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Polymicrobial antibiofilm activity of the membranotropic peptide gH625 and its analogue

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ABSTRACT

This work illustrates a new role for the membranotropic peptide gH625 and its derivative gH625-GCGKKK in impairing formation of polymicrobial biofilms. Mixed biofilms composed of *Candida* and bacterial species cause frequently infections and failure of medical silicone devices and also show a major drug resistance than single-species biofilms. Inhibition and eradication of biofilms were evaluated by complementary methods: XTT-reduction, and crystal violet staining (CV). Our results indicate that gH625-GCGKKKK, better than the native peptide, strongly inhibited formation of mixed biofilms of clinical isolates of *C. tropicalis/S. marcescens* and *C. tropicalis/S. aureus* and reduced the biofilm architecture, interfering with cell adhesion and polymeric matrix, as well as eradicated the long-term polymicrobial biofilms on silicone surface.

1. Introduction

Microbial biofilms are one of the most distributed and successful life-style on the planet Earth. In particular, most microorganisms do not exist in a free-living state but rather form biofilms in order to increase their fitness and make themselves more equipped to survive in stressful environments. Biofilms are found almost everywhere, in the soil, water and in all higher organisms including humans in which niches such as the oral cavity, gastrointestinal and urogenital tracts are colonized by microorganisms organized in biofilms.

Many infections are not caused by a single-species population of microbes but by different microbial species associated in biofilm. Polymicrobial biofilms are an infectious reservoir for a variety of microorganisms, including bacteria but even fungi interacting in a synergistic or inhibitory way on the health of patients.

Biofilms are acquiring a clinically relevant importance and represent a problem still not enough investigated; even less is known concerning the behavior of communities of such mixed microorganisms.

With this work, we highlight the importance of studying complex biofilms which more closely reflect the microbial colonies found in nature rather than the single-species biofilms that represent only a case-study in the laboratory. Particularly, we focused on biofilms formed by *C. tropicalis, S. aureus* and *S. marcescens*.

These three pathogens are independently responsible for a substantial number of infections but there is increasing evidence that they are associated as co-infecting organisms.

C. tropicalis has been considered as the second most causative agent of candidemia, especially in neoplasia patients [1,2], and is often associated with nosocomial urinary tract infections [3]. This is partly due to the virulence characteristics of this species, such as its high adhesion capacity and ability to form biofilms on epithelial and endothelial tissues [4–6]. Recently, drug-tolerant or resistant *C. tropicalis* isolates have been isolated from patients and exhibited resistance to the antifungal drugs, such as the azoles derivatives, amphotericin B, and echinocandins [7].

S. aureus is a Gram-positive bacterium responsible for a significant and increasing number of nosocomial and community-acquired infections worldwide [8]. *S. aureus* is a clinically important pathogen, with higher rates of device-related systemic infection and mortality. Its pathogenicity is due to a number of virulence factors including adhesins, toxins, coagulase, and a variety of antimicrobial resistance genes [9]. All these virulence factors, coupled with its innate ability to resist antibiotic therapy with antibiotic resistance gene expression and biofilm formation, have made *S. aureus* a significant burden for the medical community [10].

The Gram-negative *Serratia marcescens* is involved in hospital-acquired infections, particularly catheter-associated bacteremia, urinary

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tract infections and wound infections. This aerobic, mobile species belonging to the *Enterobacteriaceae* family is found in water, soil and air. Furthermore the production of a surfactant agent called "serrawettin", which is probably involved in the process of biofilm formation, contributes to its pathogenicity [11–13] and is a potential emerging MDR pathogen.

Candida tropicalis is able to associate to *Staphylococcus aureus* or *Serratia marcescens* forming dual-species biofilms which are of medical interest because of increased frequency and/or severity of the diseases which they are able to cause.

A typical emergent properties of biofilms is the high tolerance to antibiotics and other antimicrobial agents compared with planktonic bacterial cells. When embedded in biofilms or in mixed biofilms, microorganisms make their environment even less sensitive to antibacterial agents.

In addition, the widespread occurrence of antimicrobial resistance among pathogens is a global concern, and infections caused by resistant microorganisms are now frequent events in hospitalized or community patients. Not only bacteria are able to form biofilms after the acquisition of mutations which make them resistant towards antibiotics, but also the biofilm as a mode of living promotes the occurrence of genetic mutations.

