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# Synergistic and antibiofilm activity of the antimicrobial peptide P5 against carbapenem-resistant *Pseudomonas aeruginosa*

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**Abstract**. In the search for new antimicrobial molecules, antimicrobial peptides (AMPs) offer a viable alternative to conventional antibiotics, as they physically disrupt the bacterial membranes, leading to membrane disruption and eventually cell death. In particular, the group of linear α-helical cationic peptides has attracted increasing research and clinical interest. The AMP P5 has been previously designed as a cationic linear α-helical sequence, being its antimicrobial and hemolytic properties also evaluated. In this work, we analyzed the feasibility of using P5 against a carbapenem-resistant clinical isolate of *Pseudomonas aeruginosa*, one of the most common and risky pathogens in clinical practice. After antimicrobial activity confirmation in *in vitro* studies, synergistic activity of P5 with meropenem was evaluated, showing that P5 displayed significant synergistic activity in a time kill curve assay. The ability of P5 to permeabilize the outer membrane of *P. aeruginosa* can explain the obtained results. Finally, the antibiofilm activity was investigated by viability analysis (MTT assay), crystal violet and confocal imaging, with P5 displaying mild biofilm inhibition in the range of concentrations tested. Regarding biofilm disruption activity, P5 showed a higher efficacy, interfering with biofilm structure and promoting bacterial cell death. Atomic force microscope images further demonstrated the peptide potential in *P. aeruginosa* biofilm eradication, confirming the promising application of P5 in multi-resistant infections therapeutics.

**Keywords**: Biofilm; *Pseudomonas aeruginosa*; antimicrobial peptide; antibiofilm peptide; synergic activity; meropenem.

## Introduction

Antibiotic resistance is one of the main problems concerning public health and clinical practice [1]. Particularly, the development of antibiotic resistance by *Pseudomonas aeruginosa* is a major concern in the treatment of bacterial pneumonia [2]. Chronic *P. aeruginosa* infections are commonly associated with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), and effective treatment is difficult due to the development of multidrug resistance (MDR) in these bacteria [3]. Furthermore, the ability of *P. aeruginosa* to evolve into biofilm communities increases their resistance to most conventional treatments [4]. Additionally, biofilms of antibiotic-resistant *P. aeruginosa* occur with high frequency, both *in vitro* and in the lungs of CF patients [5], or directly on medical implant surfaces, such as catheters and artificial prosthesis [6].

Antimicrobial peptides (AMPs) are good candidates for the development of new therapeutic drugs [7], since they have shown antimicrobial activity against a wide range of bacterial species and since they can be *de novo* designed in order to meet the challenge of multiresistant bacteria but avoiding host side effects [8–10]. Compared with conventional antibiotics, killing of bacteria by these peptides is extremely rapid and can involve multiple bacterial cellular targets [11]. In addition, another alleged advantages of AMPs is that bacterial resistance would evolve much more slowly than against conventional antibiotics, a highly desirable property [12]. Indeed, peptides are not hindered by the resistance mechanisms that are placing currently used antibiotics in jeopardy, for example, against methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *P. aeruginosa* [7].

The efficiency of AMPs is often attributed to their ability to disrupt cellular membranes of microorganisms [13]. Particularly for cationic linear α-helical peptides, amphipathicity and helicity play a crucial role in antimicrobial activity and possible hemolytic activity [9,14]. Although the potency of these AMPs against more susceptible pathogens is normally not as strong as certain conventional antibiotics, one of their major strengths is their ability to kill multidrug-resistant bacteria at similar concentrations. Besides, eradication can occur synergistically with other peptides and/or conventional antibiotics, which might help to overcome some of the barriers that resistant bacteria have against currently used antibiotics [15]. Synergistic activity of some AMPs with certain conventional antibiotics has been proposed [16] as a promising and increasingly used approach to overcome the problem of MDR bacteria [17].

AMPs are also increasingly being considered as novel agents against biofilms, by inhibiting their formation or eradicating established ones [18–20]. Bacterial biofilms are three-dimensional multi-cell structures, in which bacteria are embedded in an extracellular matrix composed of polysaccharides, DNA and proteins [21,22]. Biofilms are important for bacterial survival in their natural environments, and protects the bacteria from the immune system and antibiotics [23]. Bacterial susceptibility to AMPs in biofilm has been shown to be lower compared to the planktonic state [24]; however,

this resistance to AMPs has not been extensively studied compared to other antimicrobial agents [21].

In this context, we analyzed the possible synergistic activity of a novel antimicrobial peptide, P5, with meropenem (member of the carbapenem class of β-lactam antibiotics) and its potential as antibiofilm agent. The cationic AMP P5 was previously designed by some of us and evaluated *in vitro* to determine its antimicrobial and hemolytic activity, together with its physicochemical properties [14,25]. The tests presented here were performed against a clinical isolate of *P. aeruginosa* resistant to carbapenems (due to the expression of the carbapenemase NDM-1). P5 showed an $IC_{50}$ of 19.1 μg/ml and demonstrated a pronounced synergistic activity with meropenem. Biofilm inhibition, eradication and bacterial viability after P5 treatment were evaluated using different methodologies (MTT, crystal violet and confocal fluorescence). Atomic force microscopy (AFM) was also applied; showing surface perturbation of P5 treated biofilms. The peptide was shown to promote biofilm disruption and bacteria-killing properties, with small effects in biofilm growth inhibition. Promising results in biofilm eradication by P5 may lead to a future use of this peptide or its derivatives against hospital-related infections.

## Materials and methods

*Peptide synthesis*

The peptide P5 (RIVQRIKKWLLKWKKLGY) was synthesized with C terminus amidation and obtained at a purity grade of >95% by HPLC (GenScript Co., Piscataway, NJ 08854, USA). The peptide design and physicochemical properties have been previously described [25][14]

*Bacterial strain and growth conditions*

*Pseudomonas aeruginosa* M13513 is a clinical isolate harboring the carbapenemase NDM-1 (*blaKPC-2*). It was kindly provided by Dr. Diego Faccone, from Servicio Antimicrobianos, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS "Dr. Carlos G. Malbran", Buenos Aires, Argentina. Bacterial cultures were grown in Cation Adjusted Muller Hinton Broth (CAMHB) as recommended by the CLSI for antimicrobial testing.

*Growth kinetics*

Growth kinetics were performed to evaluate the effect of different concentrations of P5 against *P. aeruginosa* M13513. P5 at concentrations ranging from 8 to 1024 µg/ml was incubated in CAMHB, with $5x10^6$ UFC/ml as the starting inoculum, in 96-wells polystyrene plates, with 100 µl of final volume. Bacteria without peptides and CAMHB alone were used as positive and negative controls, respectively. Plates were incubated at 37°C for 20 h in the Cell Imaging Multi-Mode Reader Cytation TM5, and measurements at 600 nm were taken every 2 hours and a half, with a previous shaking. Optical density was plotted against time, showing mean of two measures ± SD for each point. $IC_{50}$ values were determined calculating the concentration required to eliminate 50% of CFU/ml from cultures. $IC_{50}$ values were calculated as the concentration at which the best-fit trend lines crossed 50% of surviving CFU/ml from control cultures [26]. Non-linear fitting was performed using GraphPad Prism5 software.

*Synergy by killing kinetics*

The potential synergistic effect of P5 in combination with meropenem was evaluated by time-kill kinetic studies. Experiments were performed in 50 ml tubes (final volume 10 ml). *P. aeruginosa* M13513 log-phase culture was reached and diluted in CAMH to a final cell concentration of $5\times10^6$ CFU/ml. Peptide and meropenem were evaluated at 0.5×MIC (minimal inhibitory concentration), alone and/or combined. Bacteria without treatment were used as a control. Samples were incubated at 37°C, with constant shaking, for 24 h. Aliquots were taken at 0, 1, 2, 3, 4, 8 and 20 h, serially diluted in

saline solution and three drops of 50 µl per dilution were plated on LB agar. Plates were incubated at 37°C for 24h, for CFU counting. Results were plotted as Log CFU/ml *vs.* time. Reduction in two or more logarithmic units between the combined and the most active agent alone after 24h was considered as synergistic. Reduction of the starting inoculum in three or more logarithmic units was considered as bactericidal.

