Article title: In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin resistance *Staphylococcus aureus* biofilms.

Running title: Antibiotic and AMP combinations against MRSA biofilms

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are most often found in hospital and community-acquired infections. The danger of MRSA infections results from not only the emergence of multi-drug resistance but also the occurrence of strong biofilm forming bacteria. We investigated the in-vitro activities of antibiotics (daptomycin, linezolid, teichoplanine, azithromycin and ciprofloxacin) and antimicrobial cationic peptides (AMPs; indolicidin, CAMA: cecropin (1-7)-melittin A (2-9) amide, and nisin) alone or in combination against MRSA ATCC 43300 biofilms. The minimum inhibitory concentrations (MIC) and minimum biofilm eradication concentrations (MBEC) were determined by microbroth dilution technique. Antibiotic and AMP combinations were assessed using the chequerboard technique. For MRSA planktonic cells, MICs of antibiotics and AMPs were ranged between 0.125-512 and 8-16 mg/L, and the MBEC values of them were 512-5120 and 640 mg/L, respectively. With a fractional inhibitory concentration of ≤ 0.5 as borderline, synergistic interactions against MRSA biofilms were frequent with almost all antibiotic-antibiotic and antibiotic-AMP combinations. Against planktonic cells, they generally had an additive effect. No antagonism was observed. All of the antibiotics, AMPs, and their combinations were able to inhibit the attachment of bacteria at the 1/10xMIC and biofilm formation at 1xMIC. Biofilm-associated MRSA was not affected by therapeutically achievable concentrations of antimicrobial agents. Using a combination of antimicrobial agents can provide a synergistic effect, which rapidly enhances anti-biofilm activity and may help prevent or delay the emergence of resistance. AMPs seem to be a good candidate for further investigations in the treatment of MRSA biofilms, alone or in combination with antibiotics.

**Key words:** Biofilm, Antimicrobial cationic peptides, antibiotic, MRSA
Introduction

*Staphylococcus aureus* is a human pathogen that can cause a range of illnesses, from minor skin infections to life-threatening diseases such as pneumonia, meningitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It is one of the most common causes of nosocomial infections and is often the cause of postsurgical wound infections. Methicillin-resistant *S. aureus* (MRSA) strains which have become resistant to most antibiotics, are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections (16, 20, 25). The danger of MRSA infections results from not only the emergence of multi-drug resistance but also the occurrence of strong biofilm forming bacteria.

A biofilm is a cluster of microorganisms which they attached to surfaces and produced extracellular polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings. Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause several infections in patients with indwelling inert surfaces like medical devices for internal or external use. An appreciation of the role of biofilms in infection should enhance the clinical decision-making process. The microbial cells, growing in a biofilm, are physiologically distinct from planktonic cells of the same organism. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated (10, 13).

Antimicrobial cationic peptides (AMPs) are major components of the innate immune system that plays an important role in host defense against the environmental microorganisms and are widely distributed in nature, existing in organisms from insects to plants and microorganisms to mammals. AMPs have rapid action and a broad spectrum of activity against...
infectious agents including Gram negative and positive bacteria, fungi, viruses and parasites. Furthermore, cationic peptides are not affected by many antibiotic-resistance mechanisms that are limiting the use of other antibiotics (15, 33). There are many kind of AMPs were found active against bacterial biofilms (7, 8, 17, 24). Therefore, we tried to determine whether or not the indolicidin, cecropin A (1-7)-melittin A (2-9) amide (CAMA) and nisin shows in-vitro anti-biofilm activity alone or in combination with antibiotics which are routinely used in clinics against MRSA biofilms.

**Materials and methods**

**Bacterial strains:** Methicillin resistant *S. aureus* (MRSA) ATCC 43300 was used in this study and *S. aureus* ATCC 29213 was used to verify the accuracy of the microdilution test procedure for antibiotics.

**Antimicrobial substances:** Two AMPs, CAMA and indolicidin were obtained from Bachem AG, nisin was obtained from Sigma-Aldrich and all other antibiotics such as daptomycin, linezolid, teichoplanine, ciprofloxacin and azithyromycin were kindly provided by their respective manufacturers. Stock solutions from dry powders were prepared according to the manufacturers’ recommendation and stored frozen at –80°C for up to six months.

