biofilms were treated with MSC CM and protein and bacterial volume were evaluated by confocal microscopy. Results were expressed as protein volume relative to bacterial volume. Treatment with DMEM and oxacillin were included as controls, and experiments were repeated with *MRSA USA300 Δsigβ* as well. This knock-out strain contains a deletion of the *sigB* gene that leads to an increase of cysteine protease production, and thus, to protein degradation and subsequent loss of the ability to produce stable biofilms.[36] We found that MSC CM treatment of *MRSA* biofilms resulted in significantly reduced protein volume relative to bacteria, when compared with DMEM or oxacillin treatment, which were not significantly different from each other (Figure 4A(i), (ii)). As expected, *MRSA USA300 Δsigβ* formed less biofilm mass compared with the wild-type *MRSA* under control conditions (Figure 4B), and MSC CM treatment did not decrease protein volume relative to bacteria (Figure 4A(i)), further indicating that the mechanism by which MSC CM exerts its effect involves protein degradation.

Because proteins are degraded by proteases, we next screened the MSC CM for the presence of proteases, using a Human Proteome Profiler Protease Array that screens for 34 proteases. Many proteases were detected in the MSC CM (Figure S3A), and after setting an artificial threshold of 300 pixels, cathepsins A, B, and V were present in the highest concentration, followed by MMP-12 and urokinase (Figure S3A(i)). Western blot analyses of MSC CM using antibodies against cathepsins B and V confirmed the presence of these proteases in the MSC CM (Figure 4C). To provide a more direct link between the presence of these proteases in the MSC secretome and its effect on protein volume in *MRSA* biofilms, we treated MSC CM with the irreversible-binding cysteine protease inhibitor E64, which is used as a standard method to inhibit cysteine proteases including cathepsins B and V[37,38] and evaluated biofilm mass as well as protein/bacterial volume when compared with untreated MSC CM. We chose E64 because cathepsins B and V, present in the highest concentration in MSC CM (Figure S3A(i)), belong to the family of cysteine-proteases.[39,40] Treatment of *MRSA* biofilms with untreated MSC CM did significantly diminish biofilm mass when expressed relative to DMEM control (dotted line, Figure 5A(i)), which is in line with what we previously found (Figure 3D). However, this effect was abolished when MSC CM was