*Outer-membrane permeability*

Outer-membrane permeabilization activity of P5 was determined by using the NPN (1-*N*-phenylnaphthylamine) fluorescent assay, as previously described [27]. Measurements were carried on a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia), with excitation at 350 nm and emission at 420 nm. Briefly, an overnight culture of *P. aeruginosa* M13513 cells was inoculated in fresh Mueller-Hinton broth (MHB) media and incubated at 37°C under agitation until reaching an $OD_{600nm}$ of 0.1. Cells were harvested by centrifugation at 1,000×*g* for 5min, washed twice in PBS. One ml of cell suspension was mixed with an aliquot of NPN to a final probe concentration of 12 µM. Subsequently, 1ml of cell suspension was added to a 0.5 cm quartz cuvette and mixed with different final concentrations of P5. Fluorescence was recorded over time until no further increase was detectable.

*Inhibition of biofilm formation*

Inhibition of biofilm development was assayed in 96-well flat-bottom polystyrene plates. Antimicrobials were two-fold serially diluted in Mueller Hinton broth and then an ON culture of bacterial inoculum was added to reach a final concentration of $5x10^5$ CFU/ml, with a final volume of 100 µl per well. Plates were incubated at 37°C for 24 h. A control was defined as bacteria in the absence of peptide. To determine the amount of biofilm after peptide incubation, the supernatant was gently removed and the formed biofilms were washed twice with 100µl of saline solution to withdraw planktonic cells. The remaining biofilm was fixed with 100µl of methanol, for 15 min, and then stained with 100µl of crystal violet (CV) 1% (v/v), for 5 min. The dye was removed, wells were washed twice with 200µl of distilled water and the plate was dried at 37°C for 30 min. Finally, 100µl of acetic acid 33% were added, samples were homogenized by gentle agitation and absorbance was measured in a microplate reader (RT2100, Rayto Life and Analytical Sciences Co., Ltd), at 595nm.

*Eradication of pre-formed biofilm and cell viability*

*P. aeruginosa* M13513 biofilms were developed in 96-well flat-bottom polystyrene plates with CAMHB, for 24 h. After biofilm formation, supernatant was removed and 100 µl of CAMHB with two-fold serial dilutions of antimicrobials were added to each

well. Plates were then incubated for another 24 h, at 37°C. Two plates were prepared for each experiment: one was used for biofilm quantification, using the CV staining method described above; the other was used to analyze bacteria cell viability within biofilm by the formazan dye-based MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. For this purpose, media was gently removed and biofilms were washed three times with 200µl of saline solution. Then, 100µl of MTT 0.05% (w/v) were added to each well and plates were incubated at 37°C, in the dark, for 3 h. After incubation, the supernatant was withdrawn and formazan crystals were dissolved with 100µl of DMSO, being homogenized by orbital agitation for 10 min. Absorbance was measured at 570 nm, with a microplate reader (RT2100, Rayto Life and Analytical Sciences Co.,Ltd), at 595nm.

*Confocal laser scanning microscopy*

For confocal laser scanning microscopy (CLSM) analysis, biofilms were grown in µ-dish$^{35mm,\ low}$ibiTreat (80136, Ibidi, Fitchburg,WI, USA), using the same conditions of biofilm inhibition and eradication assays, with a final volume of 300µl. After P5 incubation, biofilms were stained using the live/dead staining *BacLight* bacterial viability kit (L7007, Molecular Probes, Thermo Fisher Scientific, Waltham,MA, USA). Samples were imaged with a point scanning confocal microscope Zeiss LSM 710 (Oberkochen,Germany), equipped with a Plan-Apochromat 63×/1.40 oil objective. Lasers used include an argon ($\lambda_{exc}$ = 458, 488 and 514 nm, 25 mW maximum power) and a DPSS 561-10 ($\lambda_{exc}$ =561 nm, 15 mW maximum power). For the three-dimensional representations, images of the same sample field were obtained at different *z* values (z-stack), using the same distance from the center image (± 20 µm). The adjustment of the pinhole to one airy unit was performed and images were acquired using the Zen Black edition software. The proportion of live and dead cells was determined by counting three representative images taken from each biofilm imaged, using the image analysis software Icy (http://icy.bioimageanalysis.org, [28])

*Atomic force microscopy*

Atomic force microscopy (AFM) imaging was conducted as described elsewhere [29]. Briefly, the same samples used for CLSM analysis were washed five times with distilled water and allowed to air dry at room temperature. Bacterial biofilms in the absence and presence of P5 were then scanned with a JPK NanoWizard IV (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope. Measurements were carried out in intermittent contact mode (air) using ACL silicon cantilevers (AppNano, Huntingdon, UK) with a tip radius of 6nm, a resonant frequency of approximately 190 kHz and a spring constant of 58 $N.m^{-1}$. The scan rate was set between 0.3 and 0.6 Hz and the setpoint was close to 0.3 V. Height and error signals were collected and images were analyzed with Gwyddion software.

*Statistical analysis*

Statistical evaluation was determined using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). When applicable, one-way ANOVA followed by Dunnett post-test was performed, considering differences statistically significant for $p < 0.05$. All data presented as mean ± standard error of the mean.

## Results

### Antimicrobial activity

*Bacterial growth kinetics*

*P. aeruginosa* growth curves were studied for 24 h in the presence of a range of P5 sub-inhibitory to inhibitory concentrations (Figure 1A). In these studies we could see that the peptide was active at concentrations below 64µg/ml, showing that there was no sharp threshold concentration (under which the peptide had no activity) like other AMPs, instead the antimicrobial activity was dependent on its concentration.

After 20h of peptide incubation (maximum measured), bacterial growth was evaluated for the different P5 concentrations, yielding an $IC_{50}$ (peptide concentration necessary to reduce to 50% the bacterial cell count) of 19.1 µg/ml (Figure 1B). This data is important because it give us information regarding the activity of the antimicrobial agent at concentrations below the MIC.

Figure 1A shows that until 10h of culture, even 16 µg/ml of P5 can completely inhibit bacterial growth, Afterwards, and in order to have more accurate MIC values for synergy assays, the MIC value for P5 was adjusted to 50 µg/ml testing different concentrations ranging from 32 to 64 µg/ml (data not shown). A similar procedure was performed for meropenem obtaining a MIC value of 800 µg/ml.

*Outer membrane permeabilization*

The ability of P5 to permeabilize the outer membrane of *P. aeruginosa* was determined by the NPN uptake assay. NPN is a small hydrophobic molecule that is excluded by intact bacterial outer membranes, but exhibits increased fluorescence after partitioning into disrupted outer membranes. Thus, an increase in fluorescence intensity in the presence of any agent indicates a disrupted or permeabilized bacterial outer membrane. As shown in Figure 2, the addition of P5 promoted NPN uptake across the outer membrane of *P. aeruginosa*, confirming its ability to disrupt the outer bacterial membrane. Moreover, its ability to be incorporated reaches a stagnation at 14.18 µg/ml (Figure 2B), indicating that this concentration is sufficient to obtain maximum peptide permeabilization effect.

*Synergistic activity*

The possible synergy between P5 and meropenem was evaluated in a time-kill kinetic assay. This procedure, although it is laborious and time-consuming, is more accurate than the checkerboard method, because this latter method is particularly prone to reproducibility problems because of the intrinsic error of the method (+/- one dilution) [30]. In spite of the lack of consensus, the time-kill method may be considered the gold standard for synergism evaluation, as it allows a dynamic evaluation and higher

sensitivity [29]. Figure 3 shows a complete inhibition of *P. aeruginosa* M13513 growth using the combination of P5 and meropenem at 0.5xMIC each, indicating synergy between both compounds. Besides, the synergistic combination showed bactericidal activity due to the reduction of more than 2 logarithmic units compared to the initial inoculums.