**Media:** Media used in this study were tryptic soy broth supplemented with 1% glucose (TSB-glucose, Difco Laboratories) for biofilm production, cation adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories) for minimum inhibitory concentration (MIC), minimum biofilm eradication concentration (MBEC) and microbroth checkerboard technique, and tryptic soy agar (TSA, Difco Laboratories) for minimum bactericidal concentrations (MBC) and MBEC determinations and colony counts.
MIC and MBC determinations: MICs of antibiotics and AMPs were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) (5). The MIC was defined as the lowest concentration of antibiotic producing complete inhibition of visible growth.

MBCs were determined at the end of the incubation period by removing two 10 µl samples from each well demonstrating no visible growth and plated onto TSA. Resultant colonies were counted after an overnight incubation at 37°C. The MBC was defined as the lowest concentration of antimicrobials producing at least 99.9% killing of the initial inoculums (19).

High-inoculum MIC determinations: High-inoculum MICs of antibiotics and AMPs were determined by microbroth dilution technique as described by the CLSI with the following modification (5). MRSA ATCC 43300 was diluted to give a final concentration of approximately 5×10⁷ cfu/ml instead of 5×10⁵ cfu/ml, before added to the wells. The MIC was defined as the lowest concentration of antibiotic producing complete inhibition of visible growth.

Determination of fractional inhibitory concentration index (FICI): The effects of antibiotics and AMPs in combination were assessed by using the microbroth checkerboard technique (23). Each microtitre well containing the mixture of antibiotics was inoculated with a 4-6 hours broth culture diluted to give a final concentration of approximately 5×10⁵ cfu/ml. After incubation at 37°C for 18-20 hours, the FICI was determined as the concentration at the combination divided by the single concentration. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth. With this method, synergy was defined as a FICI of ≤ 0.5, no interaction as a FICI of > 0.5–4 and antagonism as a FICI of > 4.0.
Biofilm formation: MRSA ATCC 43300 was cultured in 5 ml TSB-glucose with rotation (50 rpm) at 360°, for 24 hours at 37°C and diluted 1/50 in fresh TSB-glucose to give a final concentration of approximately 1×10^7 cfu/200 µl. This suspension was added to each well of a 96-well tissue-culture microtitre plates (Greiner) and incubated at 37°C, 24 hours. The negative control was TSB-glucose. After the incubation, the waste media was aspirated gently and the wells of the plates were washed three times with 250 µl physiological buffered saline (PBS) solutions to remove unattached bacteria and air-dried. 200 µl of 99% methanol was added per well for 15 minutes for fixation and aspirated, and plates were allowed to dry. Wells were stained with 200 µl of 0.1% crystal violet (in water) for 5 minutes. Excess stain was gently rinsed off with tap water, and plates were air-dried. Stain was resolubilized in 200 µl of 95% ethanol, shaking in orbital shaker for 30 minutes and measured at OD595 nm (11).

Biofilm attachment assay: Biofilm attachment assays were performed as previously described method with some modifications (22). The overnight culture of MRSA ATCC 43300 (diluted 1/50 to give 1×10^7 cfu/200 µl in TSB-glucose) was added to each well of a 96-well tissue-culture microtitre plates with 1/10xMIC of AMPs, antibiotics, and their combinations. The plates were incubated 1, 2, and 4 hours at 37°C. Six wells were used for each AMP, antibiotic, and combination. The positive control is MRSA ATCC 43300 in TSB-glucose without peptide or antibiotic. After the incubation, wells were washed with PBS solutions and measured at OD595 nm.

Inhibition of biofilm formation: MRSA ATCC 43300 (1x10^5 cfu/200 µl) in TSB-glucose was incubated at 37°C, 24 hours with AMPs, antibiotics, and their combinations at 1xMIC, 1/10xMIC, and 1/100xMIC in 96 well tissue-culture microtitre plates. Six wells were used for each AMP, antibiotic, and combinations. The positive control is MRSA ATCC 43300 in TSB-
glucose without peptide or antibiotic. After the incubation, wells were washed with PBS solutions and measured at OD$_{595}$ nm.

