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Synergic combinations of antimicrobial peptides (AMPs) against biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) on polystyrene and medical devices

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Highlights

1. MRSA biofilms are the most frequent cause of a catheter-related infections

2. Need of new approaches versus health care-acquired MRSA infections

3. Ability of selected AMPs combinations to eradicate preformed biofilms on PSS and CVC

4. AMPs as prophylactic or therapeutic tool for control biofilm and related infections

Abstract

Objectives: Antimicrobial research is being focused to look for more effective therapeutics against antibiotic-resistant infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). In this direction, antimicrobial peptides (AMP) appears a promising solution. The aim of the present study was to investigate the potential activity of Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 in synergic activity against MRSA biofilms developed on polystyrene surface (PSS) and central venous catheter (CVC).

Methods: The research was subdivided into distinct phases to assess the ability of AMPs to inhibit biofilm formation, to identify a possible synergy between AMPs, and to eradicate preformed biofilms on PSS and CVC using AMPs alone or in combination.

Results: The activity of the AMPs was particularly evident in the inhibition of biofilm formation on PSS and on CVC, while the eradication of preformed biofilms was more difficult and was reached only after 24h of contact. The synergic activity of AMPs combinations, selected by their FICI, has led to an improvement in the performance of all the molecules in the removal of different biofilms.

Conclusions: Overall, AMPs could represent the next generation of antimicrobial agents for a prophylactic or therapeutic tool to control biofilm of antibiotic-resistant and/or biofilm-associated infections on different medical devices.

Keywords: synergistic activity, antimicrobial peptides, *Staphylococcus aureus* (MRSA), biofilms, medical devices.

1. Introduction

Biofilms pose a serious problem for public health because the ongoing use of antibiotics increased the number of bacteria becoming resistant to them and their potential to cause severe infections in patients with indwelling inert surfaces, such as catheters for internal or external use. Different control strategies are emerging, including the identification of new antibiotics, anti-biofilm molecules, or antimicrobial peptides (AMPs). AMPs are one of the most promising antibacterial drugs that have interesting advantages and potential applications. Their property of membrane permeabilization makes them uniquely effective in the rapid killing of multidrug-resistant bacteria [**1**] and against both dormant and growing cells, irrespective of the cells' metabolic state. In addition, their charge promotes interaction with negatively charged bacterial surfaces [**2**], interfering with metabolic processes or with intracellular targets that may result in inhibition of cell wall synthesis, nucleic acid synthesis, and protein production [**3**]. Moreover, for their singular multimodal action, AMPs are less likely to promote the development of antimicrobial resistance [**4**-**6**].

Staphylococcus aureus is considered the leading cause of human disease not only in hospitalized individuals but also in individuals living in a community and responsible for a variety of diseases involving gastrointestinal, respiratory, skin and soft tissue, and bloodstream infections [**7**]. Biofilms formed by *S. aureus* are among the most frequent causes of a catheter-related infection (CRI) associated with significant morbidity and mortality [**8**]. Moreover, intraluminal colonization of the central venous catheter (CVC) is frequently caused by methicillin-resistant staphylococci embed in biofilm layer in the lumen of the catheter, resulting in catheter-related bloodstream infections. Given the cost, difficulty, and complications associated with the removal of a long-term CVC and the insertion of a new CVC at a different site, the susceptibility to AMPS of *S. aureus* organized in biofilm have been studied [**9**-**12**]. It can be noted that the activity of AMPs was assessed against bacterial infections, in association with antibiotics commonly used in clinical therapy [**13**-**15**], while their potential alone or in combination is poorly investigated.

For these reasons, in the present research we investigated the potential activity of selected AMPs (Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2), alone or in dual combinations, against MRSA biofilms developed on different surfaces. The experimental design included three distinct phases: i) the inhibition of biofilm formation on polystyrene surface (PSS) and central venous catheter (CVC); ii) the identification by checkerboard test of the possible synergies between AMPs; iii) the treatment of preformed biofilms on PSS and CVC with AMPs alone and in their combinations.

2. Materials and methods

2.1. Antimicrobial peptides (AMPs)

Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 were used in this study (**Table 1**). The stock solutions (5 mg/mL) of Citropin 1.1, Temporin A and CA(1–7)M(2–9)NH_2 were prepared in distilled water, while that of Pal-KGK-NH_2 in dimethyl sulfoxide (DMSO) (Sigma, Italy). All the solutions were

sterilized by 0.22 µm filter (VWR, Italy) and keep at 4 °C in the dark. The Minimum Inhibitory Concentration (MIC) of each AMP (**Table 2**) on planktonic microorganisms was previously determined [**1**].

2.2. Bacterial strains

Six clinical *Staphylococcus aureus* methicillin-resistant strains (MRSA), isolated from wounds and skin lesions at Microbiology Laboratory of "Ospedali Riuniti" (Ancona, Italy), were selected for this study: *S. aureus* 357426, *S. aureus* 355872, *S. aureus* 348839, *S. aureus* 354432, *S. aureus* 350355, *S. aureus* 360212. The MRSA reference strain *S. aureus* ATCC 43300 was also included. The strains were characterized for their antimicrobial susceptibility by broth micro-dilution with Vitek 2 system (bioMérieux), using the Vitek 2 Gram-positive identification cards according to the manufacturer's directions, as previously described in Ciandrini et al [**1**]. All the strains were maintained in Tryptic Soy Agar (TSA) (VWR, Milan, Italy) and stored at -20 °C in Tryptic Soy Broth (TSB) (VWR) supplemented with 20% glycerol.

2.3. Congo Red assay

The slime production was determined by Congo Red assay. For this, all the MRSA strains were cultivated on Congo Red agar prepared with Brain Heart Infusion agar (BHIA) (Oxoid, Milan, Italy), 0.08% Congo Red stain (w/v) (Sigma, Milan, Italy) and 5% sucrose (w/v) (Sigma). The medium was then autoclaved at 121 °C for 15 min. After cooling, Congo Red agar was distributed in plastic petri dishes and used for the assay until 24 h. Single colonies of each MRSA strain were streaked on the surface of Congo Red agar plates and incubated at 37 °C under aerobic condition for 24 h. The biofilm producer strains were identified as those forming black colonies while the non-biofilm producer strains as those with red colonies [**16**].

2.4. Biofilm formation inhibition on polystyrene surface (PSS) and central venous catheter (CVC)

The strains were grown in 10 ml of TSB (VWR) at 37 °C for 24 h and then the turbidity was adjusted to an $OD_{610\ nm}$ (0.13-0.15) corresponding to about 10^7 CFU/mL. Each bacterial suspension was inoculated in 24-well polystyrene plates (VWR) with the right amount of each AMP at their relative MIC and 2X MIC values (final volume 1 mL/well). Two wells were inoculated with each MRSA strain in TSB (VWR) as controls. The plates were incubated for 24 h at 37 °C to allow biofilm development. Then, the unbound bacteria were kindly removed by aspiration and the wells were washed in PBS pre-warmed at 37°C. At this point, 2 mL of Crystal Violet 0.1% (w/v) (CV, Sigma) were added for 15 minutes, after which the wells were PBS washed again and air-dried and 2 mL of ethanol 95% (Sigma) were added for 15 min. At the end, 200 µL of each sample were transferred to a 96-well plate and analyzed by spectrophotometer at 570 nm (Multiscan Ex Microplate Reader) (Thermo Scientific, Italy). Each data point was averaged from at least 8 replicate wells. All data were expressed as the mean of three independent experiments performed in duplicate.

In parallel, central venous catheter (CVC) (5Fr x 55 cm; Alfamed, Italy) were cut in 0.2 cm pieces, sterilized in 70% ethanol for 1.5 h and left to dry under flow cabinet. Then, the sterilized catheters segments were placed in 96-wells plate (VWR) and covered with each MRSA bacterial suspension (about 10^6 cells/mL) and the right

amount of each AMP at their relative MIC and 2X MIC values (final volume 0.2 mL/well). After 24 h of incubation at 37 °C, each CVC segment was gently transferred in a new sterile 96-wells plate, washed by pre-warmed PBS and analysed for biomass production as described above.