**Antibiofilm activity**

*Inhibition of biofilm formation*

Firstly, *P. aeruginosa* biofilm was allowed to develop in the presence of P5 at sub-inhibitory concentrations, ranging from 0.25×MIC to 0.5×MIC, being the total biomass presents after 24h quantified (Figure 4). Data show that P5 inhibits biofilm formation (approximately 20%) only at 0.5×MIC (Figure 4A). Even being a small reduction, it should be noticed that none of the conventional antibiotics tested (gentamycin and tobramycin) were able to inhibit biofilm formation at the concentrations tested (Figure 4B).

To corroborate the effect on biofilm inhibition, confocal images of *P. aeruginosa* biofilms incubated with P5 at 0.5×MIC were acquired (Figure 5). The 3D representation of the z-stack imaging (Figure 5A and B) shows that, for both biofilms, there was a high percentage of living cells, maintaining the live-dead proportion (Figure 5C) despite the exposure to the peptide. However, in the control group, a homogeneous biofilm structure is observed, whereas in the P5-treated sample a less dense biofilm structure can be noticed.

*Eradication of pre-formed biofilms*

After 24h of biofilm formation, the different antimicrobial compounds (P5, tobramycin or gentamycin) were added and incubated for another 24h (Figure 6). The concentrations chosen for each compound ranged from the MIC to 8×MIC. In the CV assays, we could observe that P5 is active in reducing pre-formed biofilm mass, reaching a 50% reduction at higher concentrations tested (Figure 6A). It is worth to notice that neither of the conventional antibiotics tested showed biofilm disruptive activity at equivalent concentrations (Figure 6B). This ability of P5 to disrupt a pre-formed biofilm should correlates with the low number of viable bacterial cells present in the biofilm. For this reason, we determined the cell viability for each concentration tested after peptide or conventional antibiotics incubation (Figure 7). The results obtained show that P5 is able to diminish cell viability for concentrations above the MIC (Figure 7A), with a reduction of 80% of viable bacteria at 2 to 8 × MIC. Therefore, P5 exhibit sigh killing capacity for the bacteria present inside the biofilm. Tobramycin and gentamycin also showed antimicrobial activity on the concentration range tested, despite being less effective than P5 (Figure 7B). Tobramycin yielded a 20% reduction

in bacterial viability (80% of viable cells) for all concentrations tested. As for gentamycin, its maximum effect in reducing bacterial cell viability was 50% at 8×MIC, the highest concentration tested.

The P5 effects on the biofilm architecture were also addressed by confocal microscopy, using a z-stack 3D reconstruction. The biofilm was allowed to develop for 24h, and afterwards was treated with P5, in concentrations ranging from 4 to 8×MIC (Figure 8). The biofilm was then stained to label live and dead bacterial cells, as previously described in materials and methods. The pre-formed biofilm was completely disrupted by P5 at 8×MIC (Figure 8B), with a loss of homogeneity relative to the control biofilm (Figure 8A). Additionally, the number of dead cells present in the biofilm substantially increased, as evidenced by the live/dead relation (Figure 8C), with live cells representing ~90% of total counts in the control biofilm, but droping to ~5 % in the P5-treated biofilm, in a good agreement with MTT data.

Finally, in order to better visualize the effects of P5 in pre-formed biofilms, the evaluation of their surface was also performed by AFM (Figure 9). Conditions were maintained between confocal and AFM imaging, using the same samples for both assays. Looking at the results obtained for the control biofilm (Figure 9A), we could conclude that biofilm was fully formed, with a complex 3D structure, evidenced by bacterial cell density. As for the P5-treated biofilm (Figure 9B), loss of complexity and structure can be easily noticed, corroborating the results obtained by confocal imaging (Figure 8). The center and right images of Figure 9B also depict bacterial cell damage, with loss of integrity, probably due to P5 activity towards *P. aeruginosa*.

## Discussion

AMPs have gained increasing attention as potential novel antimicrobial drug alternatives for combating infections caused by conventional antibiotic-resistant bacteria and/or associated to biofilms [31]. Thus, the design or identification of novel AMPs with such potential is an important goal, creating advances and overcoming the current antibiotic resistance worldwide problem [20]. In this work, we analyzed the possible synergistic and antibiofilm activity of the designed antimicrobial peptide P5, which has been previously the subject of biophysical studies, confirming its preference to interact with bacterial cells, contrary to eukaryotic membranes [14].

Being one of the AMPs features their rapid activity and targeting, it is important to follow peptide action over time [32]. MIC is commonly determined 24h post-inoculation, which may mask any differences in susceptibility. If the AMP acts in the first hours, bacterial resistance can occur even at small percentages of the total population, but this small number of bacteria may be enough to promote cell growth after a few hours. Due to this, growth kinetic experiments with different P5 concentrations were performed, showing a direct relationship between concentration and growth inhibition (Figure 1). This indicates that the peptide apparently does not display a "threshold" concentration over which it has activity and below which is inactive. From the same data, we could determine the $IC_{50}$ of P5 for *P. aeruginosa* M13513 being 19.1 μg/ml. This value was considered for all further experiments.

Keeping in mind that the biophysical characterization of P5 activity toward negatively charged membranes was already performed [14], outer-membrane permeabilization of planktonic bacteria cells was also evaluated. We could observe that a stagnation in the promoted permeabilization is reached at 14.18μg/ml of P5 (Figure 2B), in a good agreement with $IC_{50}$ and MIC determinations, confirming its ability to target *P. aeruginosa* cells. This property is not exclusive of this AMP, with several peptides showing the ability to permeabilize the outer-membrane of Gram-negative bacteria [33,34]. Considering that we were testing P5 antibiofilm activity, it is important to have data where bacteria permeabilization is demonstrated, supporting further studies.

A plausible and accepted strategy to treat drug-resistant-associated infections is using a combination of antimicrobial agents, particularly with different mechanisms of action, which would hamper the emergence of resistance. P5 in combination with meropenem showed not only synergistic activity but also a bactericidal activity for the combination.

As pointed above, our outer membrane permeabilization experiments showed that P5 in a concentration below 0.5xMIC (i.e. 25 μg/ml) can easily permeabilize the membrane of the cell. This membrane permeabilization activity could explain the synergistic effect observed with meropenem, which requires entry into the cytoplasmic space to acetylate the PBPs in order to interfere with the formation of peptidoglycan in the cell wall. The synergistic activity found for these two compounds highlights the promising possible combination of these two molecules in *P. aeruginosa* infections. Further work should be

performed in order to analyze different combinations of P5 and carbapenems in *vitro* and *in vivo*, together with pharmacokinetic experiments, to determine the possible clinical dose.

Synergy between AMPs and carbapenems has been suggested for other peptides [8] and was hypothesized for colistin (a cyclic polypeptide used in clinical practice also known as polymixin E), whose synergistic activity with meropenem it is believed to rely on its capacity to change the permeability of the outer membrane of the cell wall, which in turn allows meropenem to act inside the bacteria [35].

The cyclolipopeptide analog of polymyxin AMP38, also display synergy with the carbapenem imipenem, but it did not show anti-biofilm activity, except when administered in combination with imipenem at 62 ug/ml [36]. Other AMPs, like the α-helical AMP PL-5 [37] or the synthetic protegrin IB-367 have been shown to display synergy with imipenem in *P. aeruginosa*, evaluated with the checkerboard method, although no antibiofilm activity has been reported in this bacteria to date.