**Minimum biofilm eradication concentration (MBEC):** Measurements of the antimicrobial susceptibilities of MRSA ATCC 43300 biofilms were performed as previously described MBEC assay with the following modifications (3). The 24 hours biofilms in a 96 well tissue-culture microtitre plates were washed three times with 250 µl PBS solutions and air-dried. Serial two-fold dilutions ranging from 640 to 0.06 mg/L for AMPs and 5120 to 5 mg/L for antibiotics were prepared in CAMHB. 200 µl of each concentration was added to each corresponding well and plates were incubated 24 hours at 37°C. After the incubation, antibiotics were aspirated gently and plates were washed two times with sterile PBS solutions, wells were scraped thoroughly, with particular attention to well edges. Well contents were removed, placed in 1 ml PBS solution, placed in a sonicating waterbath (Bandelin sonopuls HD 2200) for 5 minutes to disrupt the biofilm, and 100 µl samples were plated on TSA. Colonies were counted after 24 hours at 37°C. The MBEC was defined as the lowest concentration of AMP or antibiotic that prevented bacterial regrowth.

**Antibacterial activities of combinations on biofilm:** The effects of antibiotics and AMPs in combination against biofilms were determined by using the modified microbroth checkerboard technique (23). The 24 hour biofilms in a 96 well tissue-culture microtitre plates were washed three times with 250 µl PBS solutions and air-dried. Combinations of antibiotics and AMPs were tested at five concentrations starting from their 2xMIC. After incubation of biofilms (including 1x10$^7$ cfu/ml cells) with antibacterial combinations at 37°C for 24 hours, antibiotics were aspirated gently and plates were washed two times with sterile PBS solutions, wells were scraped thoroughly, with particular attention to well edges. Well contents were removed, placed in 1 ml PBS solution, placed in a sonicating waterbath for 5 minutes to disrupt the biofilm, and 100 µl...
samples were plated on TSA. Colonies were counted after 24 hours at 37°C. The FICI was determined as the concentration at the combination divided by the single concentration. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth. With this method, synergy was defined as a FICI of ≤ 0.5, no interaction as a FICI of > 0.5–4 and antagonism as a FICI of > 4.0.

Statistically analysis: All experiments were analyzed using two independent assays. To determine MIC, MBC, MBEC, and FICI, when the results were different in both experiments, we made another test for final result. For the biofilm attachment and inhibition of biofilm formation assays, the results are shown as the mean ± standard deviation of two independent experiments. One-way ANOVA with the Bonferroni multiple comparison test was used to compare differences between the control and antimicrobial-treated biofilms. A \( p \) value < 0.001 was considered statistically significant.

Results

Susceptibility: The in vitro activities of the studied antibiotics and AMPs against the MRSA ATCC 43300 planktonic cells, high-inoculum planktonic cells and biofilms are summarized in Table 1. The MIC values of the antibiotics against the quality control strain \( S. aureus \) ATCC 29213 were within the accuracy range in CLSI throughout the study (6). There was no major difference between bactericidal and inhibitory concentrations of the bactericidal antibiotics and AMPs. The MBC values were generally equal to or two-times greater than those of the MIC values (data not shown).

Checkerboard: The results of combination studies, where antibiotics were used in combination with AMPs and antibiotics against the MRSA ATCC 43300 planktonic cells and biofilms are shown in Table 2 and Table 3. With a FICI of ≤ 0.5 as borderline, synergistic
interactions were frequent with almost all of the combinations against the MRSA ATCC 43300 biofilm. No antagonism was observed with any combination.

**Biofilm attachment assay:** When we incubated the 1/10xMIC of AMPs, antibiotics, and their combinations with MRSA ATCC 43300 in a 1, 2, and 4 hours at 37°C for the adherence to the wells of tissue-culture microtitre plates, all of the antimicrobial agents, alone or in combination, were inhibited the biofilm attachment according to time (Fig. 1).