2.5. Checkerboard test

Checkerboard assays were performed in 96 polypropilene wells (VWR) as described by Jorge et al. [**14**]. Planktonic cells (5 x 10^5 CFU/mL) of each strain were inoculated in Mueller Hinton II with different combinations of two peptides (concentrations ranges from 0.03 µg/mL to 64 µg/mL) and the plates were incubated at 37°C for 24 h. The Fractional Concentration Index (FICI) was determined following the indication of Jorge et al. [**14**] and, for each combination, the FICI was calculated and interpreted as synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1), indifferent (1 < FICI ≤4) and antagonist (FICI > 4.0).

2.6. Eradication of preformed biofilms from polystyrene surface (PSS) and central venous catheter (CVC)

Preformed biofilms, obtained as described above, were once PBS washed and left in contact for a relative brief time, specifically 4 and 6 h, to simulate a short-term therapeutic approach with Citropin 1.1, Temporin A, Pal-KK-NH_2 and CA (1-7)M(2-9)NH_2 alone (at 2X MIC and 4X MIC) or with their combinations at FICI concentrations. In order to mimic a possible long-term therapeutic approach, the contact time of AMPs alone or in combination was prolonged for 24 h. In the case of AMPs alone, peptides were used at low concentration (2X MIC), while when used in combinations related FICI concentrations were utilized. For each plate, two wells were treated with physiological saline (negative controls). At each time point, treated and untreated samples of PSS and CVC were PBS washed and the eventual reduction in biomass production was verified by CV staining as described above.

2.7. Statistical analysis

Statistical analysis was performed using the Prism 5.0 program (GraphPad Software, Inc., La Jolla, USA). All data were expressed as the mean of three independent experiments performed in duplicate. The assumptions for parametric test were checked prior carrying out the analysis. When the assumptions for parametric test were not respected, non-parametric tests (Mann Whitney, Kruskall-Wallis) with Dunn's multiple comparing tests were used. The significance level was always considered with $\alpha = 0.05$.

3. Results

3.1. Slime production

Among the MRSA screened to evaluate their ability to produce “slime”, only *S. aureus* 357426, *S. aureus* 355872, *S. aureus* 348839 and the reference strain were defined as “slime producers”, resulting in numerous black colonies on Congo Red Agar plates (**Fig. 1**). For this reason, these strains were selected for all the subsequent experiments.

3.2. Effectiveness of AMPs in inhibiting biofilm formation on PSS and CVC

The activity of each tested AMP at MIC values on planktonic microorganisms resulted negligible (data not shown), while more interesting results were observed at 2X MIC values (**Fig. 2**). In the case of biofims developed on PSS, the most active AMP resulted to be Citropin 1.1 that inhibited the biofilm formation of all MRSA strains ($p < 0.001$), compared to the relative untreated controls, at concentrations (2X MIC) ranging from 32 to 128 µg/mL. A similar trend was observed for CA(1-7)M 2-9)NH_2 that at 16 µg/mL induced a statistically significant reduction ($p < 0.001$) for all the strains with the exception of *S. aureus* 348839 (2X MIC 32 µg/mL). In the case of Temporin A (2X MIC 16 µg/mL), statistically significant reductions ($p < 0.001$) were observed for *S. aureus* 357426 (OD_{570} 0.022) and *S. aureus* ATCC43300 (OD_{570} 0.047) compared to the relative controls (OD_{570} 0.502 and 0.344 respectively). On the contrary, Pal-KGK-NH_2 at 2X MIC concentrations ranging from 2 to 64 µg/mL resulted ineffective in inhibiting biofilm formation of all the tested MRSA (**Fig. 2 A**).

In the case of biofilms formed on CVC, the efficacy of AMPs was lower than that found in biofilms developed on polystyrene. Indeed, the peptides showing a statistically significant biomass reduction were Temporin A and Citropin 1.1. In particular way, the incubation of *S. aureus* 357426 and *S. aureus* ATCC 43300 with Temporin A (2X MIC 16 µg/mL) determined OD_{570} values equal to 0.112 and 0.154 respectively ($p < 0.05$). The presence of Citropin 1.1 (2X MIC 64 µg/mL) caused a biomass reduction for the reference strain with an OD_{570} of 0.152 compared to control (OD_{570} = 0.347) (**Fig. 2 B**).

3.3. Determination of FICI indices

The FICIs on planktonic bacteria indicated that CA(1-7)M(2-9)NH_2 in combination with Temporin A and Citropin 1.1 resulted always synergic with FICI ranging from 0.25 to 0.5, while in the combinations with Pal-KGK-NH_2 in some cases the FICI resulted to be indifferent (1.5 for *S. aureus* 357426 and 2.5 for *S. aureus* 355872). On the contrary, the combinations of Pal-KGK-NH_2 with Temporin A or Citropin 1.1 showed FICI mostly antagonist or inhibitory, with a synergism observed only for *S. aureus* 357426 (FICI 0.38). Similarly, the combination Citropin 1.1 with Temporin A showed synergic activity for *S. aureus* 357426 (0.38) and *S. aureus* 355872 (0.31) but resulted to be additive against the other two strains (**Table 3**).

3.4. Eradication of preformed biofilms on PSS and CVC using AMPs alone

The effectiveness of AMPs at 2X MIC values after 4 and 6 h of contact was negligible (data not shown), whereas data relative to 4X MIC were reported in **Figure 3-4**. As regard the biofilms preformed on PSS, the treatment for 4 h with Temporin A resulted in a remarkable removal of biofilms formed by *S. aureus* 357426 ($OD_{570\ nm}$ 0.347) (4X MIC 32 µg/mL) and *S. aureus* 348839 ($OD_{570\ nm}$ 0.311) (4X MIC 16 µg/mL) compared to the untreated controls ($OD_{570\ nm}$ 0.463 and 0.480 respectively) ($p < 0.01$) (**Fig. 3 A**). Similarly, after 6h of contact a noticeable reduction of biomass was also observed in biofilm formed by *S. aureus* 355872 ($OD_{570\ nm}$ 0.376) (4X MIC 16 µg/mL) in comparison to the related control ($OD_{570\ nm}$ 0.563) ($p < 0.01$) (**Fig. 3 B**). Citropin 1.1 was active after 4 h of contact, resulting able to remove biofilms of all the examined strains after

6 h of exposure. Specifically, after 4h of contact, the biomass of *S. aureus* 355872 biofilm reached an OD of 0.408 (4X MIC 64 µg/mL) compared to 0.523 of the untreated control ($p<0.01$); as expected, in the case of *S. aureus* 348839 and the reference strain (4X MIC 64 and 128 µg/mL), OD of 0.203 and 0.314 respectively were measured, lower than the ODs of the related untreated biofilms (0.480 and 0.544) ($p<0.01$) (**Fig. 3A**). In any case, the most marked removal activity was registered after 6 h of treatment with Citropin 1.1, with statistically significant OD_{570} values against all the MSRA strains compared to the related untreated biofilms ($p <0.01$ and $p <0.001$) (**Fig. 3B**). CA(1-7)M(2-9)NH_2 showed to be effective after 4 h in remove biofilms produced by *S. aureus* 357426 ($OD_{570\ nm}$ 0.177) (4X MIC 32 µg/mL), *S. aureus* 348839 ($OD_{570\ nm}$ 0.203) (4X MIC 32 µg/mL) ($p <0.01$) and *S. aureus* ATCC 43300 (OD 570 nm 0.363) (4X MIC 32 µg/mL) ($p<0.05$) in comparison to the OD values of the untreated controls (0.463, 0.480 and 0.544 respectively); moreover, after 6 h of contact, this peptide resulted to be effective against all clinical isolates ($p <0.001$) (**Fig. 3 B**). On the contrary, Pal-KGK-NH_2 (4X MICs ranging from 4 to 128 µg/mL) did not result to be particularly effective in remove biofilms from PSS after 4 and 6 h of treatment (**Figs. 3 A and 3 B**).