Biofilms are social communities of bacteria that involve several interactions. Generally, biofilm-associated bacteria, such as *P. aeruginosa*, cause chronic infections that may persist for decades. Consequently, biofilm-associated infections treatment has become an important part of antimicrobial chemotherapy, as they are not affected by conventional antibiotic therapeutic concentration [38]. In the last decade, growing interest has been devoted to the possible use of AMPs as antibiofilm agents [31,39–41]. In this context, some AMPs have been reported to prevent biofilm formation and/or to eradicate established ones, and in some cases mechanisms beyond these antibiofilm effects have been hypothesized [31,42].

In order to explore P5 antibiofilm activity, inhibition of biofilm formation was tested. Inhibition activity of P5 at 0.5×MIC was modest (20% inhibition), without a significant difference at 0.25×MIC (Figure 4). These data correlate with the confocal microscopy images obtained for the biofilm, depicting slight differences in biofilm height, relative to the control (Figure 5).

It is worth to notice that for tobramycin and gentamycin, the biofilm biomass increases at 0.25×MIC and 0.5×MIC. A possible explanation could be that the cells that remain able to grow must face a very hostile environment and synthesize a larger amount of exoproducts than usual, probably in the attempt to protect themselves. *P. aeruginosa* produces alginate exopolysaccharide (naturally anionic), so the presence of this matrix may explain the slow penetration of fluoroquinolones and aminoglycosides. It has been reported that sub-MIC concentrations of β-lactam antibiotics induce increased alginate synthesis in *P. aeruginosa* biofilms [43,44]. In this work, we observed that sub MIC concentrations of two aminoglycosides (tobramycin and gentamycin) also induced this phenomenon (figure 4B).

A different situation occurs for established biofilms, which tend to be disrupted upon P5 incubation. In this case, the peptide promoted a significant disruption in pre-formed

biofilms, decreasing their biomass in approximately 50% (Figure 6). Although the crystal violet staining enables a good estimate of biofilm mass, it is not informative on bacteria viability, being impossible to demonstrate bactericidal or directed activity of the peptide towards the biofilm [45]. For that reason, bacterial metabolism viability was also evaluated by the MTT assay. Data revealed a significant decrease in bacterial metabolism of biofilm-associated cells after P5 treatment, demonstrating that the peptide extensively affects these bacteria (Figure 7). It is generally accepted that metabolic activity reduction analyzed by the MTT assay may be due to cell death. Nevertheless, in the case of biofilms, that correlation may not be so straightforward, since biofilm-associated bacteria can enter a reversible dormant status, in which cells are metabolically inactive [46]. Therefore, confocal imaging of live/dead cell staining was used to determine P5 effect on biofilm viability. Loss of bacterial cell viability was confirmed (Figure 8C), with the number of dead cells increasing to ~90% when incubated with P5 (8xMIC). By observing the 3D biofilm architecture (Figure 8), it was also possible to visualize a loss of structure and homogeneity in the biofilms treated with P5. To further confirm this, AFM imaging was also performed. As previously indicated, P5 promoted biofilm disruption (Figure 9B), with severe damage to biofilm architecture and bacterial cells.

Other synthetic cationic AMPs, derived from natural peptides such as the human cathelicidin LL-37 and the bovine peptide indolicidin [47], have been identified as biofilm inhibitory compounds [48]. Unlike P5, LL-37 at subinhibitory concentrations displayed a strong biofilm inhibition (approx. 80%), but a mild biofilm eradication in a *P. aeruginosa* culture (50% after 4 days treatment) [47], just the reverse of P5. The AMP obtained from frog´s skin ocellatin-PT3 was also reported to inhibit the proliferation of 48-h mature MDR *P. aeruginosa* biofilms in concentrations up to 10× MIC, although no biofilm rupture was observed [18].

The immunomodulatory peptide IDR (innate defense regulator)-1018 prevented *P. aeruginosa* biofilm development at subinhibitory concentrations and eradicated or reduced existing biofilms at 10 µg/ml [49], evaluated using a flow cell apparatus and then monitoring biofilm formation for 3 days (different experimental conditions from those used in this work)

In conclusion, this study shows that P5, a *de novo* designed antimicrobial peptide, displays interesting synergistic activity with meropenem, biofilm disruptive activity and biofilm-associated bacteria-killing properties. This AMP displays relatively low toxicity evaluated *in vitro* in a human erythrocytes hemolytic assay [14] and also *in vivo*, when administered by instillation to Balb/c mice at 10 mg/kg (data not shown, manuscript under review).

Besides further experiments will be required, these results suggest that this molecule might be an interesting candidate for future drug development strategies, alone or in combination with carbapenems, with a particular interest in chronic *P. aeruginosa* infections, commonly associated with biofilm formation.

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## Figures legends

**Figure 1. Growth kinetics of P. aeruginosa M13513 incubated with different P5 concentrations.** (A) Growth kinetics upon incubation of P5 at sub-MIC (8-32 µg/ml) and above MIC (64 µg/ml) concentrations, monitored for a total time of 20h. Control (ctrl+) was defined as the growth kinetics of P. aeruginosa in the absence of any antimicrobial molecule. (B) Logarithmic growth curve at 20h of incubation with different P5 concentrations. Non-linear fitting was performed using GraphPad Prism software, obtaining an $IC_{50}$ of 19.1 µg/ml. MIC value for P5 was previously determined as 50 µg/ml, after testing concentrations ranging from 32 to 64 µg/ml (data not shown). One representative experiment of n=3.

**Figure 2. Outer-membrane permeabilization of P. aeruginosa M13513 cells.** Bacterial cells were incubated with NPN in the presence of different P5 concentrations. (A) NPN uptake by P. aeruginosa incubated with different concentration of P5, measured by an increase in fluorescence intensity after probe partition into the hydrophobic core of the outer membrane. Data presented as mean ±SD. Statistical comparison using one-way ANOVA followed by a Dunnett's post-test for multiple comparisons vs. control (absence of peptide); *p<0.05, ***p<0.001,N=3. (B) Representation of NPN uptake kinetics after the addition of 14.18mg/ml of P5 (dashed line in panel A).

**Figure 3. Synergistic activity of P5 and meropenemagainstP. aeruginosaM13513**. Bacteria were grown for 24 h with 0.5×MIC of P5, 0.5×MIC of meropenem, or both at 0.5×MIC each. **GC**: growth control. The decrease in 2 logarithmic units for the combined formulation (P5 + Mer), comparing it to the most active agent (Mer), indicates a synergistic effect. One representative experiment of n=3

**Figure 4. Inhibition of P. aeruginosa M13513 biofilm formation.**(A) Bacteria were incubated with P5 at 0.25×MIC, 0.5×MIC, or (B) with the control conventional antibiotics tobramycin (T) or gentamycin (G). After 24h of biofilm growth, dishes were washed, and total biomass was quantified using crystal violet. One-way ANOVA followed by Dunnett's multiple comparison test; *p<0.05. N=3 independent experiments

**Figure 5. Confocal microscopy evaluation of the inhibition of P. aeruginosa M13513 biofilm formation**. Bacteria cells were incubated for 24 h with P5 at 0.5×MIC. Staining was obtained with SYTO9 (green fluorescence, live) and propidium iodide (red fluorescence, dead). (A) and (B) present 3D biofilm representations (200X) of the control and P5-treated, respectively. (C) Evaluation of viable and dead cells obtained through image analysis is represented in bars, depicting mean ± SD.