**Inhibition of biofilm formation:** All of the studied AMPs, antibiotics, and their combinations have shown the significant inhibitor activity against MRSA ATCC 43300 biofilm formation at 24 hours according to their concentrations (Fig. 2).

**Discussion**

The anti-biofilm activities of antibiotics are becoming an important part of treating biofilm-related infections, such as chronic wounds or catheter-associated infections caused by MRSA. In this study, we investigated the in vitro activities of clinically available antibiotics and AMPs alone and in combination against MRSA planktonic cells (5 × 10^5 [standard] or 5 × 10^7 [high] cfu/ml per inoculum) and biofilms. Here, we show that the planktonic MRSA ATCC 43300 cells are susceptible to all of the examined antibiotics, except azithromycin, demonstrating MIC values between 0.125–1 mg/L. On the other hand, when we considering the anti-biofilm activities of these antibiotics, MBEC values ranged between 512–5120 mg/L and the MBEC/MIC ratio was between 10–8000-fold. Similar results have been noted by other researchers (18, 28). These results suggest that these high values might be due to not only the biofilm structure but also the differences between the planktonic and biofilm bacteria counts (5 x 10^5-5 x 10^7 cfu/ml). We tried to determine the antibiotic’s activities against high-inoculum planktonic cells, and the MIC values were found to be 80 mg/L, except for azithromycin. These findings indicate that the size
of the inoculum could change the MIC values by 80–640-fold in planktonic cells. Accordingly, the MBEC/MIC ratio was found to change between 5–26-fold.

When we determined three AMP’s antimicrobial and anti-biofilm activities against MRSA ATCC 43300, we found that the MIC, high-inoculum MIC, and MBEC values of them were within the ranges of 8–16, 20–40, and 640 mg/L, respectively. According to these results, the MBEC/MIC ratios with standard and high-inoculum MICs were within the range of 40–80 and 16–32-fold, respectively. Although the MIC values of the AMPs were not as low as the other antibiotics that were used in this study, it was notable to see that AMPs demonstrated activities with similar MIC, high-inoculum MIC, and MBEC values against MRSA and that there were not very high MBEC/MIC ratios like antibiotics. The differences between antibiotic and AMP activities might be due to their different structures and antibacterial mechanisms.

Because AMPs have desirable properties that make them excellent prospects for use as antimicrobial agents, they are one of the most preferred classes of antimicrobial substances for future use as treatments of serious infections, either alone or in combination with antibiotics, without demonstrating significant resistance problem (9). Among these, indolicidin, which is encoded by a member of the cathelicidin gene family and is classified as a cationic antimicrobial tridecapeptide amide, was isolated from cytoplasmic granules of bovine neutrophils (27). It is one of the shortest, a 13-residue AMP with extremely high tryptophan content that exhibits broad-spectrum antimicrobial and hemolytic activities (14). The antimicrobial actions of indolicidin are different from well-defined channel formation, by creating pores through the cell membranes or totally disintegrate the membrane structures (12). CAMA is a cecropin-melittin hybrid peptide that contains portions of the amino acid sequences of cecropin-A and melittin with differing properties. A series of hybrid peptides were created that consisted of the amphipathic \( \alpha \)-helical N-terminal region of cecropin-A and the hydrophobic N-terminal \( \alpha \)-helix of the bee venom peptide,
melittin. These hybrids form ion-permeable channels in model lipid membranes. Hybrid peptides demonstrate improved antimicrobial activities against Gram-positive bacteria with a significant reduction of the toxicity that come from melittin (2, 29). Nisin, which is a 34-residue peptide that can be isolated from the non-pathogenic bacterium *Lactococcus lactis*, belongs to a special group of AMPs called bacteriocins that because of having unusual amino acids (lanthionine and methyl lanthionine residues) emerged as one of the most extensively studied lantibiotics. Nisin demonstrates rapid bactericidal activities against Gram-positive bacteria, including multidrug-resistant pathogens. It is characterized by a dual mode of action against cell membranes. The first mode of action is characterized by the nonspecific recognition and binding of anionic lipids. The second was described as specific recognition and binding of lipid II. The complexes then aggregate, incorporate additional peptides, and form a pore within the bacterial membrane (26, 30, 34).