As regards the eradication of biofilms preformed on CVC, after 4 h of exposure to the examined AMPs at 4X MIC values, the biofilms resulted not compromised in their biomass and only Citropin 1.1 and CA(1-7)M(2-9)NH_2 were able to affect biofilms of *S. aureus* 355872 (OD 0.213 and 0.226) and the reference strain (OD 0.252 and 0.249) compared to the related untreated biofilms (OD 0.329 and 0.391)($p<0.05$) (**Fig. 4 A**). On the contrary, the eradication activity has been observed after 6 h treatment with Temporin A (4X MICs values), which removed the biofilms of all the examined MRSA strains ($p <0.05$). Similarly, Citropin 1.1 (4X MIC 128 µg/mL) induced a statistically significant reduction on biofilms of *S. aureus* 357426 (OD 0.108) ($p <0.01$), and CA(1-7)M(2-9)NH_2 (4X MIC 128 µg/mL) showed a marked activity against all the MRSA strains ($p <0.05$; $p <0.01$), while Pal-KGK-NH_2 did not determine a significant biomass reduction (**Fig. 4 B**).

A general improvement in the "performance" of each molecule at their 2X MIC values was observed against biofilms preformed on PSS and CVC when the contact time was extended to 24 h (**Fig. 5 A-B**). Indeed, as regards PSS preformed biofilms, Temporin A induced a significant decrease in biomass values ($p <0.01$) for all strains tested, and Citropin 1.1 was effective against *S. aureus* 357426 (OD_{570} 0.252) and *S. aureus* ATCC 43300 (OD_{570} 0.160) compared to the related controls (OD_{570} 0.572 and 0.525). Concerning CA (1-7)M(2-9)NH_2, a statistically significant reduction was observed in the case of *S. aureus* 357426 ($p <0.01$) and, noticeable, the long-term treatment with Pal-KGK-NH_2 resulted in a statistically significant reduction in biofilms of *S. aureus* 355872, *S. aureus* 348839 and the reference strain ($p <0.05$) (**Fig. 5 A**). A Similar trend was observed in the case of CVC preformed biofilms, with a remarkable decrease in biomass values in most of the examined strains after 24 h of contact with the different AMPs at 2X MIC concentration ($p <0.01$) (**Fig. 5 B**).

3.5. Eradication of preformed biofilms on PSS and CVC using AMPs in synergic combination

The two combinations CA(1-7)M(2-9)NH_2 with Temporin A and CA(1-7)M(2-9)NH_2 with Citropin 1.1 showed a remarkable eradication of biofilms generated on PSS in comparison to that observed with every

single AMP alone ($p<0.05$). This is particularly evident in the case of *S. aureus* 348839 biofilms treated with CA(1-7)M(2-9)NH_2 with Temporin A that reached an OD of 0.121 in comparison to 0.495 and 0.290 obtained with CA(1-7)M(2-9)NH_2 and Temporin A alone respectively. Similarly, in the biofilms of *S. aureus* 355872 treated with the same combination, the recovered biomass showed an OD of 0.279, lesser than 0.358 and 0.312 reached by the single AMP (CA(1-7)M(2-9)NH_2 and Temporin A respectively) (**Fig. 6 A**). Analogously, regarding biofilms of *S. aureus* 357426 treated with the second combination (CA(1-7)M(2-9)NH_2 with Citropin 1.1), the recovered biomass showed an OD of 0.135 in comparison to 0.235 and 0.252 OD values obtained by CA(1-7)M(2-9)NH_2 and Citropin 1.1 alone respectively. A similar trend was observed in the case of *S. aureus* 348839 biofilms treated with CA(1-7)M(2-9)NH_2 combined with Citropin 1.1 (OD 0.130) (**Fig. 6 A**).

Concerning the activity of AMPs combinations against biofilms formed on CVC, it can be noted that their efficacy was higher compared to those observed with every single AMP, but lesser if compared with the efficacy observed toward biofilms created on PSS (**Fig. 6 B**). Indeed, in the case of *S. aureus* 355872 biofilms treated with CA(1-7)M(2-9)NH_2 with Temporin A an OD of 0.179 was observed in comparison to 0.405 and 0.406 obtained with CA(1-7)M(2-9)NH_2 and Temporin A respectively. Similarly, in the biofilms of *S. aureus* 348839 treated with the second combination (CA(1-7)M(2-9)NH_2 with Citropin 1.1), the recovered biomass showed an OD of 0.171, lesser than 0.382 and 0.493 OD reached by the single AMP (CA(1-7)M(2-9)NH_2 and Citropin 1.1 respectively).

4. Discussion

Many AMPs have been discovered or synthesized in recent years as alternatives to antibiotics to contrast the problem of antimicrobial resistance [**11**,**17**,**28**]. Besides the characteristics of effective membrane-targeting, fast bactericidal action, low immunogenicity, low cytotoxicity and low risk of resistance, some of these AMPs possess anti-biofilm activity also against *S. aureus* [**19**]. The pathogenic effect of this microorganism can be attributed to different virulence factors, such as adhesion to cells, toxins, enzymes and chemotactic factors production [**20**,**21**]. In addition, *S. aureus* creates biofilms on the surfaces of catheters (intravenous catheters, urinary catheters, dialysis catheters etc.) and implanted medical devices (fluid shunts, joint prostheses and pacemakers) resulting resistant to antibiotics and innate host defense [**19**].

In the present work, the potential activity of four AMPs, Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2, against MRSA biofilms developed on PSS and CVC was assessed. As regards the ability to inhibit biofilm formation on these surfaces, we can point out that the best "performances" of the examined AMPs have been observed against biofilms developed on PSS compared to those developed on CVC. These data stressed the problem of methicillin-resistant staphylococci in biofilms formed on catheters and the need to find molecules that can inhibit their development [**22**]. Among the infections associated with medical devices, those involving the use of venous catheters are particularly relevant, because bacteria can migrate along the external and/or internal surface and form biofilms representing the starting point of infections [**23**]. The venous catheter replacement procedure can easily be associated with an infectious risk for the patient, so

the prevention of biofilm formation becomes an important strategy for controlling the associated infections. Indeed, a prophylactic approach can prevent biofilm development by killing planktonic cells theoretically able to create a biofilm, and, on the other hand, by blocking the adhesion or inhibiting the growth of cells already present in the early biofilm [**24**]. In this sense, our data evidenced the potential of the selected APMs at their 2X MIC values to inhibit biofilm formation also on surfaces particularly subjected to bacterial colonization, such as CVC. Considering that these molecules normally show MBC values against planktonic cells equal to 2X MIC values [**14,26**], this finding suggests that the observed activity of AMPs against MRSA biofilms is very likely corresponding to MBC values, thus ascribing their action to the killing capacity. Indeed, different mechanisms were assigned to AMPs but is generally accepted that they act mainly on cytoplasmic membrane. In the case of a bacterial biofilm, other possible mechanisms could be the interaction of AMPs with the matrix leading to its disruption or the interference with bacterial quorum sensing and, as consequence, with the adhesion of bacteria on solid surfaces [**25**].

The following step in our research was to evaluate the ability of selected AMPs to eradicate preformed biofilms from both the chosen surfaces (PSS and CVC). In this case, the biofilm removal activity was evaluated after a relatively short exposure times (4 and 6 h) using AMPs at their 2X MIC and 4X MIC values, as well as after a prolonged time (24 h) with AMPs at the lower concentration of 2X MIC. The reason leading to this choice was related to verifying if the active concentration of AMPs could be reduced prolonging the time of exposure. Our data revealed the higher capacity of the chosen AMPs at 4X MIC in removing biofilms from PSS surface compared to that observed in CVC in the short time of exposure, as already noted for biofilm formation inhibition on the same surfaces. Also at this high concentration, we can hypothesize a killing activity of AMPs on bacteria organized in biofilms, as observed at 2X MIC values during biofilm formation. Interestingly, the prolonged exposure for 24 h at lower concentrations (2X MIC) led to an improvement in the anti-biofilm effect of Temporin A, Citropin 1.1 and Pal-KGK-NH_2 on both the examined surfaces, revealing an effect time-dependent, and thus opening a new opportunity for therapeutic approaches.