**Figure 6. Eradication of pre-formed P. aeruginosa M13513 biofilms.** After 24h of bacteria growth and biofilm formation, P5 (A) or conventional antibiotics (B) were added at supra-MIC concentrations for another 24h. Concentrations tested ranged from the MIC to 8×MIC. The biofilm after peptide or conventional antibiotic (T, tobramycin; G, gentamycin) treatment was measured using crystal violet. One-way ANOVA followed by Dunnett's multiple comparison test; *p<0.05, ***p<0.0001. N=3 independent experiments

**Figure 7. P. aeruginosa M13513 cell viability in pre-formed biofilms.**P5 (A) or conventional antibiotics (B) treatment after 24h of pre-formed bacterial biofilms.Concentration tested ranged from 2 to 8×MIC. Cell viability (metabolic activity) was assessed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. One-way ANOVA followed by Dunnett's multiple comparison test; *p<0.05,**p<0.001, ***p<0.0001.T, tobramycin; G, gentamycin. N=3 independent experiments

**Figure 8. Confocal microscopy evaluation of pre-formed P. aeruginosa M13513 biofilms eradication.** Biofilms were grown for 24h and then treated with P5 at 8×MIC. Live/dead staining was performed by SYTO9 and PI (live/green and dead/red fluorescence, respectively). 3D biofilm representation (200X) of untreated control cells (A) and cells treated with P5 (B). Evaluation of viable and dead cells (C) is represented in bars, depicting mean ± SD.

**Figure 9. Atomic force microscopy evaluation of pre-formed P. aeruginosa M13513 biofilms eradication.** Biofilms were grown for 24 h and then treated with P5 at 8 × MIC. AFM height images of untreated control cells (A) and cells treated with P5 (B). Representative images of bacteria are shown for each treatment.

## Authors statement

Authors' individual contributions:

Melina Martinez: Investigation; Methodology; Data curation and Formal analysis

Sónia Gonçalves: Investigation; Methodology and data curation

Mário R. Felício: Investigation; Methodology and data curation

Patricia Maturana: Investigation and Methodology

Nuno C. Santos: Resources and Writing – review & editing

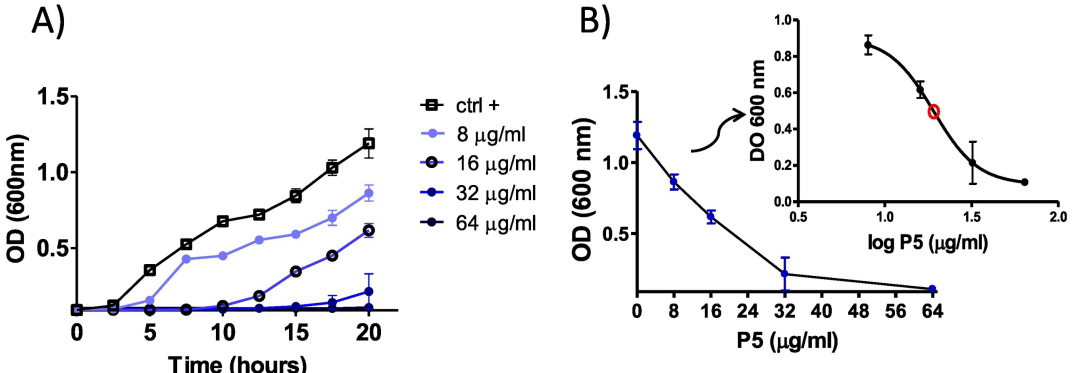
Liliana Semorile: Resources and Writing – review & editing

Axel Hollmann: Investigation; data curation, Resources and Writing – review & editing

Paulo C. Maffía: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing

# Highlights

- The antimicrobial peptide P5 displays synergistic activity with meropenem in a carbapenem-resistant strain of P. aeruginosa
- P5 permeablizes the membrane of P. aeruginosa
- P5 eradicates P.aeruginosa biofilms and displays bactericidal activity against biofilm associated bacteria


A)
OD (600nm)
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20
Time (hours)
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8 µg/ml
16 µg/ml
32 µg/ml
64 µg/ml
B)
OD (600 nm)
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0.5
0
8
16
24
32
40
48
56
64
P5 (µg/ml)
DO 600 nm
1.0
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0.6
0.4
0.2
0.0
0.5
1.0
1.5
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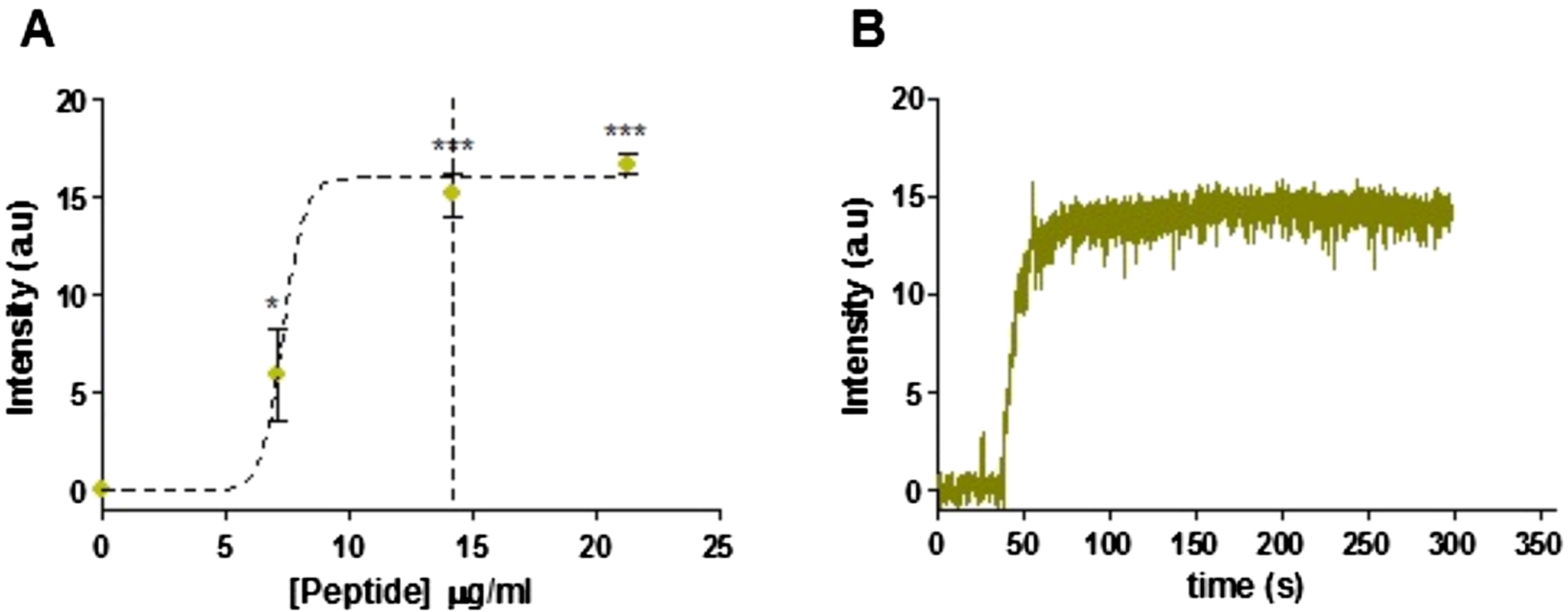
log P5 (µg/ml)