Prevention, weakening, disruption and direct killing represent four main strategies to treat biofilms but it seems that the most efficient method is the mechanical or surgical removal of the biofilm.

For implants or, general, for non-accessible surfaces such mechanical removal is obviously not possible; thus a different strategy is required.

A possible strategy to treat biofilms and multidrug-resistant infections could be the use of alternative compounds such as antimicrobial peptides. Antimicrobial peptides (AMPs) are short cationic molecules characterized by the presence of both hydrophobic and hydrophilic residues, producing amphipathic structures; AMPs are found in both the animal and plant world, with activities against a broad spectrum of microorganisms [14,15]. Several AMPs have been also demonstrated to have antibiofilm activities better than that of conventional antibiotics [16,17].

AMPs with their pore-forming activity target growing cells as well as dormant populations; they might reduce the potential to develop bacterial resistance and represent an advantage of these peptides.

Both early biofilm adhesion and eradication of mature polymicrobial biofilm are crucial steps; thus, the best approach for reducing the number of these infections may rely on the development of novel antimicrobial peptides able to interfere with both processes.

Treatment of biofilms requires well-penetrating antibiotics to ensure a sufficient concentration of drug at the site of infection.

Thus, in order to design novel antimicrobial peptides effective against polymicrobial biofilms, we selected themembranotropic peptide gH625 which has been proved to be able to interact with membrane bilayers, transiently and locally disrupting the bilayer [18,19].

gH625 is characterized by the presence of a high content of alanine, glycine and leucine residues which is responsible of its intrinsic conformational flexibility and ability to adopt different secondary structures in different environments [20]. Furthermore, the presence of aromatic residues, allows strong interactions with phospholipid groups at the membrane interface, contributing significantly to the insertion of the peptide among the lipids. The peptide gH625 was also proved to be effective as a drug delivery carrier for its ability to interact with membrane bilayers without displaying toxic effect on eukaryotic cells but it has not been evaluated yet for its antimicrobial or antibiofilm activity.

Aiming at this, in this work, we used both gH625 and its modified sequence obtained by the addition of a sequence of lysine residues, which should promote selective interactions with the negative charges of bacterial membrane bilayers. The modified peptide, named gH625-GCGKKKK, was also shown to have enhanced ability to cross membrane bilayers [21].

Subsequently, we probed the ability of the native gH625 and its analogue to prevent or reduce *in vitro* the formation of monomicrobial and polymicrobial biofilms of clinical isolates of *C. tropicalis* and *S. aureus* and *S. marcescens*. We also tested the ability of gH625-GCGKKKK to eradicate mature polymicrobial biofilms grown on silicone, which is a material widely used in the biomedical field for its biocompatibility and mechanical properties, but it is prone to be colonized in the long term by biofilm producer microorganisms.

In this work, we grew polymicrobial biofilms on silicone platelets and simulated a typical long-term colonization occurring on silicone medical devices *in vivo*; then, we probed our peptide gH625-GCGKKKK in eradication showing its efficient anti-biofilm action.

2. Materials and methods

2.1. Synthesis and characterization of gH625 and gH625-GCGKKKK

Peptides were synthetized by the Fmoc-solid-phase method as previously reported [22], using a rink amide MBHA (0.54 mmol/g) resin. Consecutive deprotection (30% piperidine in dimethylformamide, for 10 min, twice) and double coupling (4 equivalents of amino acid, 4 equivalents of 1-hydroxybenzotriazole, 4 equivalents of 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate for 40 min) steps were performed. A solution of trifluoroacetic acid/H_2O/thioanisole/ethanedithiol/anisole 85/5/5/3:2 v/v) was used for side chain deprotection and cleavage from the resin. Peptides were precipitated in cold ethylic ether and the crudes were analysed by HPLC–MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 20 to 80% in 15 min. Purifications were performed by preparative RP-HPLC. All purified peptides were obtained with good yields (50–60%).

2.2. Strains and growth conditions

The *Candida tropicalis*, *Staphylococcus aureus*, and *Serratia marcescens* strains used in this study were clinical isolated from University Hospital "Luigi Vanvitelli" (Naples, Italy).

C. tropicalis was isolated from a systemic infection and maintained on Sabouraud dextrose agar.

S. aureus was obtained from a patient with an otitis infection and maintained in Trypticase Soy Agar (TSBA). The multi-resistant *S. marcescens* clinical isolate was device-related infection isolate and maintained on Luria–Bertani (LB) agar.