The activity of AMPs is often reported associated with antibiotics to improve their effectiveness against biofilm-related infection treatment [**9,15**,**26,27**]. Wu and collaborators [**28**] reported as the association of different AMPs with azithromycin resulted in an increased antimicrobial effect on multi-drug resistant bacteria such as *S. aureus*. Similarly, Jorge et al. [**14**] investigated the synergistic effect of colistin and AMPs against two important pathogens, such as *Pseudomonas aeruginosa* and *S. aureus*, in single- and double-species biofilm cultures. Moreover, considering that bacteria in biofilms are embedded in a self-produced extracellular matrix constituted by a mixture of polymeric substances, the combination of AMPs with enzymes, EPS inhibiting agents, chelating agents, and matrix disaggregating agents resulted in a potentiating anti-biofilm activity [**29-31**]. On the contrary, only a few reports described the application of AMPs combined with peptide-based molecules or with other AMPs [**32-34**] and for this reason, in the last part of the present study, the selected AMPs were tested in combination with each other in order to assesses the real potential of AMPs to treat MRSA biofilm-associated infection. Two synergic AMPs combinations were selected and used on preformed biofilms, not considering those combinations showing additive, indifferent or antagonistic activity.

Effectively, different FICIs of the same combinations were obtained toward the selected MRSA strains (**Table 3**). This is particularly evident in the combinations of PAL-KGK-NH_2 with Temporin A or with Citropin 1.1 that were antagonistic against *S. aureus* 355872 but indifferent against *S. aureus* 348839; similarly, PAL-KGK-NH_2 with CA(1-7)M(2-9)NH_2 resulted to be indifferent against *S. aureus* 355872 but synergic against *S. aureus* 348839 and *S. aureus* ATCC 43300. This behaviour, even if difficult to explain, was also observed by Jorge et al. [**14**] that, testing the combination colistin with three different AMPs against several strains of S. *aureus*, found both indifferent and additive effects of the same combination. It can be observed that in *S. aureus* the two-component regulatory system (GraRS) appears involved in the responses to antimicrobial peptides, though with a not well-defined mechanism(s) [**36**]. In our case, having no additional information on the genetic profiles or metabolic characteristics other than the production of slime of the examined MRSA strains, a possible explanation of the apparently contradictory reported data might be the different susceptibility demonstrated by the strains to the AMPs in the antimicrobial assay (**Table 2**).

As regard the comparison of AMPs activity alone or in dual synergic combinations against biofilms on PSS or CVC, we can observe that, in general, the biofilms developed on polystyrene surface were more susceptible to the antimicrobial effect of AMPs compared to those on CVC, both in term of biofilm formation inhibition as well as eradication activity. Indeed, CA(1-7)M(2-9)NH_2 with Temporin A and CA(1-7)M(2-9)NH_2 with Citropin 1.1, differently from each single AMPs, were able to significantly reduce the biomass of all the examined MRSA strains after 24 h of exposure. This effect is less evident against biofilms developed on CVC and it can be hypothesized that, on this particularly narrow surface, the presence of the extracellular matrix surrounding the bacterial population constitutes an actual impediment to peptide penetration into the biofilm structure [**35**]. A particular interesting data was the observed ability of the tested microorganisms to colonize, after 24 h of incubation without the presence of AMPs, the surface of CVC in a way sometimes higher than that observed on PSS. This aspect assumes considerable importance because underlines the ability of MRSA strains to grow and organize on smooth and very little surfaces, such as central venous catheters considered difficult to colonize, and as possible consequence, to cause infections [**37**].

Overall, the obtained results indicating that synergistic combinations of AMPs are able to remove microorganisms organized in biofilm on polystyrene and venous catheters with an increased efficacy compared to the single tested molecule. For these reasons, AMPs and their combinations represent a promising template for the development of novel antimicrobials as a prophylactic or therapeutic tool for control biofilm of antibiotic-resistant strains and biofilm-associated infections.

Declarations

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Ethical approval not required.

Conflicts of interest None to declare.

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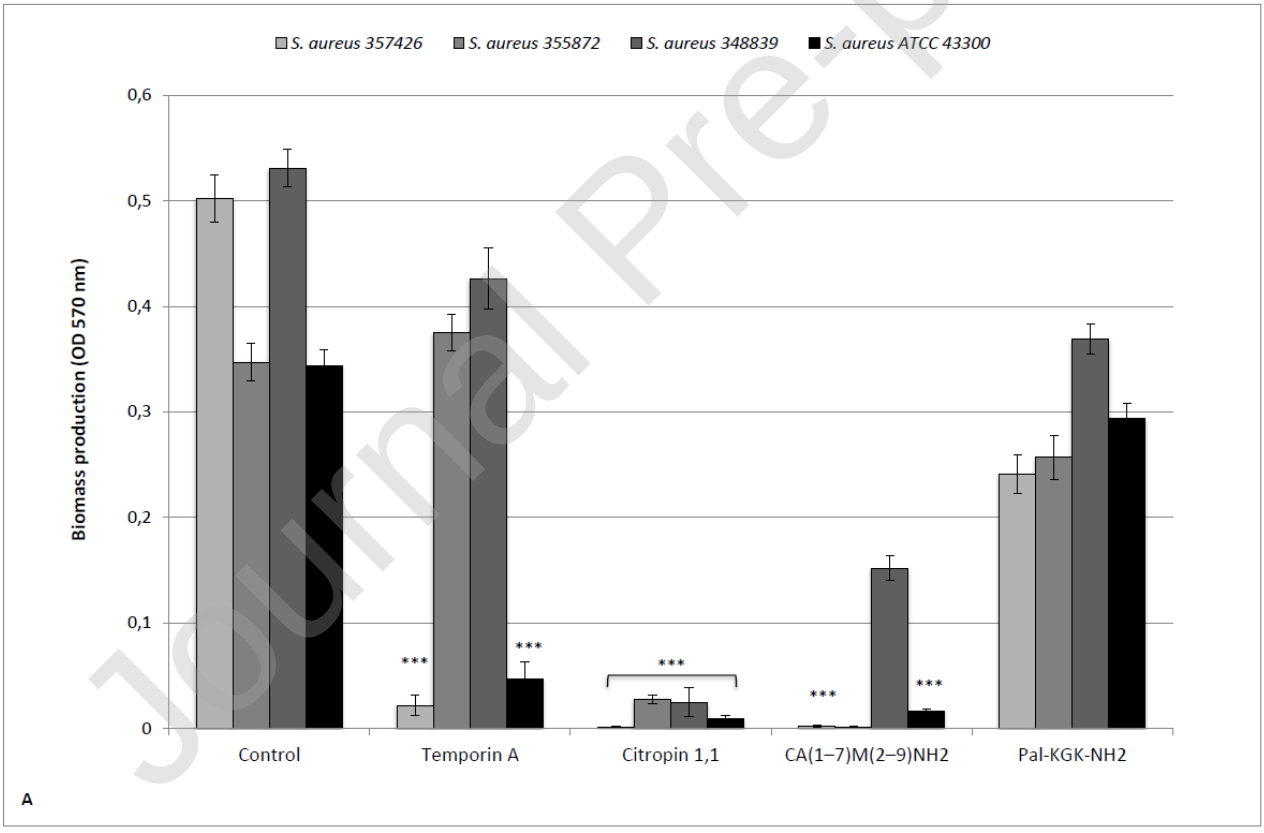
Figure Legends

Fig. 1 Results of the colony colour of MRSA strains grown on Congo Red agar. Black colonies identified *slime* producer strains (+), while red colonies not *slime* producer strains (-).

MRSA strains	Colonies on Congo Red agar (*slime* producer)
S. aureus 357426	Dark (+)
S. aureus 355872	Dark (+)
S. aureus 348839	Dark (+)
S. aureus 354432	Red (-)
S. aureus 350355	Red (-)
S. aureus 360212	Red (-)
S. aureus ATCC 43300	Dark (+)

Dark colonies (*slime* producer MRSA) Red colonies (not *slime* producer MRSA)

Fig. 2 Effect of Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 at their 2X MIC values on biomass production of MRSA strains during biofilms development (24 h at 37°C) on polystyrene (**A**) and central venous catheters (**B**). Data represent mean values of three independent experiments performed in duplicate and asterisks denote values statistically significant (*P<0.05, **P<0.01, ***P<0.001).