Figure 1


A
B
Intensity (a.u)
[Peptide] µg/ml
time (s)
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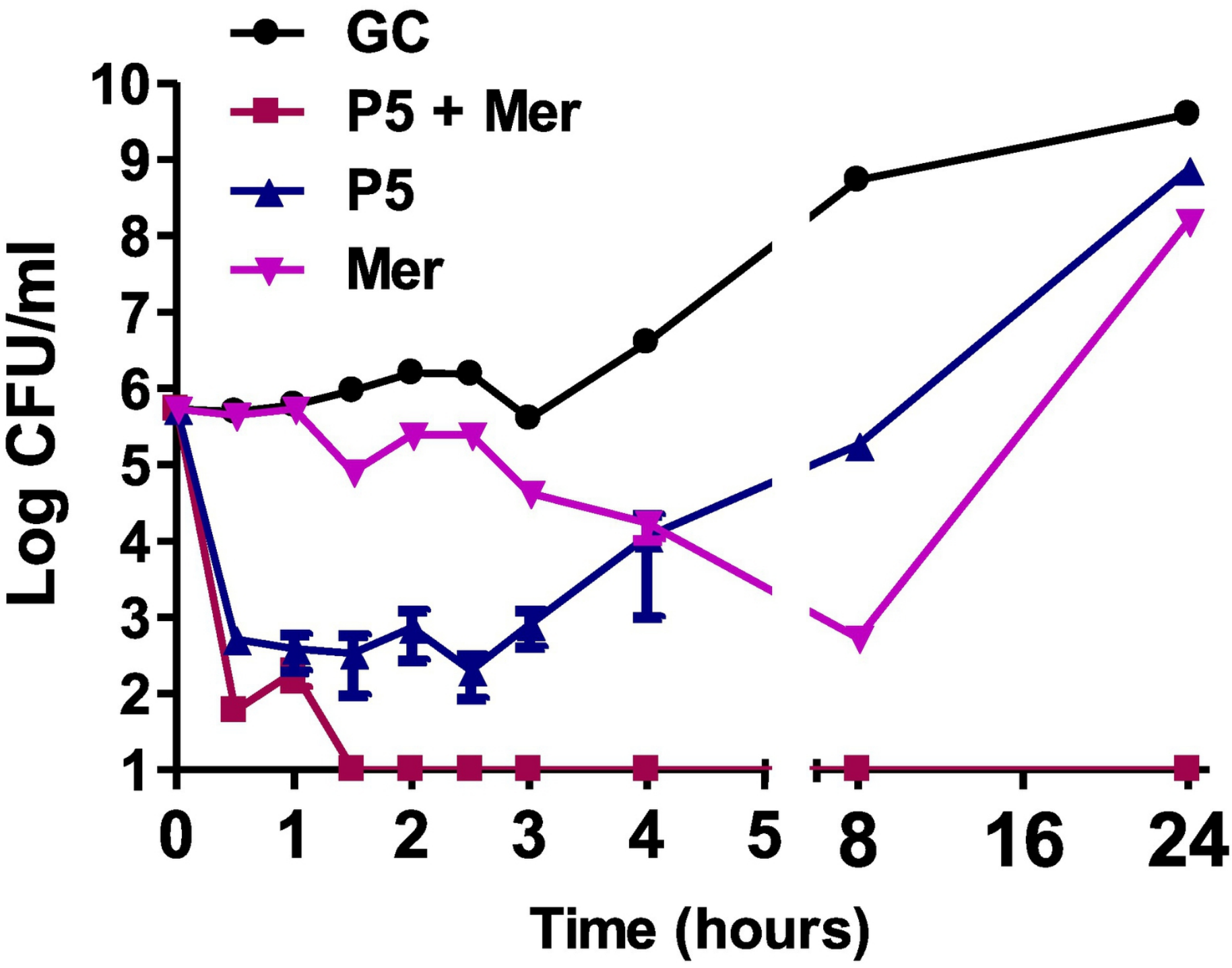
200
250
300
350

Figure 2


GC
P5 + Mer
P5
Mer
Log CFU/ml
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9
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3
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1
0
1
2
3
4
5
8
16
24
Time (hours)

Figure 3

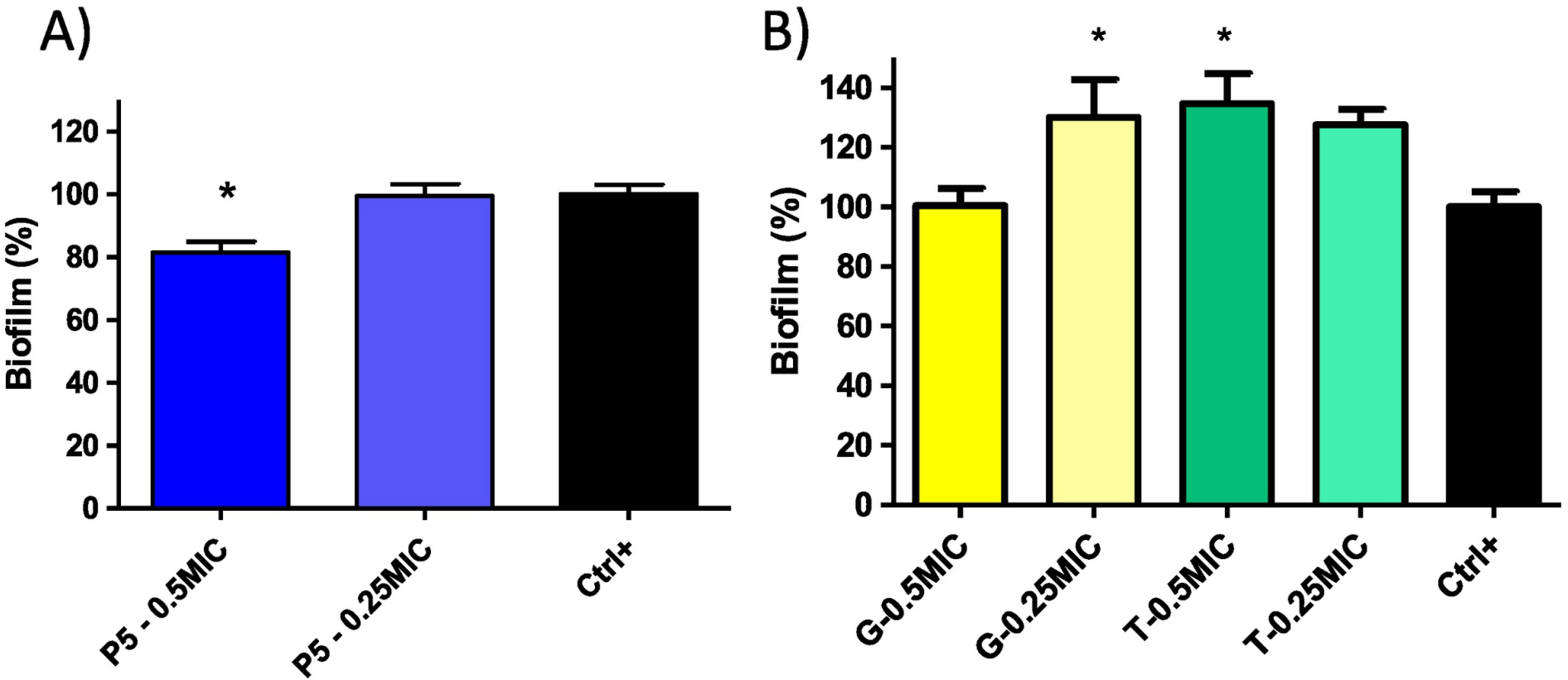
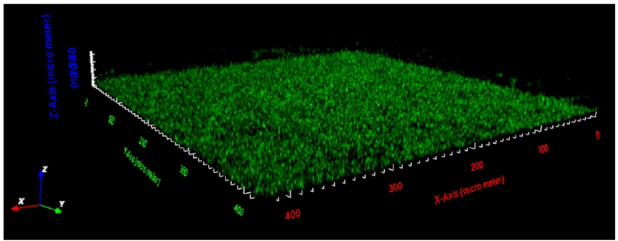
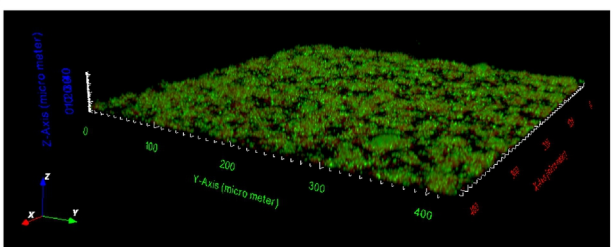

A)
Biofilm (%)
120
100
80
60
40
20
0
*
P5 - 0.5MIC
P5 - 0.25MIC
Ctrl+
B)
Biofilm (%)
140
120
100
80
60
40
20
0
*
*
G-0.5MIC
G-0.25MIC
T-0.5MIC
T-0.25MIC
Ctrl+