2.3. Monomicrobial and polymicrobial biofilms detection

Tryptic soy broth (TSB) supplemented with 0.1% glucose was used for all microorganisms.

Overnight candidal or bacterial cultures were grown in TSB 0.1%, and diluted to 1×10^6 cfu/ml and 1×10^8 cfu/ml with TSB respectively. We formed a mixed biofilm at a final concentrations of 10^6 cfu/ml.

100 μl of monomicrobial suspensions and or 100 μl of the two mixtures of *C. tropicalis* and *S. aureus* or *C. tropicalis/S. marcescens* and *C. tropicalis/S. aureus* were loaded into polystyrene 96-well microplate and incubated at 37 °C for 24 h to allow the production of biofilm. Biofilm biomass was evaluated with crystal violet (CV) staining [23]. The metabolic activity of biofilms was calculated or determined as metabolic activity using a 2, 3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5 carboxanilide (XTT) reduction assay [20].

In details, after 24 or 48 h, wells were washed with PBS and stained with crystal violet (CV, 0.1% w/v) solution for 30 min. The plate was washed with PBS and incubated in acetic acid (30% v/v). The absorbance at 570 nm was detected with a spectrophotometer (DR5000, HACH). In the case of XTT reduction assay, wells were washed and

incubated for 3 h with 10 µl XTT working reagent (Kit cell Counting Kit-8 Enzolife Science, Switzerland) at 37 °C. The resulting absorbance was read at 450 nm.

2.4. Biofilm formation on medical grade silicone

Biofilms were formed on silicone as described elsewhere [24]. In brief, medical grade silicone plates (diameter: 8 mm, thickness: 3 mm, Websinger, Austria) were steam sterilized at 121 °C (20 min) and placed into wells of 48-well microplate. 250 µl monomicrobial suspensions and 250 µl of two mixtures of *C. tropicalis/S. aureus* and *C. tropicalis/S. marcescens* were pipetted into wells. The plates were incubated at 37 °C for 24 h. After removing the non-adherent cells after 24 h, the culture medium was replaced by fresh TSB 0.1% glucose. This procedure was repeated every 24 h for a total incubation period of 7 d. The plates were, subsequently, rinsed with PBS and stained with crystal violet, as previously described.

2.5. Minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of gH625 and gH625-GCGKKKK, microtitre plate assay was performed following the method described by Clinical and Laboratory Standards Institute [25]. Briefly, 100 µl of nutrient broth containing each strain was introduced into each well of 96- well microplate and different concentrations of gH625 and gH625-GCGKKKK modified (5 µM–50 µM) were introduced into the wells and incubated for 24 h at 37 °C. The growth of each strain was measured by the turbidity method using an ELISA plate reader at 590 nm wavelength. MIC was estimated as the lowest concentration of gH625 and gH625-GCGKKKK capable of inhibiting the growth of each microbial pathogen.

2.6. Biofilm-Prevention assay

A biofilm inhibition assay was used to evaluate the ability of the peptides gH625 and gH625-GCGKKKK to prevent or reduce monomicrobial and polymicrobial biofilms formation. In inhibition assays, peptides were incubated with the cells before the biofilm development. Peptides were tested in the biofilm prevention assay in a range of concentrations (1–50 µM) which were lower than MIC value evaluated in the test with planktonic microorganism. Plates were then incubated for 24 h to allow biofilm formation. Wells were washed three times with 200 µl PBS to facilitate removal of planktonic cells before quantification by the crystal violet assay as described before.

2.7. Biofilm-eradication assay

A biofilm-eradication assay was used to evaluate the ability of gH625and gH625-GCGKKKK to disrupt the mature biofilms. An initial monomicrobial and polymicrobial biofilms were allowed to form for 48 h. Wells were then washed three times with 200 µl PBS to facilitate removal of non -adherent cells before addition of peptides who were added once the biofilm was formed at the same concentrations used for the Biofilm-Prevention Assay (1–50 µM). Plates were then incubated for further 24 h to allow peptides to act. Biofilms quantification were done by XTT assay as described above.

2.8. Scanning electron microscopy (SEM)

Biofilms were formed in multi-well plates as previously described. After 24 h, wells were washed with PBS (three times) to remove planktonic cells. The slides were prepared for scanning electron microscopy (SEM) using a previously published protocol [26]. Briefly, the slides were placed in 3% glutaraldehyde at 4 °C, then washed with PBS and post-fixed in 1% aqueous solution of osmium for 90 min at room temperature. Then, samples were dehydrated in a series of graded alcohols, dried to the critical drying point, and finally coated with gold. Specimens were evaluated with a scanning electron microscope (QUANTA 200 ESEM FEI Europe Company, the Netherlands).