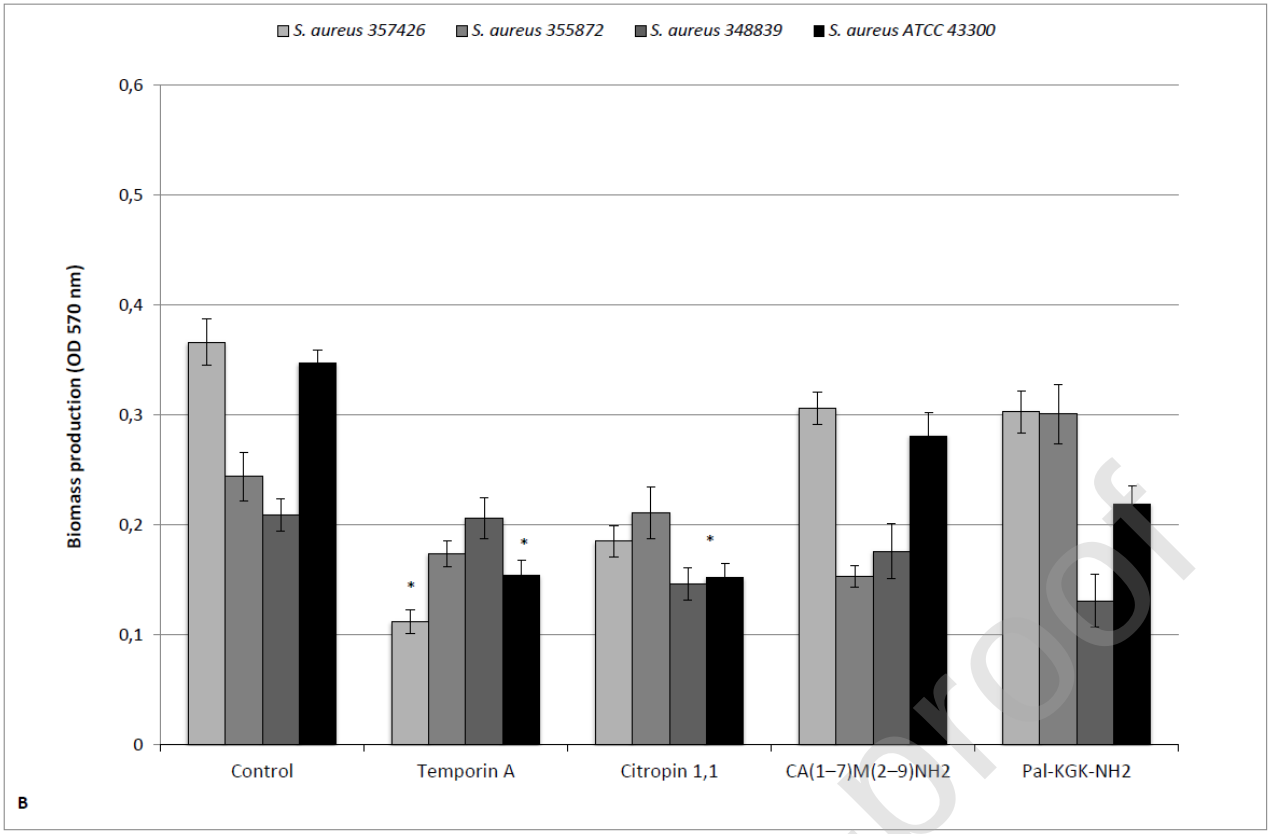


Fig. 3 Eradication of MRSA preformed biofilms on polystyrene after 4 h (**A**) and 6 h (**B**) of contact with Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 at their 4X MIC values. Data represent mean values of three independent experiments performed in duplicate and asterisks denote values statistically significant (*P<0.05, **P<0.01, ***P<0.001).

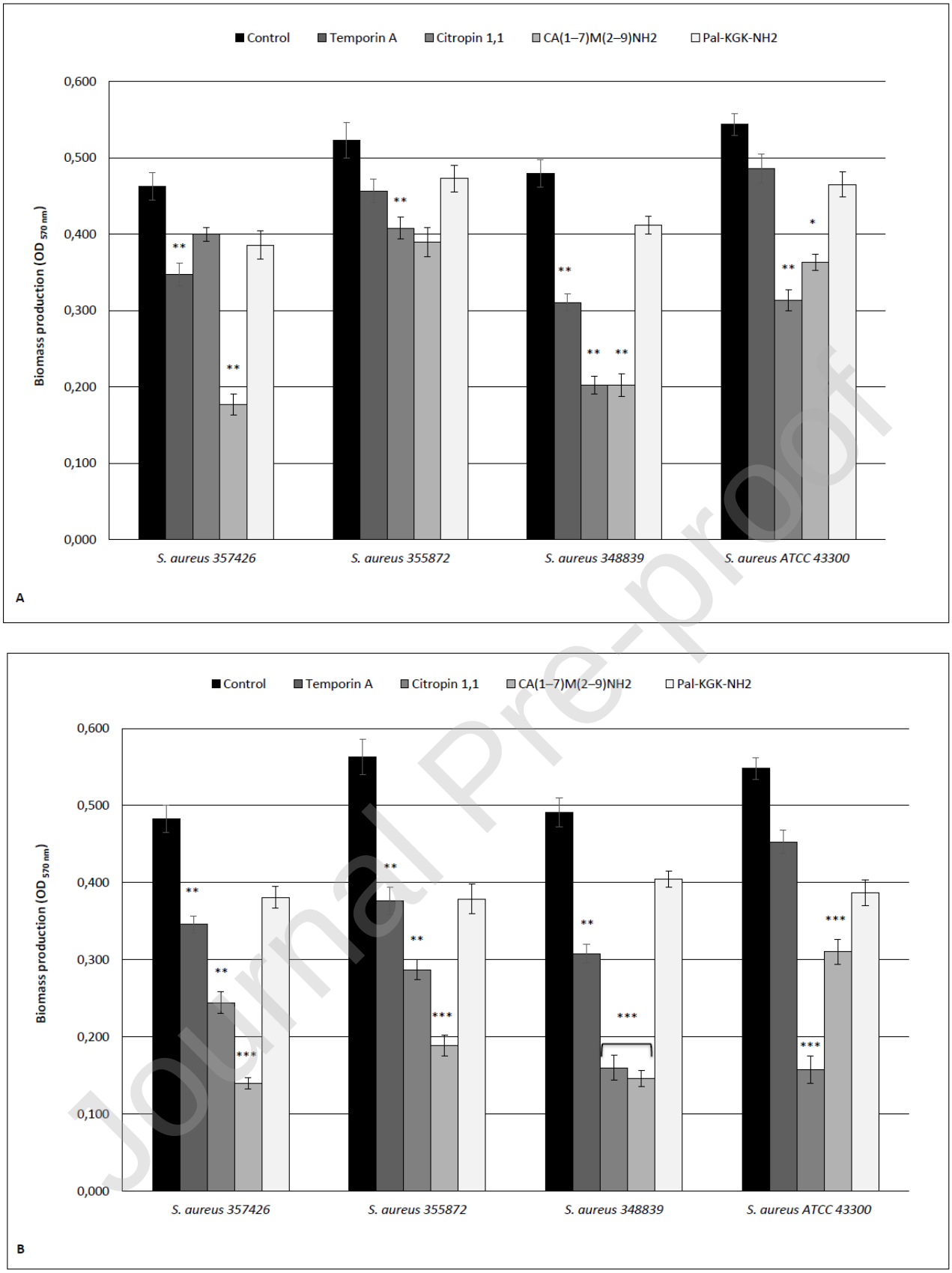
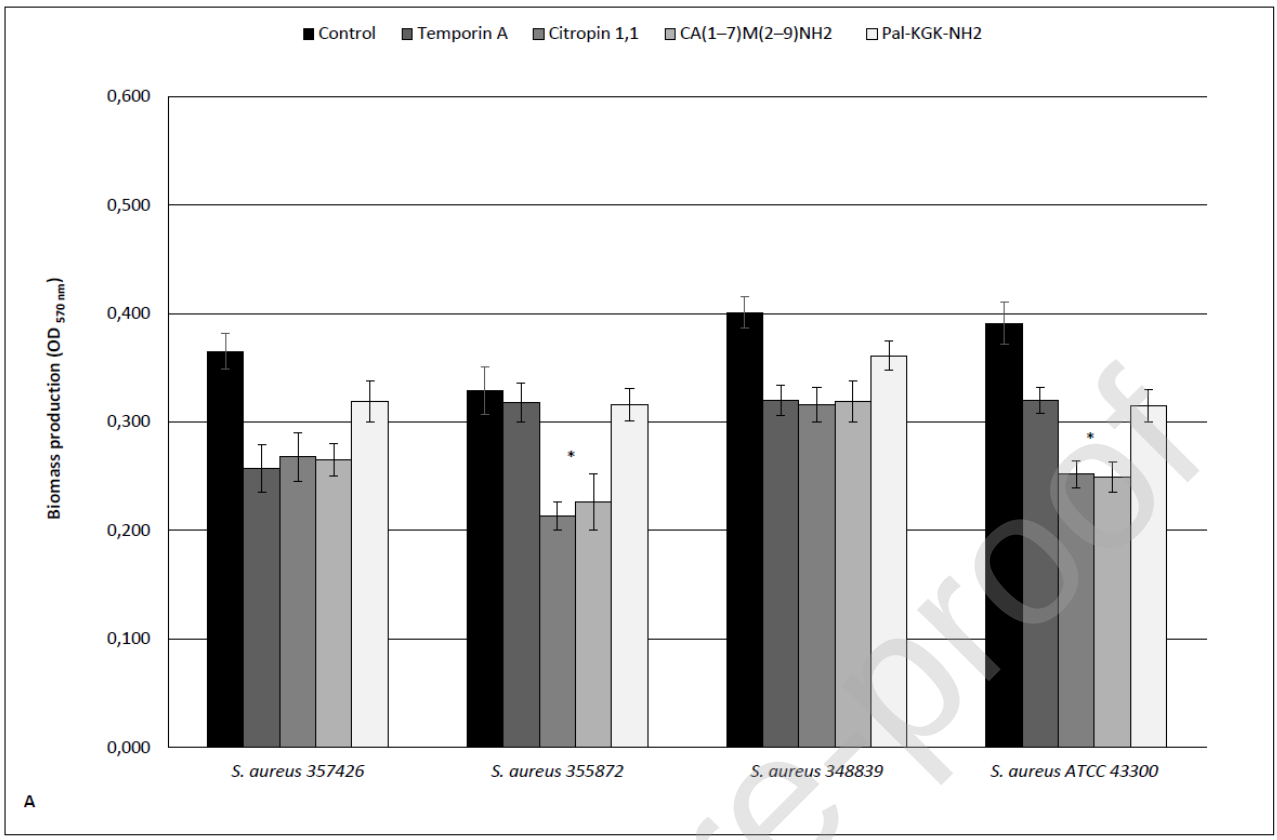