As shown by our findings, which are similar to those noted by others (18, 28), biofilm-associated bacteria do not affect therapeutically achievable concentrations of antimicrobial agents because of antimicrobial tolerance, persister cells, slow-growing cells, and the exopolysaccharide matrix. This biofilm-associated resistance, which is an excellent type of bacterial resistance, is provided by the sedentary life in the biofilms (1). The development of anti-biofilm therapeutics has generally focused on interfering the quorum sensing, inhibiting adhesion, enhancing dispersion, bacteriophage-based treatments, and a new options for the species-specific control of biofilms by selectively targeting antimicrobial peptides (4). An another feasible way to overcome biofilm-associated resistance is synergistic effects, which are provided by the use of antimicrobial agents in combination can result in the rapid increase in anti-biofilm activities and help to preventing or delaying the emergence of resistance.
Because animal and human data are difficult to obtain, decisions about the selection of optimal combinations are based on in vitro information. Microbroth checkerboard is the most simple and widely used technique, even if it does not always reliably show addition when agents were combined, to assess antimicrobial combinations (23, 31). Using the microbroth checkerboard technique with adequate controls and replication, synergistic interactions are frequently seen when using both antibiotic-antibiotic and AMP-antibiotic combinations to treat MRSA ATCC 43300 biofilm. When we evaluated the same combinations against planktonic cells, they generally demonstrated additive effects and synergism was rarely observed.

In addition to antimicrobial activities, AMPs serve as an anti-resistance compound to classic antibiotics (32). They are able to interact with bacterial membranes, creating ion-permeable channels which lead increased cytoplasmic membrane permeability and bacterial cell death. They may also allow the entry of other substances, like antimicrobial agents, to the inside of the cell (15). This mechanism explains how AMPs act in synergy with conventional antibiotics against planktonic cells. On the other hand, antimicrobial agents exhibit different mechanisms in order to overcome biofilm resistance, such as the specific physiological properties of biofilms that limit the efficacy of antibiotics and prevent the antimicrobial substance from reaching its target by limited diffusion or repulsion (21). The different checkerboard results that have been achieved against planktonic cells and biofilm could be explained by the differences between antibacterial and anti-biofilm mechanisms that used by antimicrobial agents.

The attachment of bacteria to the solid surface is critical for biofilm formation. Thus, many continuing studies are targeting the inhibition of this essential step. In this study, all of the antibiotics, AMPs, and their combinations were able to inhibit the attachment of bacteria at 1/10xMIC. Especially azithromycin, CAMA, and their combinations with other antibiotics or AMPs significantly decreased the attachment of MRSA to the surface in 1–2 hours ($p < 0.001$).
These inhibitions suggest the potential interactions between these AMPs and antibiotics with bacterial adhesions (8).

We were also able to determine that antibiotics, AMPs, and their combinations significantly inhibit biofilm formation, especially at 1xMIC and sub-MIC ($p < 0.001$). Although the inhibition of mature biofilm is very difficult and required to achieve very high concentrations of antimicrobial agents, the inhibition of biofilm formation during the early stages seems more applicable. Interestingly, the inhibition of biofilm formation using single antimicrobial agent and antibiotic-antibiotic or AMP-antibiotic combinations were not significantly different. These findings are comparable with the additive interactions against planktonic MRSA cells which provided from microbroth checkerboard assays, while synergism was observed against biofilm. These results suggest that the mechanisms involved in the eradication and inhibition of biofilms are very different.