2.9. Fluorescence microscopy

Morphology of mixed biofilms was observed, after staining with fluorescent dyes. Mixed biofilms were rinsed with PBS and fluorescent stains were added directly to chamber slides [27]. The following stains were used: SYTO 9 (Life Technologies Corporation, Eugene, OR) stains bacteria in green; Calcofluor white (Sigma Aldrich) stains fungal cell walls in blue. The final concentrations of stains were as follows: 5 µM for SYTO 9 and 0.25 mg/ml for Calcofluor white. The slides were examined and photographed with a Leica CTR 6500 UV microscope equipped with a Leica Application Suite.

2.10. Statistical analysis

Results are given as mean ± dev. std. Differences between samples and control group were determined by two-tailed Student's t-test. The significance of the difference among groups was evaluated by one-way and two-way analysis of variance (ANOVA) and further statistical post-hoc comparisons with Tukey's multiple comparison test. Differences were considered significant when $\alpha < 0.05$.

3. Results and discussion

The examined clinical isolates of *C. tropicalis*, *S. marcescens* and *S. aureus*, were able to adhere to polystyrene and form single species, as well as fungal-bacterial species, biofilms. In Fig. 1, total adhered biomass of mono- and polymicrobial biofilms after 24 h is reported.

Single biofilms of the two bacterial isolates resulted less dense than

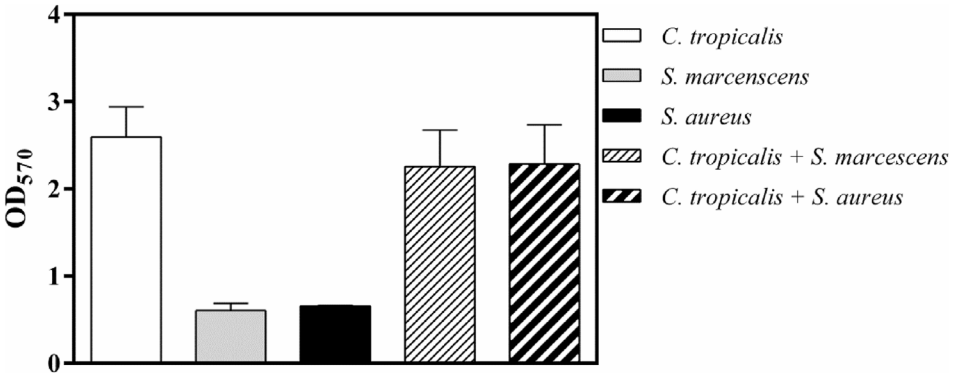

Fig. 1. Total biomass of monomicrobial and polymicrobial biofilms of the clinical isolates adhered on polystyrene (n = 3, ± st. dev.).

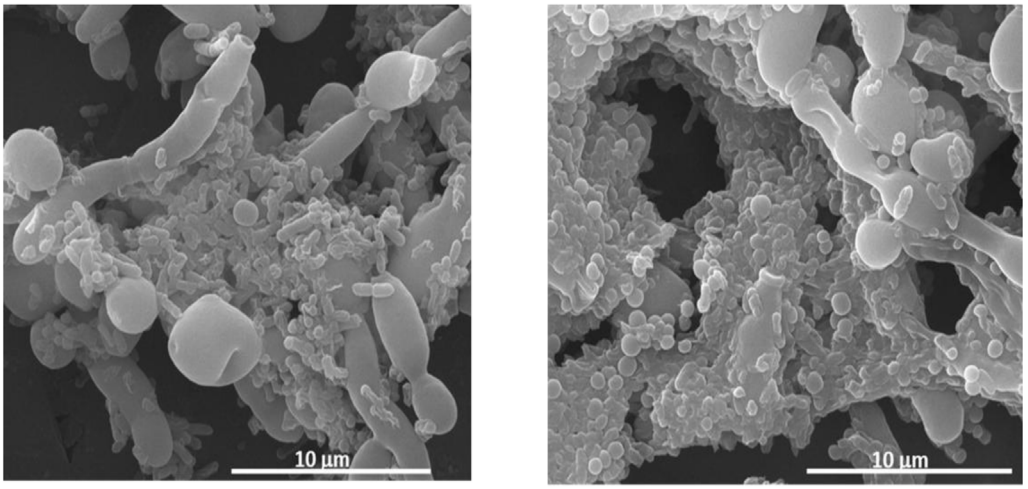

Fig. 2. SEM observation of 48 h mixed biofilm of *C. tropicalis* and *S. marcescens* (left) and *C. tropicalis* and *S. aureus* (right). Bar = 10 µm.

the fungal biofilm, whilst the polymicrobial biofilms had an intermediate value.