Fig. 4 Eradication of MRSA preformed biofilms on central venous catheters after 4 h (**A**) and 6 h (**B**) of contact with Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 at their 4X MIC values. Data

represent mean values of three independent experiments performed in duplicate and asterisks denote values statistically significant (*P<0.05, **P<0.01, ***P<0.001).



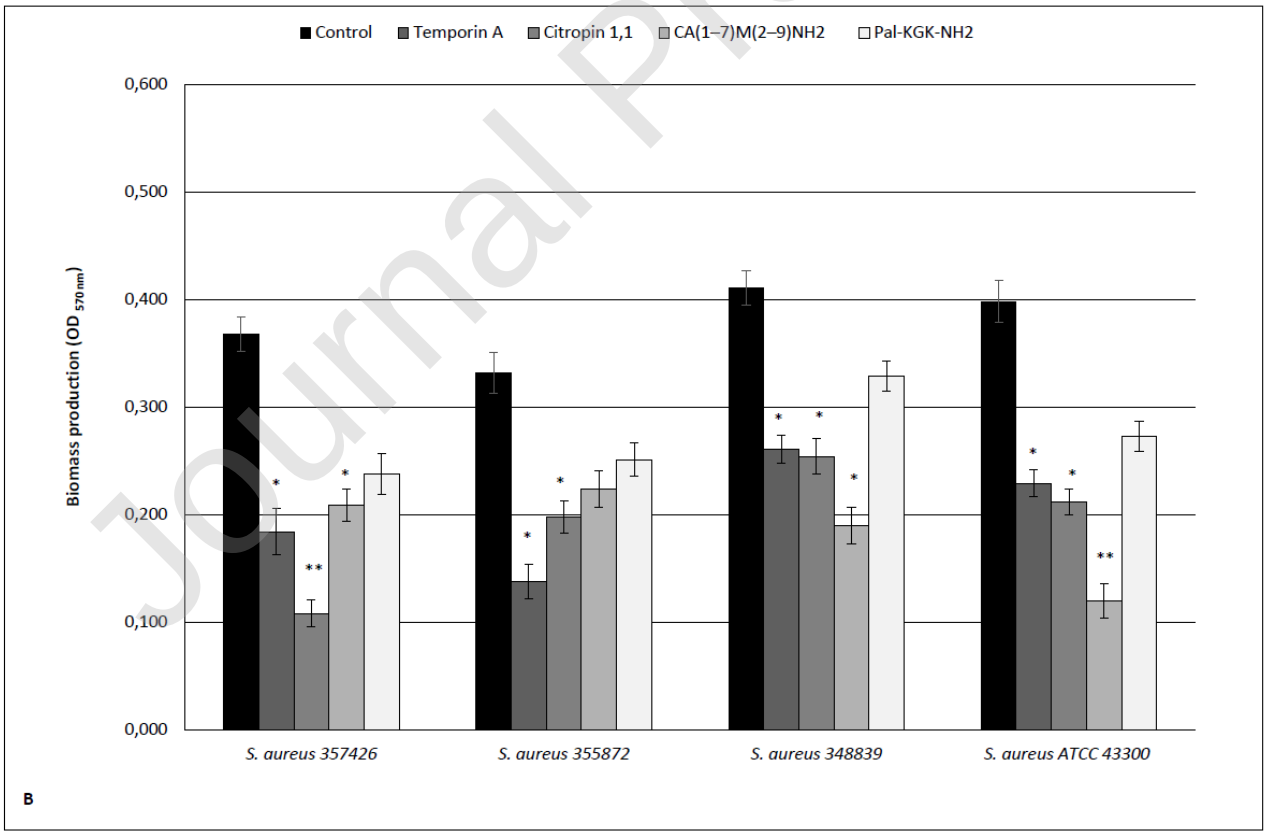
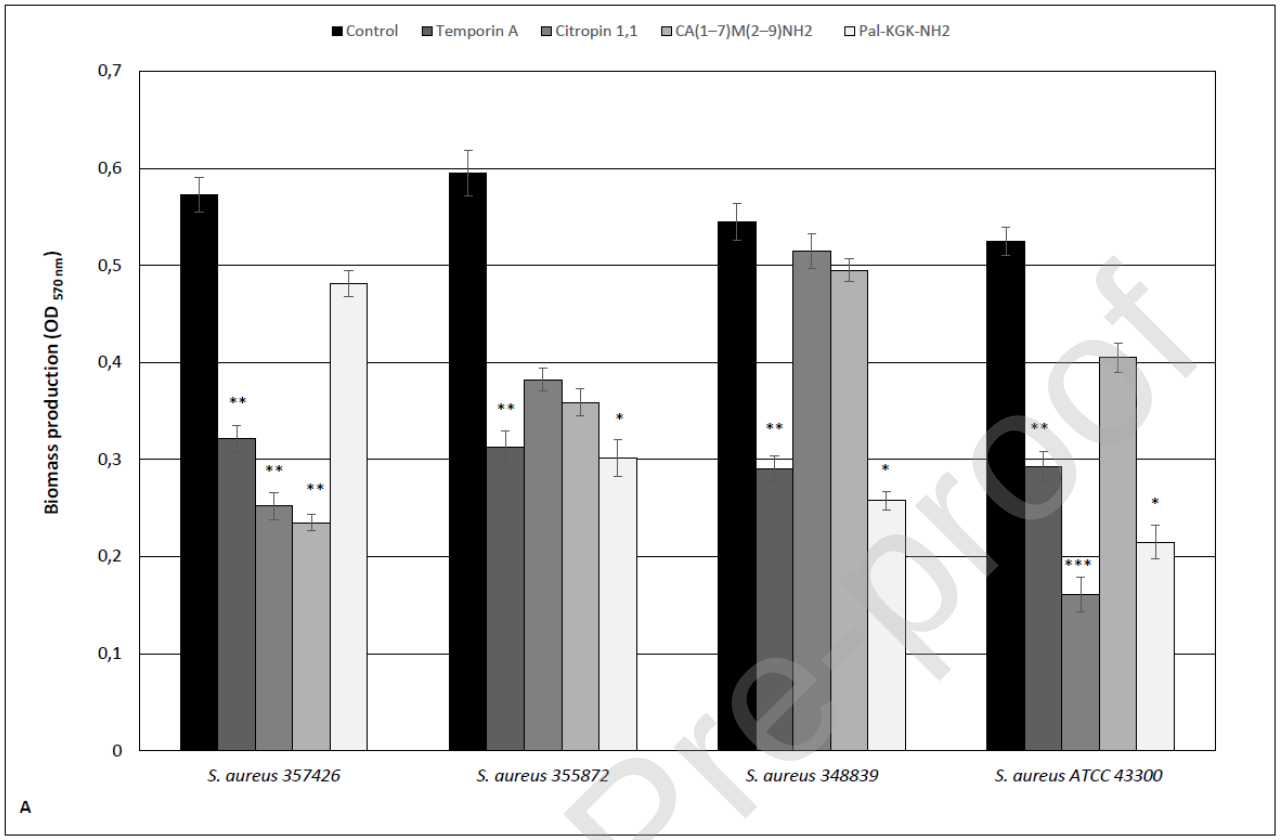