**Acknowledgements**

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**References**


TABLE 1. In vitro antibacterial activities of antibiotics and AMPs against MRSA ATCC 3934300.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>MICs (mg/L)</th>
<th>MBEC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard inoculum*</td>
<td>High inoculum**</td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td>0.125</td>
<td>80</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Teichoplanine</td>
<td>0.25</td>
<td>80</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.5</td>
<td>80</td>
</tr>
<tr>
<td>Azithyromycin</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td><strong>Cationic peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indolicidin</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>CAMA</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Nisin</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

* Standard inoculum was 5x10^5 cfu/ml, ** High inoculum was 5x10^7 cfu/ml.
TABLE 2. In vitro activities of antibiotics in combination with AMPs against MRSA ATCC 43300 planktonic cells and biofilm.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Indolicidin</th>
<th>CAMA</th>
<th>Nisin</th>
<th>Indolicidin</th>
<th>CAMA</th>
<th>Nisin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daptomycin</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>0.50</td>
<td>0.375</td>
<td>0.375</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>0.375</td>
<td>0.375</td>
</tr>
<tr>
<td>Teichoplanine</td>
<td>2.0</td>
<td>2.0</td>
<td>0.75</td>
<td>0.375</td>
<td>0.50</td>
<td>0.25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.0</td>
<td>0.75</td>
<td>1.0</td>
<td>0.50</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.75</td>
<td>0.50</td>
<td>1.25</td>
<td>0.625</td>
<td>1.125</td>
<td>1.125</td>
</tr>
</tbody>
</table>
TABLE 3. In vitro activities of antibiotics in combination with antibiotics against MRSA ATCC 43300 planktonic cells and biofilm.

<table>
<thead>
<tr>
<th>Antibiotic combinations</th>
<th>Planktonic cells</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daptomycin-Teichoplanine</td>
<td>0.75</td>
<td>0.50</td>
</tr>
<tr>
<td>Daptomycin-Linezolid</td>
<td>2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Daptomycin-Ciprofloxacin</td>
<td>0.75</td>
<td>0.375</td>
</tr>
<tr>
<td>Daptomycin-Azithromycin</td>
<td>1.25</td>
<td>0.375</td>
</tr>
<tr>
<td>Teichoplanine-Linezolid</td>
<td>0.50</td>
<td>0.75</td>
</tr>
<tr>
<td>Teichoplanine-Ciprofloxacin</td>
<td>1.0</td>
<td>0.375</td>
</tr>
<tr>
<td>Teichoplanine-Azithromycin</td>
<td>1.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Linezolid-Ciprofloxacin</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>Linezolid-Azithromycin</td>
<td>0.75</td>
<td>0.50</td>
</tr>
<tr>
<td>Ciprofloxacin-Azithromycin</td>
<td>1.0</td>
<td>0.25</td>
</tr>
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</table>
FIG. 1. Inhibition of MRSA ATCC 43300 attachment to the surface in the studied antimicrobials. A) Antibiotics and AMPs alone; B) antibiotic + antibiotic combinations; and C) AMP + antibiotic combinations. Control bars indicate MRSA without any antimicrobial agent as 100% accepted. Each well of the 96-well plates contain 1/10xMIC of AMPs, antibiotics, or their combinations with 1×10⁷ cfu/200 µl MRSA in TSB-glucose. The plates were incubated for 1, 2, or 4 hours at 37°C. Six wells were used for each AMP, antibiotic, and their combinations. Each experiment is representative at ≥ 2 independent tests, and the error bars indicate the standard deviation. All differences between the control and antimicrobial-treated biofilms are statistically significant (p < 0.001).
FIG. 2. Inhibition of MRSA ATCC 43300 biofilm formation in the studied antimicrobials. A) Antibiotics and AMPs alone; B) antibiotic + antibiotic combinations; and C) AMP + antibiotic combinations. Control bars indicate MRSA without any antimicrobial agent as 100% accepted. Each well of the 96-well plates contain 1xMIC, 1/10xMIC, or 1/100xMIC of AMPs, antibiotics, or their combinations with 5×10^5 cfu/200 µl MRSA in TSB-glucose. The plates were incubated for 24 hours at 37°C. Six wells were used for each AMP, antibiotic, and their combinations. Each experiment is representative at ≥ 2 independent tests, and the error bars indicate the standard deviation. All differences between the control and antimicrobial-treated biofilms are statistically significant (p < 0.001).