Following further 24 h incubation in culture medium, the mixed biofilms became mature, showing a characteristic dense network of both fungal (both yeast-like and elongated forms) and bacterial cells, surrounded by an abundant polymeric matrix, as shown at SEM observation (Fig. 2). In the case of *Candida*, the occurrence of hyphae and pseudo-hyphae, together with the extracellular matrix is indicative of biofilm maturation, and represents a main virulence factor of the fungus.

In both mixed biofilms the bacterial components are predominant with respect to the fungal, as also shown after total plate count on selective media of the sessile cells of both types, following their isolation from the biofilms which showed that bacterial cells overgrew fungal cells up to three orders of magnitude. Apparently, during biofilm maturation, the bacteria overtake the fungus, which largely appears covered by them.

The tight association between *Candida* and the bacterial cells is visible also by fluorescence microscopy, following differential fluorescent staining (Fig. 3): the bacterial aggregates adhered to *C. tropicalis* hyphae are clearly evidenced.

Before testing gH625 and gH625-GCGKKKK for their ability in prevention and eradication of the monomicrobial and polymicrobial

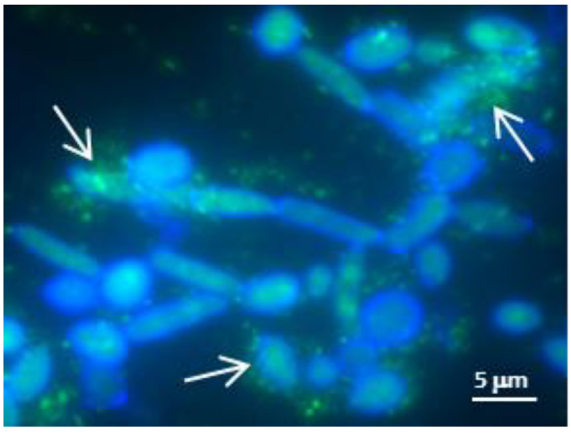

Fig. 3. Dual-species biofilm of *C. tropicalis* and *S. marcescens* stained with SYTO 9 and Calcofluor white fluorescent dyes. *S. marcescens* and *C. tropicalis* are stained green and blue, respectively. *S. marcescens* adhered to *C. tropicalis* hyphae are indicated with arrows. Bar = 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Minimal Inhibition Concentration of gH625 and gH625-CGCKKKK on planktonic cells.

Strain	Origin	gH625 (µM)	gH625-GCGKKKK (µM)
Candida tropicalis	Blood	> 50	50
Serratia marcescens	Device-related infection isolate	25	25
Staphylococcus aureus	Ear swab	50	50

biofilms, we determined the MIC values against the planktonic cells of the three clinical isolates (Table 1). Both peptides are active against *S. marcescens* but display a minor activity against the fungus *C. tropicalis* and the bacterium *S. aureus*.

The effects of gH625 and gH625-GCGKKKK on the prevention of the mono- and polymicrobial biofilm formation are reported in Fig. 4. The peptide gH625 does not display any effect on *C. tropicalis* biofilm at all the tested concentrations, whereas its modification gH625-GCGKKKK was very effective against *C. tropicalis* biofilm with 90% of inhibition. Both peptides show a similar activity with *S. marcescens* and *S. aureus* biofilms even if gH625-GCGKKKK is a little more effective.

In the case of the polymicrobial biofilms, we observed a greater inhibition with the presence of gH625-GCGKKKK which is able to inhibit the formation until 80% at a concentration of 50 µM.