Fig. 5 Eradication of MRSA preformed biofilms on polystyrene (**A**) and central venous catheters (**B**) after 24 h of contact with Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 at their 2X MIC values. Data represent mean values of three independent experiments performed in duplicate and asterisks denote values statistically significant (*P<0.05, **P<0.01, ***P<0.001).



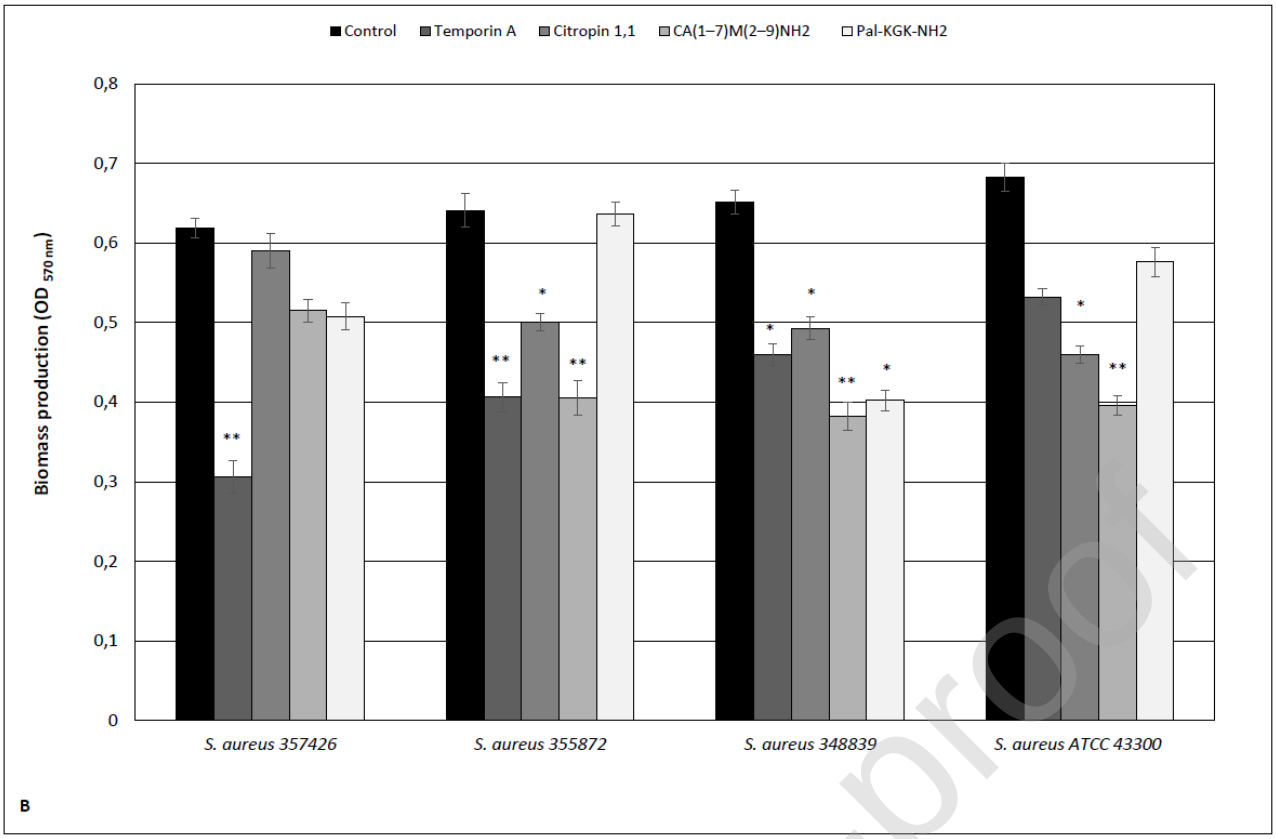
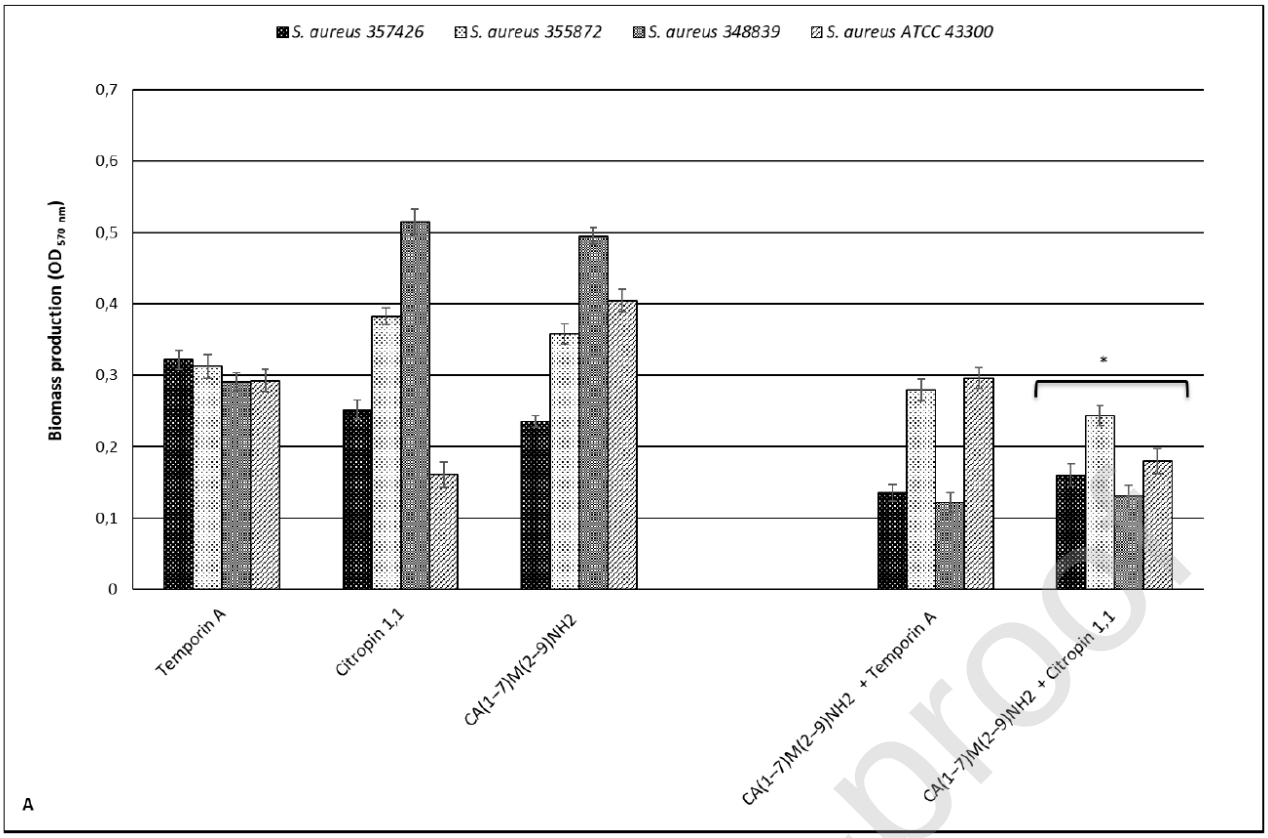


Fig. 6 Synergic effect of AMPs combinations (CA(1–7)M(2–9)NH_2 with Temporin A and CA(1–7)M(2–9)NH_2 with Citropin 1.1) applied for 24 h at their related FICI concentrations toward MRSA preformed biofilms on polystyrene (**A**) and central venous catheters (**B**) in comparison to the effect of each single AMP. Asterisks denote values statistically significant (*P<0.05).