Figure 4

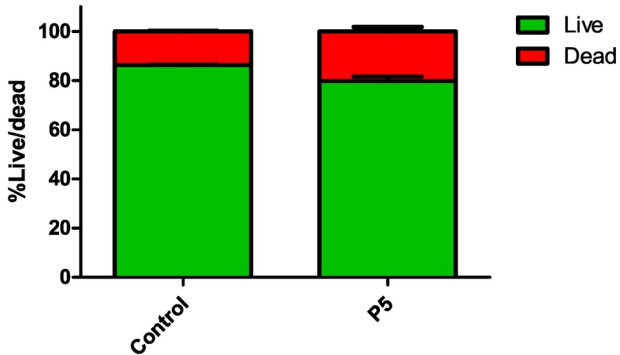
A)


Z-Axis (micro meter)
X-Axis (micro meter)

B)


Z-Axis (micro meter)
Y-Axis (micro meter)

C)


%Live/dead
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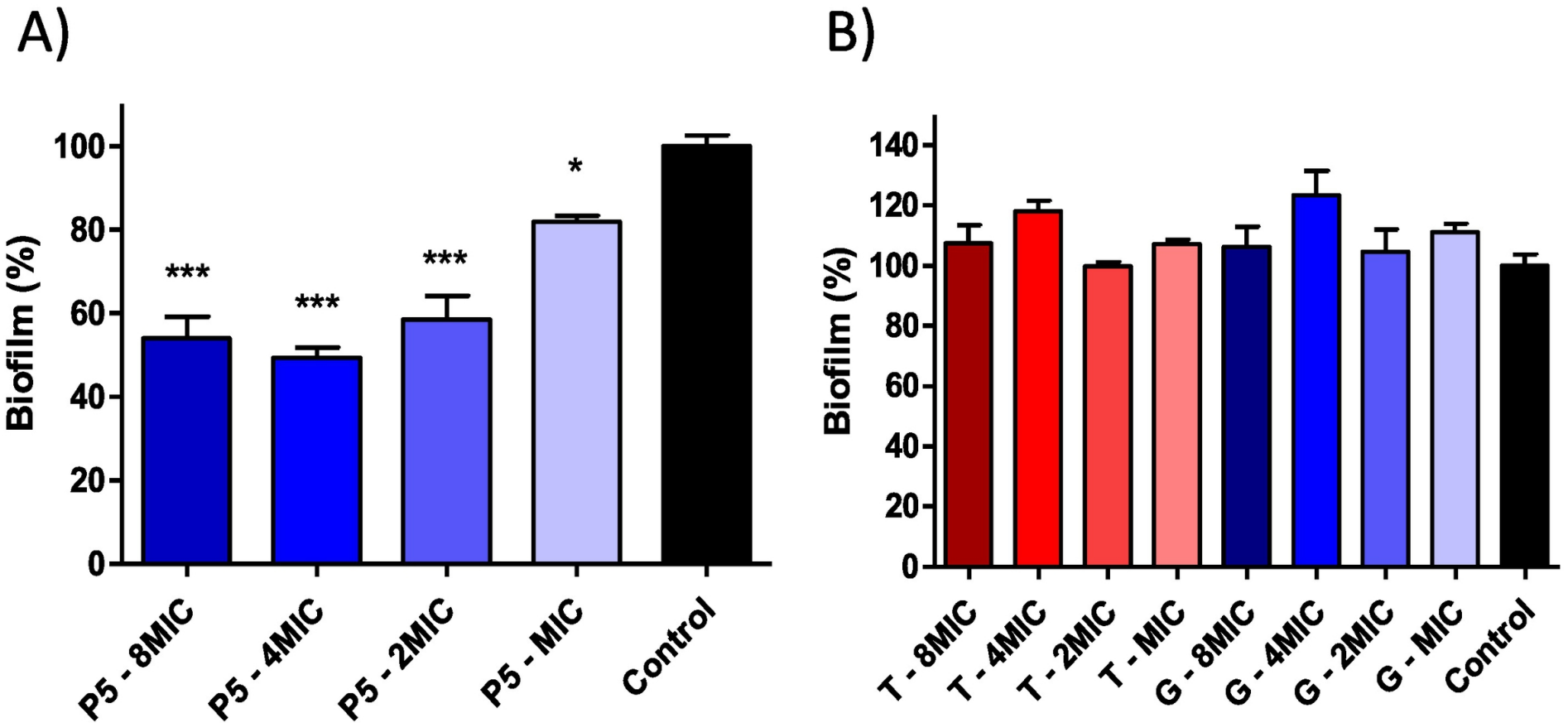
60
40
20
0
Control
P5
Live
Dead

Figure 5


A)
Biofilm (%)
100
80
60
40
20
0
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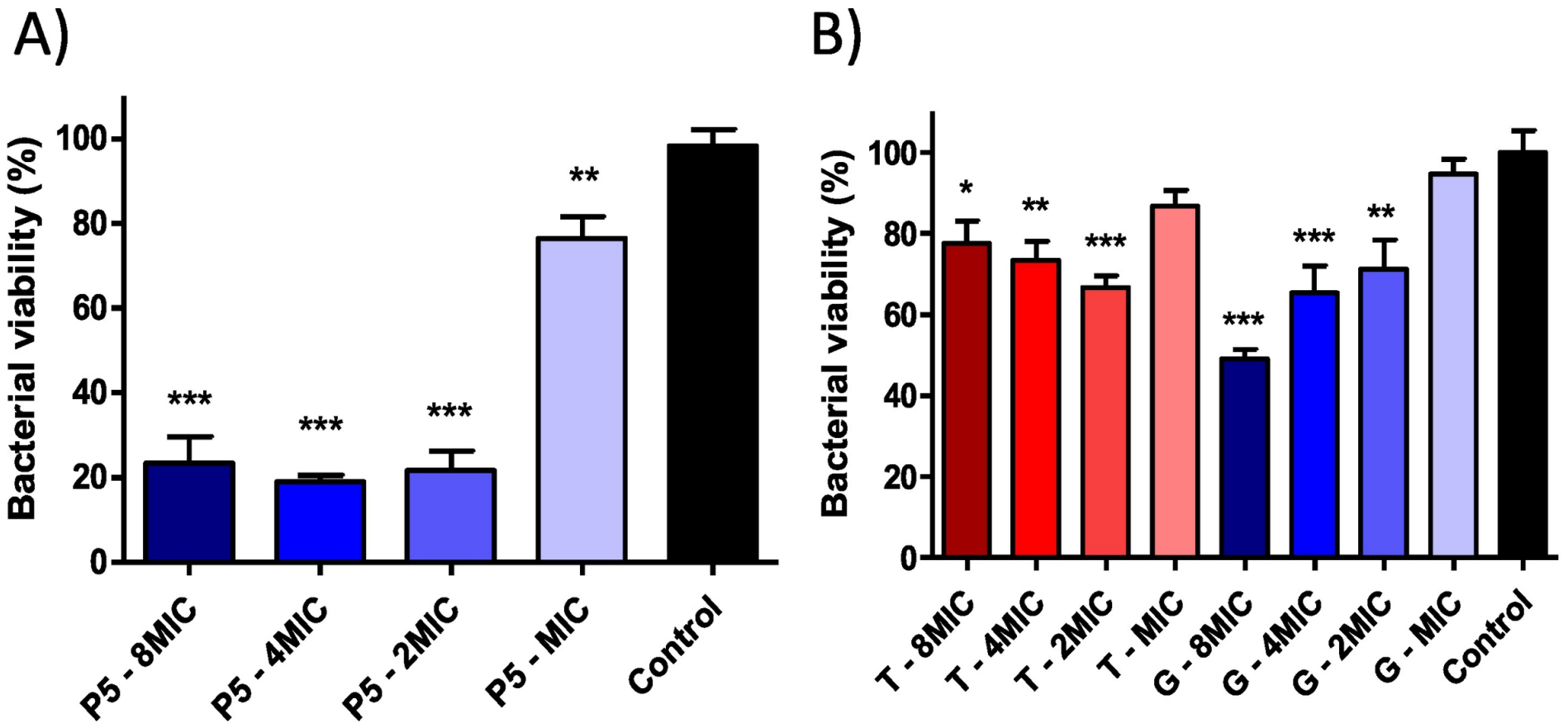
*
P5 - 8MIC
P5 - 4MIC
P5 - 2MIC
P5 - MIC
Control
B)
Biofilm (%)
140
120
100
80
60
40
20
0
T - 8MIC
T - 4MIC
T - 2MIC
T - MIC
G - 8MIC
G - 4MIC
G - 2MIC
G - MIC
Control