In the eradication of mono- and polymicrobial biofilms, both peptides resulted very effective. In particular, as shown in Fig. 5, the eradication percentages of gH625-GCGKKKK against the polymicrobial biofilms were very significant, achieving a value higher than 50% already at the lowest concentration used (5 µM). The results presented in this paper show that the modification of the peptide gH625 consisting in the addition of a sequence of lysine residues has improved capacity of penetration and interactions with the microbial membranes. The modified gH625-GCGKKKK is an antibiofilm agent more potent than gH625 in the inhibition process; in fact, it is active in the case of *C. tropicalis,* against which, gH625 did not perform any inhibitory activity. However, both the peptides are effective in the eradication even more than in prevention test.

The explanation why both peptides are more active in the eradication process lies in their chemical nature. gH625 is a peptide derived from the viral glycoprotein gH and rich in hydrophobic residues. gH625 is deeply characterized through several physico-chemical techniques and is able to interact with model membranes, penetrate in the lipid bilayer and induce fusion between membranes [22,28,29]. In little details, this viral fusion peptide was proved to undergo conformational

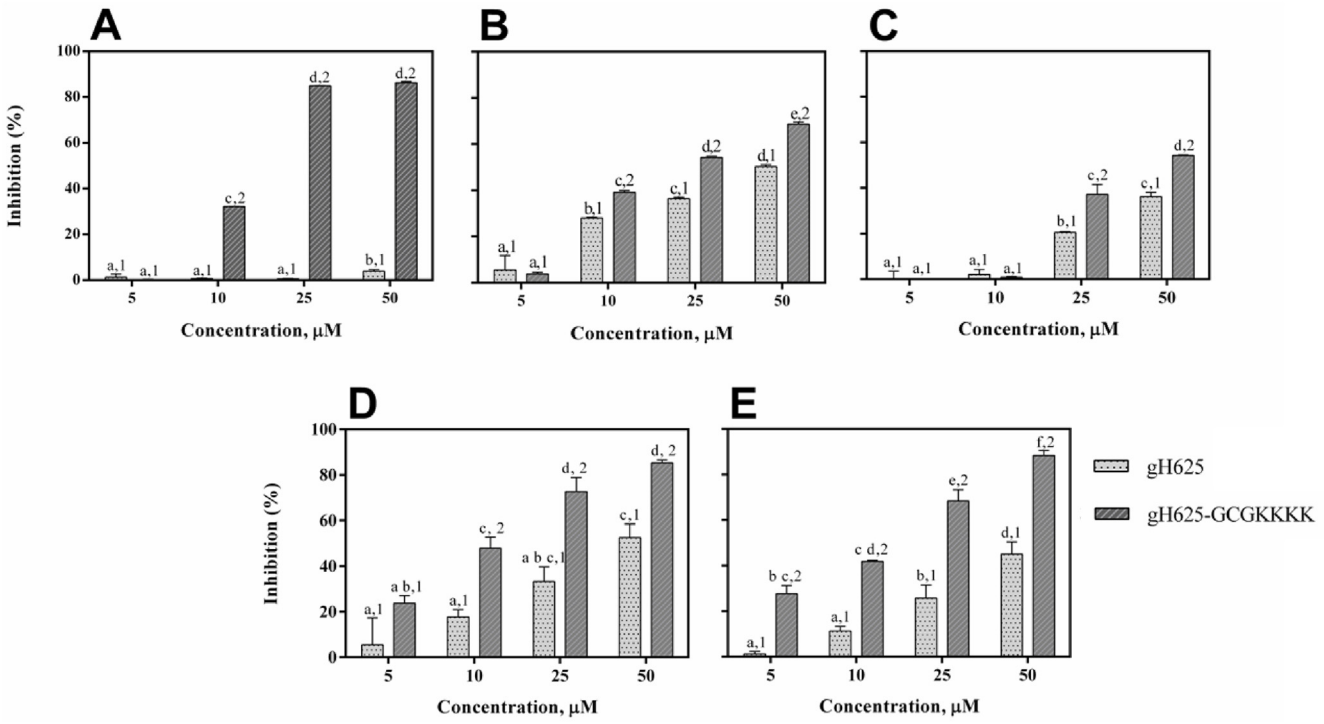

Fig. 4. Prevention of biofilm formation of: (A) *C. tropicalis* (n = 3, ± st. dev.), (B) *S. marcescens* (n = 3, ± st. dev.), (C) *S. aureus* (n = 3, ± st. dev.), (D) *C. tropicalis* and *S. marcescens* (n = 3, ± st. dev.) (E) *C. tropicalis* and *S. aureus* (n = 3, ± st. dev.).
Letters (a–f) indicate significant differences between treatments at concentrations (5, 10, 25 and 50 μM), while numbers (1 and 2) significant differences between the same concentration; the level of significance was set at $\alpha = 0.05$.