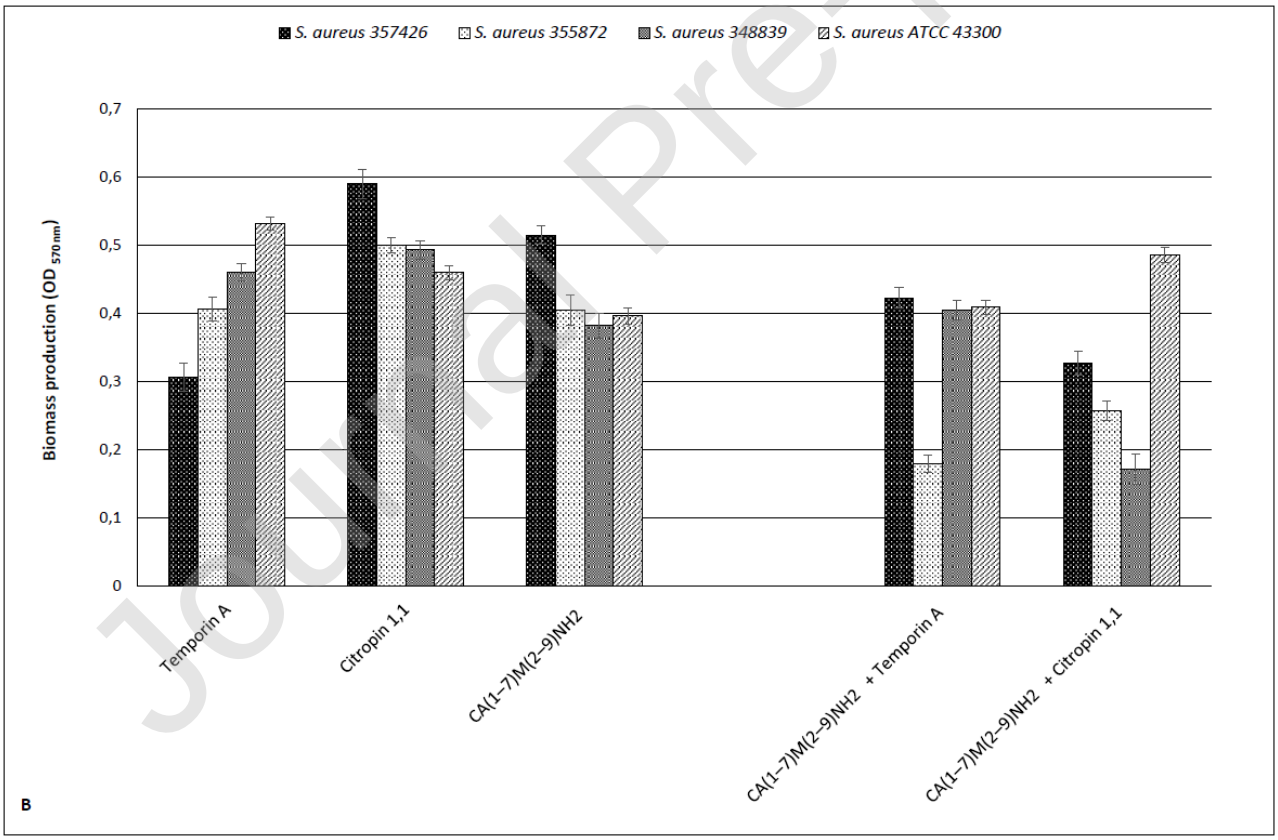


Table 1 Peptide sequences of the four antimicrobial peptides (AMPs) used in this study.

Peptide	Sequence	Reference
Temporin A	Phe-Leu-Pro-Leu-Ile-Gly-Arg-Val-Leu-Ser-Gly-Ile-Leu-NH_2	[38]
Citropin 1.1	Gly-Leu-Phe-Asp-Val-Ile-Lys-Lys-Val-Ala-Ser-Val-Ile-Gly-Gly-Leu-NH_2	[38]
CA(1–7)M(2–9)NH_2	Cecropin A-melittin hybrid peptide [CA(1-7)M(2-9)NH_2]	[39]
Pal-KGK-NH_2	Palmitoyl-Lys-Gly-Lys-NH_2	[40]

Table 2 MICs of Temporin A, Citropin 1.1, CA(1–7)M(2–9)NH_2 and Pal-KGK-NH_2 as previously assessed by Ciandrini and collaborators [1]. Data represented MIC values in μg/mL.

MRSA	Temporin A	Citropin 1.1	CA(1–7)M(2–9)NH_2	Pal-KGK-NH_2
S. aureus 357426	8	32	8	4
S. aureus 355872	4	16	8	1
S. aureus 348839	4	16	8	8
S. aureus 350355	4	64	16	16
S. aureus 360212	8	64	16	32
S. aureus ATCC 43300	8	32	8	32

Table 3 Antimicrobial activities of the dual AMPs combinations against four MRSA clinical strains. Data are expressed as Fractional Concentration Index (FICI) determined by checkerboard test on planktonic microorganisms. In grey are evidenced the two combinations with synergic activity toward all the examined strains.

MRSA and AMP combinations	FICI	Activity
S. aureus 357426		
CA(1–7)M(2–9)NH_2 + Temporin A	0.25	Synergic
CA(1–7)M(2–9)NH_2 + Citropin 1.1	0.5	Synergic
CA(1–7)M(2–9)NH_2 + PAL-KGK-NH_2	1.5	Indifferent
PAL-KGK-NH_2 + Temporin A	1.13	Indifferent
PAL-KGK-NH_2 + Citropin 1.1	0.38	Synergic
Citropin 1.1 + Temporin A	0.38	Synergic
S. aureus 355872		
CA(1–7)M(2–9)NH_2 + Temporin A	0.38	Synergic
CA(1–7)M(2–9)NH_2 + Citropin 1.1	0.48	Additive
CA(1–7)M(2–9)NH_2 + PAL-KGK-NH_2	2.5	Indifferent
PAL-KGK-NH_2 + Temporin A	4.01	Antagonistic
PAL-KGK-NH_2 + Citropin 1.1	4.06	Antagonistic
Citropin 1.1 + Temporin A	0.31	Synergic
S. aureus 348839		
CA(1–7)M(2–9)NH_2 + Temporin A	0.5	Synergic
CA(1–7)M(2–9)NH_2 + Citropin 1.1	0.5	Synergic
CA(1–7)M(2–9)NH_2 + PAL-KGK-NH_2	0.5	Synergic
PAL-KGK-NH_2 + Temporin A	1.0	Indifferent
PAL-KGK-NH_2 + Citropin 1.1	2.0	Indifferent
Citropin 1.1 + Temporin A	0.63	Additive
S. aureus ATCC 43300		
CA(1–7)M(2–9)NH_2 + Temporin A	0.26	Synergic
CA(1–7)M(2–9)NH_2 + Citropin 1.1	0.5	Synergic
CA(1–7)M(2–9)NH_2 + PAL-KGK-NH_2	0.5	Synergic
PAL-KGK-NH_2 + Temporin A	0.5	Synergic
PAL-KGK-NH_2 + Citropin 1.1	1.13	Indifferent
Citropin 1.1 + Temporin A	0.56	Additive