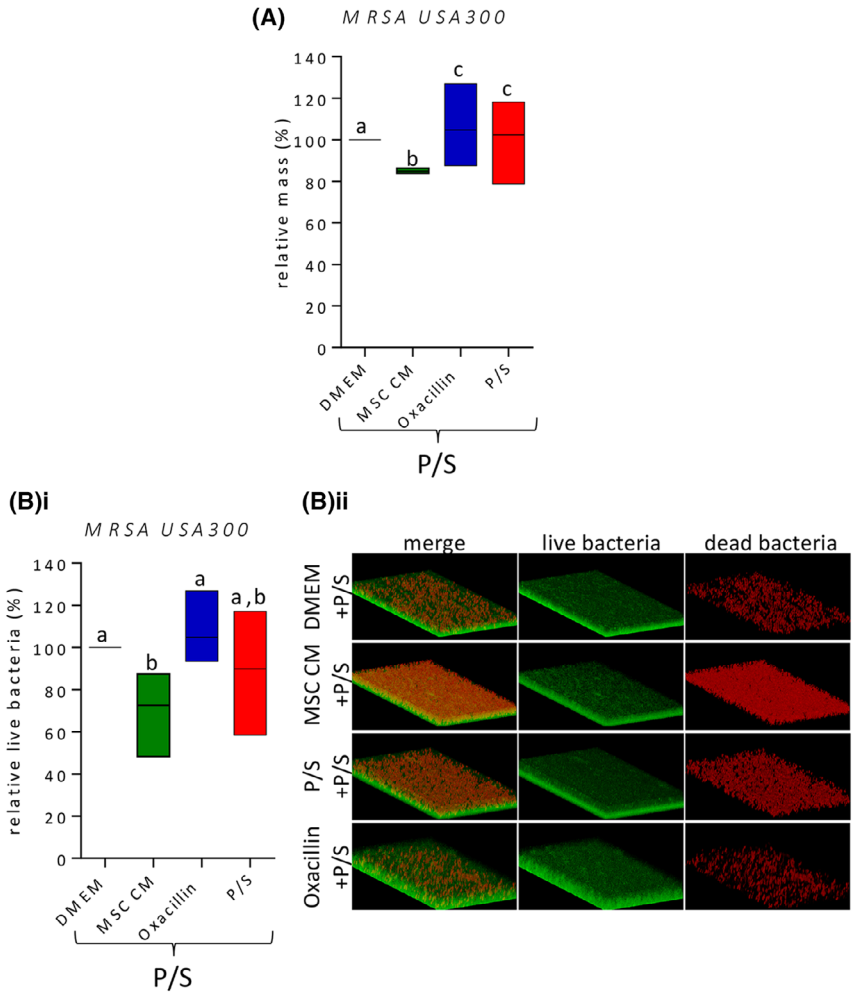


FIGURE 6 The conditioned medium (CM) of equine mesenchymal stromal cell (MSC) increases the efficacy of penicillin/streptomycin (P/S) against methicillin-resistant *Staphylococcus aureus* (*MRSA*) *USA300* in biofilms. Relative biofilm mass of mature biofilms of *MRSA USA300*, A, and relative live bacteria, B, was determined after incubation with Dulbecco's modified Eagle medium (DMEM), MSC CM, 1% P/S, and 50 μg/mL oxacillin for 24 hours, and challenging a second time with 1% P/S for 4 hours. Absorbance of bacterial cultures was measured at 550 nm, A, and live bacteria were analyzed by confocal microscopy, B(i), and expressed relatively to DMEM (negative control) which was set to 100%. Different letters indicate statistically significant ($P < .05$) differences; n = 3. Representative confocal images. Green panel shows live bacteria, red panel shows dead bacteria, merge panel shows composition of live and dead bacteria, B(ii)

treated with E64, with no significant difference in biomass relative to DMEM control but a significant difference in biofilm mass when compared with untreated-MSC CM (Figure 5A(i),(ii)). No difference in relative biofilm mass was observed when DMEM control was used in the presence or absence E64 (data not shown). Similarly, the reduction in protein volume relative to bacteria when biofilms were exposed to untreated MSC CM was less pronounced when E64-treated MSC CM was used, albeit this did not reach statistical significance (Figure 5B(i),(ii)).

Collectively, these results demonstrate that the MSC secretome contains various proteases and that it negatively impacts *MRSA* biofilms by degrading proteins via cysteine protease activity.

3.5 | MSC CM significantly decreases the tolerance of *MRSA* biofilms to Abx

Based on our findings that proteases secreted by MSC can diminish *MRSA* biofilms by reducing extracellular protein content, which normally protects bacteria against conventional Abx in biofilms,[35] we decided to evaluate whether pretreatment of biofilms with MSC CM would improve the efficacy of Abx against *MRSA*.

To this end, mature *MRSA* biofilms were pretreated with MSC CM, DMEM (negative control) or the Abx P/S and oxacillin for 24 hours. All biofilms were then treated with P/S for another 4 hours, after which biofilm mass was evaluated and expressed relative to DMEM control. As previously shown in Figure 3D, biofilm mass was not reduced upon treatment with P/S or oxacillin, and likewise, when these Abx-treated biofilms were exposed to another round of P/S treatment, no significant difference in biofilm mass was observed compared with the DMEM control (Figure 6A). Interestingly, when the biofilms were pretreated with MSC CM, a significant inhibition of biofilm mass was observed after P/S treatment (Figure 6A). Because evaluating the biofilm mass by itself does not provide any information regarding the effect on bacterial survival within the biofilm, we also determined the percentage of live bacteria, as well as the percentage of bacterial growth. Similar to the results on biofilm mass, pretreatment of *MRSA* biofilms with MSC CM, but not any of the Abx, followed by P/S treatment, resulted in a significant reduction of live bacteria, as determined by confocal microscopy (Figure 6B). To evaluate bacterial growth, bacteria were collected from the pretreated/treated biofilms, transferred to fresh TS broth, and bacterial growth was measured 4 hours later. Bacteria collected from biofilms that were pretreated with MSC CM, followed by P/S treatment, showed a significantly decreased growth when expressed relative to DMEM control (Figure S3B). In contrast, bacteria grew to the same level as DMEM control when collected from biofilms that were pretreated with any of the Abx, followed by P/S treatment (Figure S3B).

Taken together, these data demonstrate that pretreatment of biofilms with MSC CM, which leads to reduced protein content by MSC-secreted proteases, can improve the efficacy of Abx that are otherwise ineffective in the treatment of mature *MRSA* biofilms.

4 | DISCUSSION

The present study is the first to show that MSCs secrete proteases that inhibit biofilm formation and dissolve mature biofilms of various cutaneous wound-related bacteria. Although the inhibiting effect of MSC-secreted factors, collected as CM, on the growth of planktonic bacteria was not as strong when compared with conventional Abx, the MSC CM was superior to Abx in reducing bacterial growth in biofilms. Importantly, we could demonstrate that biofilms of *MRSA* are dissolved by MSC-secreted proteases. Moreover, we found that Abx unable to inhibit the growth of *MRSA* in biofilms, became effective when biofilms were pretreated with MSC secretome, most likely through its protein-degrading effects. Collectively, our results suggest that the MSC secretome could represent an alternative adjunct treatment for various wound-related bacteria, most notably Abx-resistant bacteria such as *MRSA*, not only by direct killing of bacteria via AMPs, as we and others previously demonstrated,[9,41,42] but also by enhancing the antimicrobial efficacy of Abx through biofilm degradation by MSC-secreted proteases.