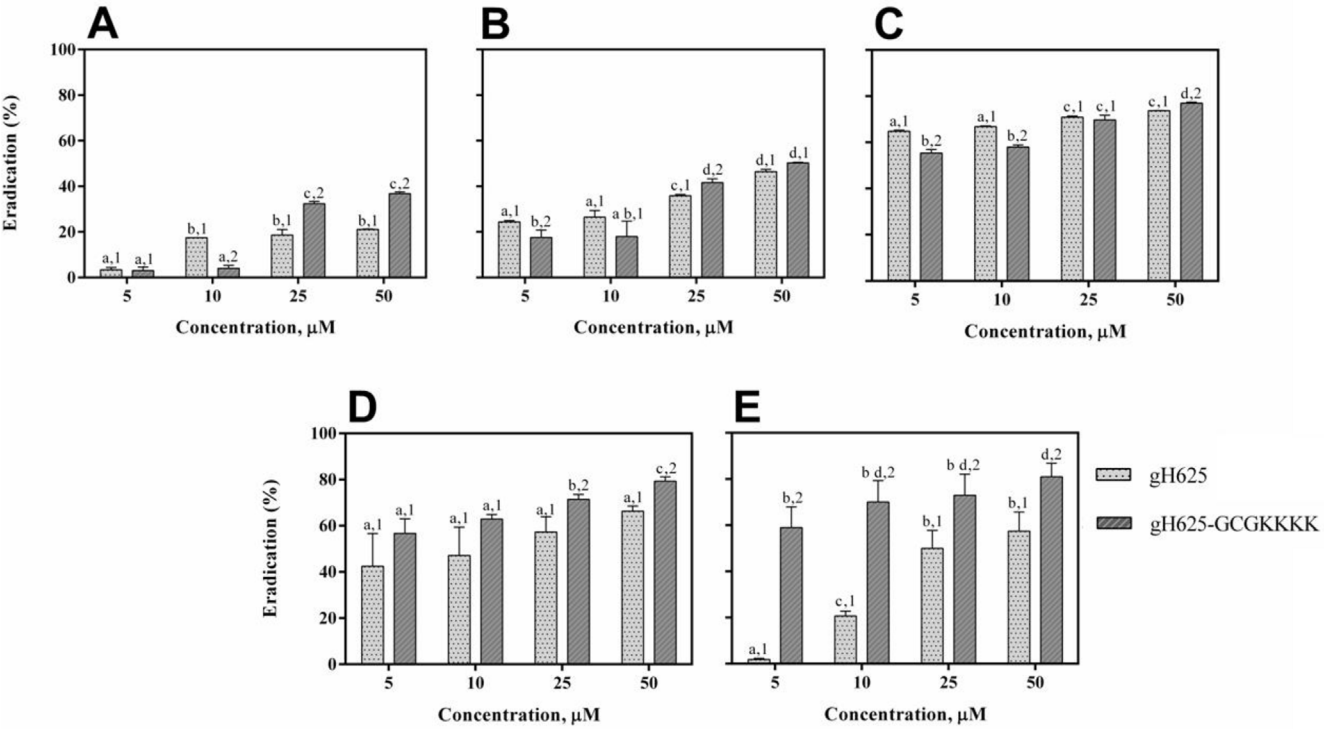

Fig. 5. Eradication of mature biofilms of: (A) *C. tropicalis* (n = 3, ± st. dev.), (B) *S. marcescens* (n = 3, ± st. dev.), (C) *S. aureus* (n = 3, ± st. dev.), (D) *C. tropicalis* and *S. marcescens* (n = 3, ± st. dev.) (E) *C. tropicalis* and *S. aureus* (n = 3, ± st. dev.).
Letters (a–e) indicate significant differences between treatments at concentrations (5, 10, 25 and 50 μM), while numbers (1and 2) significant differences between the same concentration; the level of significance was set at $\alpha = 0.05$.