Figure 6


A)
Bacterial viability (%)
100
80
60
40
20
0
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P5 - 8MIC
P5 - 4MIC
P5 - 2MIC
P5 - MIC
Control
B)
Bacterial viability (%)
100
80
60
40
20
0
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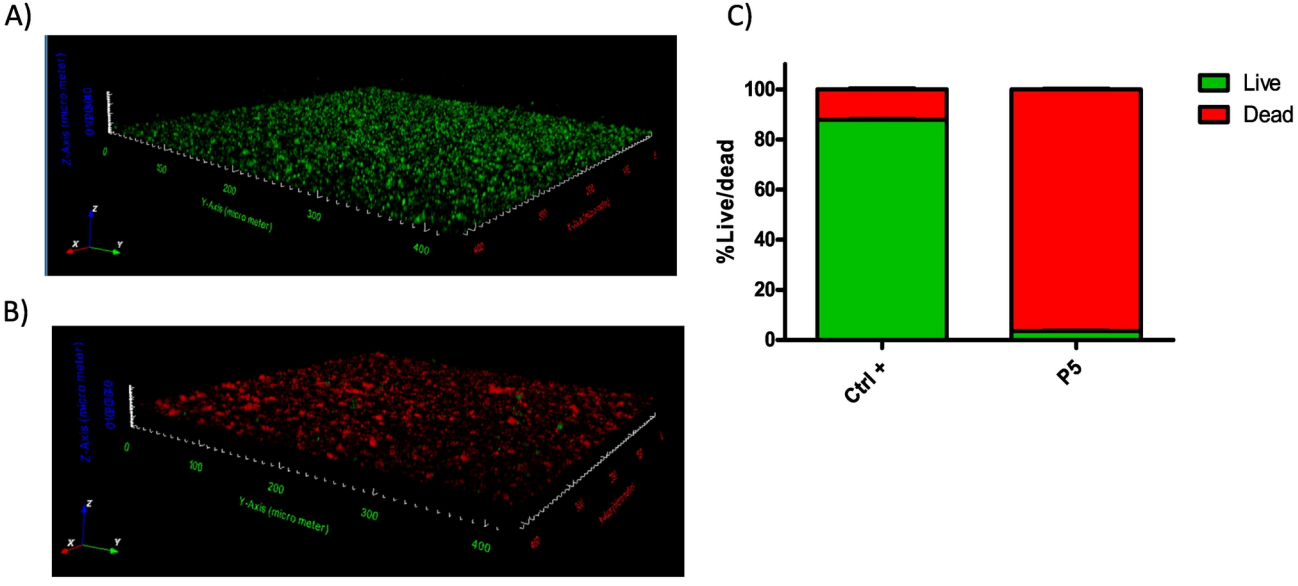
T - 8MIC
T - 4MIC
T - 2MIC
T - MIC
G - 8MIC
G - 4MIC
G - 2MIC
G - MIC
Control

Figure 7


A)
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C)
Z-Axis (micro meter)
Y-Axis (micro meter)
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P5

Figure 8

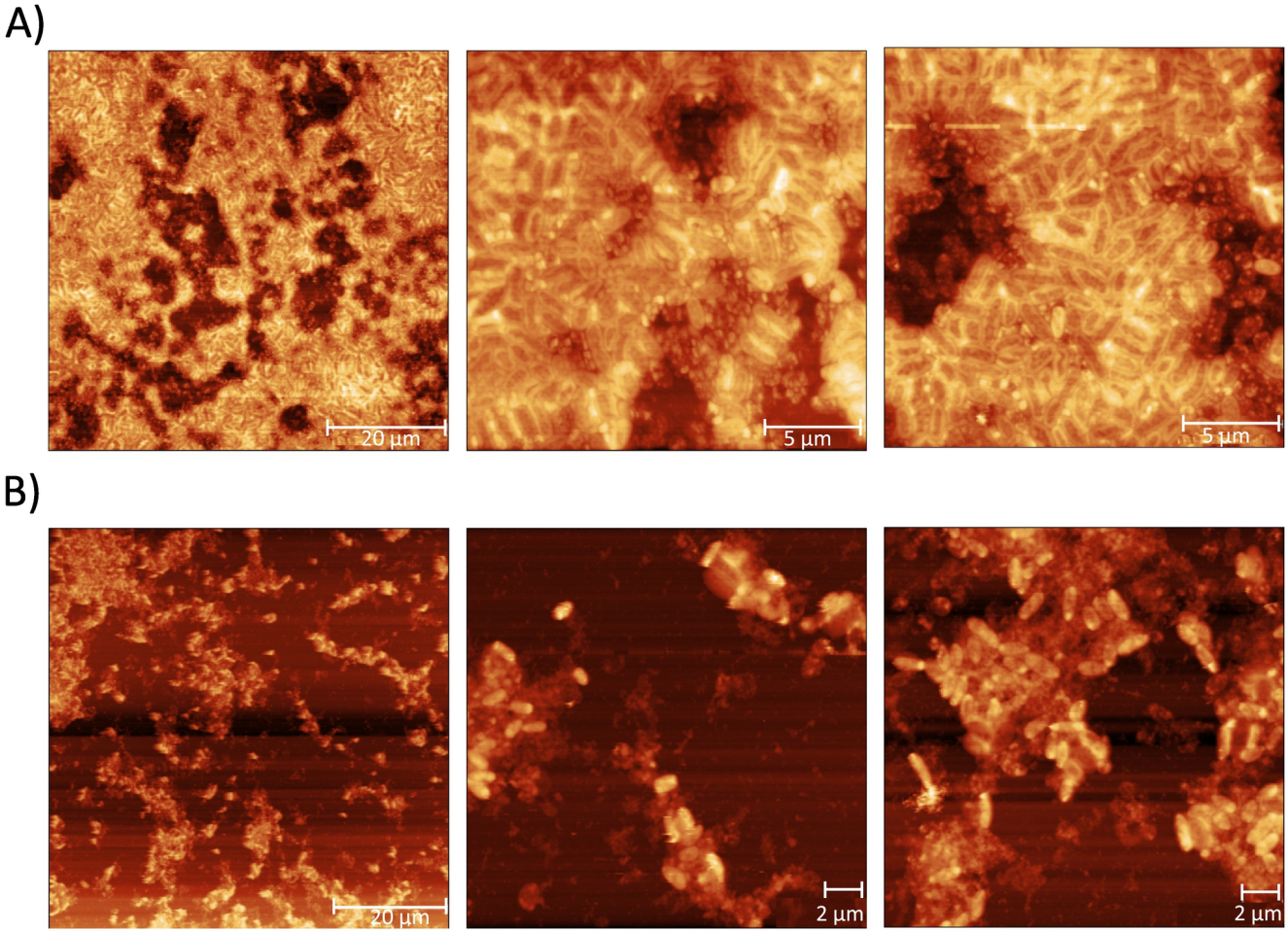


Figure 9