To our knowledge, there are no published reports on the efficacy of human MSC against *MRSA* biofilms. There is one report showing that intravenously injected, preactivated murine MSC migrate to the infection side in a murine infected wound model. Furthermore, they showed that MSC injections in combination with antibiotic treatments were effective in clearing *MRSA* biofilms in their murine wound model, as well as in a canine clinical trial using canine MSC for the treatment of spontaneous chronic multi drug-resistant wound infections.[43] However, this study did not address the mechanism by which these cells exerted their effects on Abx-resistant bacteria in biofilms. In this present study, we used the horse model of cutaneous wound healing[6] to explore underlying mechanisms of how MSC can alter biofilms.

We found that proteases secreted by equine MSC are responsible for the degradation of proteins in *MRSA* biofilms. Specifically, we found cysteine proteases, such as cathepsins B and V, to be present in high concentrations in the MSC secretome. These results are in line with previous findings that endogenous cysteine proteases from *MRSA* can dissolve biofilms, while other proteases are not effective.[35] The activity of cysteine proteases can be twofold. On the one hand, cysteine proteases can interact directly with proteins in the extracellular matrix leading to protein degradation. On the other hand, cysteine proteases can act indirectly by cleaving other *MRSA* pro-proteases in a highly regulated protease cascade that would lead to complete biofilm dissociation if activated.[36] Future experiments are planned to determine which of these two mechanisms are preferentially used, if not both, by equine MSC-derived cysteine proteases.

In addition to *MRSA*, we demonstrated that mature biofilms of *P. aeruginosa*, *S. aureus*, and *S. epidermidis* were reduced by MSC CM as well. This is particularly encouraging because *Pseudomonas* and *Staphylococci* were the two most prevalent bacterial genera in a study investigating 2963 chronic human skin wounds,[44] and wounds infected with *P. aeruginosa* and *S. aureus* in particular showed wound enlargement and healing delay.[45,46] We also found that biofilms of less pathogenic bacteria were less affected by the MSC secretome.

If the MSC secretome indeed reduces mostly pathogenic bacteria in wounds, this could potentially result in a more nonpathogenic wound colonization that would lead to a better healing outcome.[47] However, there is some contradiction in the field what exactly defines a healthy skin or wound microbiome, and further studies are needed to investigate the impact of the MSC secretome on the microbiota in infected wounds.[48] Surprisingly, while mature *P. aeruginosa* biofilms were affected by MSC CM, the formation of these biofilms was not inhibited by the MSC secretome. One potential explanation for this is that developing *P. aeruginosa* biofilms are more reliant on extracellular DNA than extracellular matrix, as previously described,[49] and thus, would be less affected by MSC-secreted proteases.

Finally, and importantly, we found that *MRSA* biofilms showed an increased susceptibility to Abx after pretreatment with MSC CM, while pretreatments with Abx did not improve the outcome of the second Abx treatment. This indicates that disruption of the extracellular matrix is important for Abx to reach single individual bacteria and subsequently kill them. Because Abx tolerance in biofilms facilitates Abx resistance, it is necessary that bacteria in biofilms are eliminated quickly.[16,19] Moreover, Abx overuse fosters Abx resistance, and aggressive antimicrobial chemotherapy could harm the patient due to drug side effects.[50,51] The ability of the MSC secretome to enhance the efficacy of Abx, in combination with other important properties of MSC-secreted factors including the promotion of angiogenesis, tissue repair, and wound healing,[3,5,23] collectively support the potential of the MSC secretome as a new therapeutic approach in chronic cutaneous wound management.

4.1 | Summary

In summary, we have demonstrated for the first time that the equine MSC secretome is effective against bacteria both in planktonic form and biofilms, including the antibiotic-resistant *MRSA*, and contains active proteases that destabilize biofilms by protein degradation, a "mode of action" that can potentially be extrapolated to human MSC.

4.2 | Limitations

Although widely utilized in the field, the in vitro microtiter dish biofilm assay used in this study is rather simplistic and gives only little information about biofilm behavior in clinically relevant in vivo situations. Still, it is a valuable tool for the study of the early stages in biofilm formation and due to its high throughput nature, excellent for analyzing novel treatment approaches.[24] When performing these assays, we included penicillin/streptomycin as a positive Abx control. Although these Abx are not the most clinically relevant, they were chosen based on their highest inhibitory effect on biofilms when compared with more clinically relevant Abx such as gentamicin, amoxicillin, and oxacillin (Figure 1).

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

C.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. S.G., R.M.H.: collection and/or assembly of data. G.R.V.d.W.: conception and design, data analysis and interpretation, manuscript writing, administrative support.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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