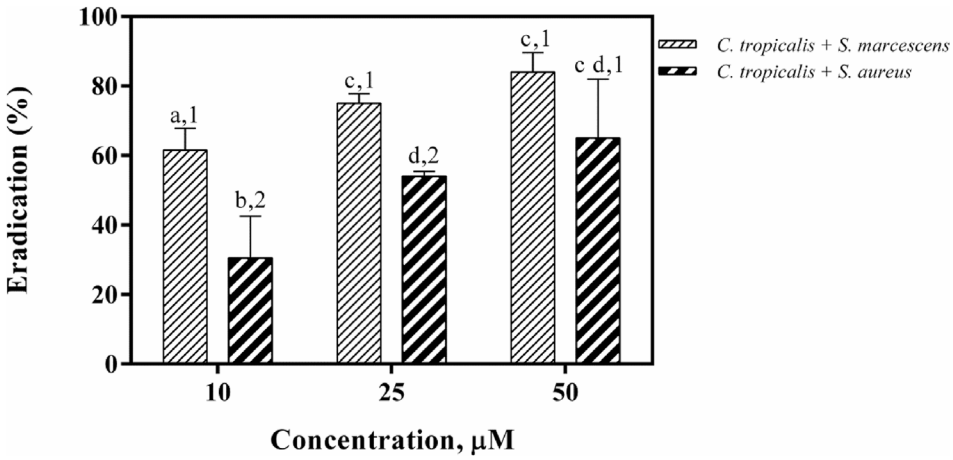

Fig. 6. Eradication of polymicrobial biofilms of *C. tropicalis* and *S. marcescens* and *C. tropicalis* and *S. aureus* formed on silicone.
Letters (a–d) indicate significant differences between treatments at concentrations (10, 25 and 50 μM), while numbers (1and 2) significant differences between the same concentration; the level of significance was set at $\alpha = 0.05$.

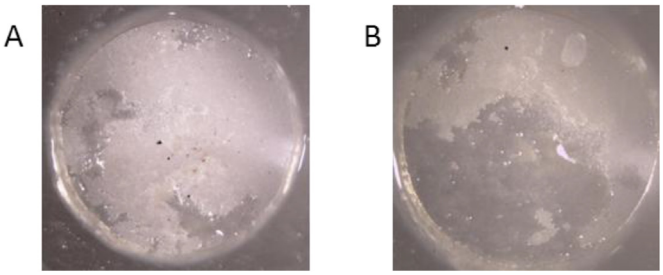

Fig. 7. Silicone platelet (8 mm diameter) colonized by mixed biofilm of *C. tropicalis* and *S. marcescens* (A) and after treatment with 50 μM gH625-GCGKKKK (B).

changes and assume an amphipathic helical structure once it reaches and binds the membrane. The amphipathic helix is a critical feature for promoting perturbation, penetrating in the lipid bilayer and inducing fusion. Thanks to its nature, it may confer super hydrophobicity to the surface where the microbes are grown and display antinfective activity, which prevents from the attachment and formation of biofilm.

Furthermore, the data presented here show that eradication activity is stronger with gH625-GCGKKKK. This may be explained considering the fact that bacterial membranes have negatively charged phospholipids, which could interact with the positively charged residues (lysine) of the peptide. As the difference in capability of eradicating the biofilm between the two peptides is not significant we believe that the hydrophobic interaction plays a critical role.

We also tested the capability to eradicate the two polymicrobial biofilms grown on silicone platelets (Fig. 6). Even on this material, the biofilms were very sensitive towards the action displayed by gH625-GCGKKKK, achieving 80% eradication for the biofilm of *C. tropicalis* and *S. marcescens*. (Fig. 7).

However, it is worth noticing that the anti-biofilm activity of the two peptides was displayed at concentrations much lower than the MIC concentrations determined, this suggesting that the mechanisms of action of the peptides may be not only the result of a “classical” antimicrobial effect, but also an interference with the so-called “biofilm lifestyle” [30].

Overall, these results are remarkable. Indeed, the majority of antimicrobial peptides reported in literature for their action against bacteria and fungi have a net positive charge and very small number of examples report on hydrophobic/membranotropic peptides capable to display this same activity. For instance, TAT is a peptide derived from a viral glycoprotein, likewise gH625, but it possesses more positive than hydrophobic amino acid residues [31]. Furthermore, gH625 is also able to act as a cell-penetrating peptide [32], which may explain its ability to eradicate a preformed biofilm even though it has a low antimicrobial activity.

Thus, in the scenario of possible applications, we could consider to utilise these peptides both to destroy a pre-existing biofilm and as carrier of other anti-infective agents for a synergistic anti-biofilm effect.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.micpath.2018.09.027.

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