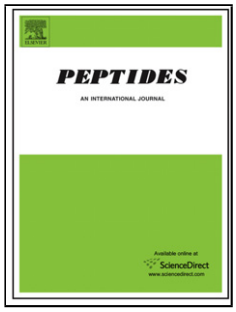

Title: Inhibition and destruction of *Pseudomonas aeruginosa* biofilms by antibiotics and antimicrobial peptides

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Article title: Inhibition and destruction of *Pseudomonas aeruginosa* biofilms by antibiotics and antimicrobial peptides.

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Abstract

Pseudomonas aeruginosa is one of the major nosocomial pathogen that can causes a wide variety of acute and chronic infections *P. aeruginosa* is a dreaded bacteria not just because of the high intrinsic and acquired antibiotic resistance rates but also the biofilm formation and production of multiple virulence factors. We investigated the *in vitro* activities of antibiotics (ceftazidime, tobramycin, ciprofloxacin, doripenem, piperacillin and colistin) and antimicrobial cationic peptides (AMPs; LL-37, CAMA: cecropin(1-7)-melittin A(2-9) amide, melittin, defensin and magainin-II) alone or in combination against biofilms of laboratory strain ATCC 27853 and 4 clinical strains of *P. aeruginosa*. The minimum inhibitory concentrations (MIC), minimum bactericidal concentration (MBC) and minimum biofilm eradication concentrations (MBEC) were determined by microbroth dilution technique. The MBEC values of antibiotics and AMPs were 80->5120 and 640->640 mg/L, respectively. When combined with the LL-37 or CAMA at 1/10xMBEC, the MBEC values of antibiotics that active against biofilms, were decreased up to 8-fold. All of the antibiotics, and AMPs were able to inhibit the attachment of bacteria at the 1/10xMIC and biofilm formation at 1x or 1/10xMIC concentrations. Time killing curve studies showed 3-$\log_{10}$ killing against biofilms in 24-h with almost all studied antibiotics and AMPs. Synergism were seen in most of the studied combinations especially CAMA/LL-37+ciprofloxacin against at least one or two strains' biofilms. Since biofilms are not affected the antibiotics at therapeutic concentrations, using a combination of antimicrobial agents including AMPs, or inhibition of biofilm formation by blocking the attachment of bacteria to surfaces might be alternative methods to fight with biofilm associated infections.

Key words: Biofilm; inhibition; destruction; antimicrobial cationic peptides; *P. aeruginosa;* time kill curve; MBEC

1. Introduction

Pseudomonas aeruginosa is a major nosocomial pathogen that can cause a wide variety of acute and chronic infections, such as bacteraemia, pneumonia, intra-abdominal wound, burn and urinary tract infections. Patients with burn wounds, urinary tract infections, acquired-immune deficiency syndrome (AIDS), lung cancer, chronic obstructive pulmonary disease, bronchiectasis, or cystic fibrosis, or those with indwelling inert surfaces are especially vulnerable to such infections [15,27]. *P. aeruginosa* is a dreaded bacteria with high intrinsic and acquired antibiotic resistance rates; it also forms biofilms and produces multiple virulence factors. Biofilm-associated *P. aeruginosa* strains can cause several types of infection in patients with indwelling inert surfaces like medical devices for internal or external use. Because the bacteria in biofilms become more resistant to antibiotic treatment and the actions of the host immune system, the treatment of biofilm-associated infections is extremely difficult [16,20].

A biofilm is a microbial community that is attached to abiotic surfaces and produces extracellular polysaccharides. It is characterized by the growth-dependent accumulation of multi-layered cell clusters that are in turn surrounded by an extracellular polysaccharide matrix. Microbial cells that grow in biofilms are physiologically distinct from planktonic cells of the same organism. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behaviour, and large groups of genes are differentially regulated [6,10,13]. Finally, the bacteria in biofilms become more resistant to antibiotics and the host's immune defence mechanisms as they adapt to changing conditions together instead of as single cells.

Antimicrobial cationic peptides (AMPs) have attracted attention as alternative or complementary antibiotics due to their prospective potency, rapid action, and broad spectrum of activities against Gram-negative and -positive bacteria, viruses, fungi, and parasites. AMPs are a major component of the innate immune systems of most living organisms, including

insects, plants, microorganisms, and mammals. In addition to protect against environmental microorganisms, they exhibit multiple mechanisms of action and, consequently, a low potential to induce de novo resistance, which allows the limited use of other antibiotics [12,29].

AMPs seem to be a good candidate for treating biofilms, either alone or in combination with antibiotics [9,11,21,23]. In this study, we tried to determine the *in vitro* activities of multiple antibiotics (ceftazidime, tobramycin, ciprofloxacin, doripenem, piperacillin, and colistin) and AMPs (LL-37, cecropin A [1-7]-melittin A [2-9] amide [CAMA], melittin, defensin, and magainin II) alone or in combination against biofilms of standard and clinical *P. aeruginosa* strains.

2. Materials and methods

2.1. Bacterial strains:

P. aeruginosa laboratory strain ATCC 27853 (Rockville, MD, USA) and four clinical isolates obtained from specimens submitted to the routine Clinical Microbiology Laboratories of Istanbul University, Istanbul Faculty of Medicine were tested. Clinical strains were isolated from different patient with acute sepsis and the isolates were identified with API 20 NE (BioMerieux, France) diagnostic kits.

2.2. Antimicrobial substances:

Five AMPs (LL-37, CAMA, melittin, defensin, and magainin II) were obtained from Bachem AG (Bubendorf, Switzerland). Antibiotics (ceftazidime, tobramycin, ciprofloxacin, doripenem, piperacillin, and colistin) were kindly provided by their respective manufacturers. Except for doripenem, stock solutions from dry powders were prepared at a concentration of 1280 mg/L for AMPs and 5120 mg/L for antibiotics according to the manufacturers' recommendation and stored at –80°C for up to 6 months before use. Doripenem solutions were prepared on the day of use.

2.3. Media:

Tryptic soy broth supplemented with 1% glucose (TSB-glucose, Difco Laboratories, Franklin Lakes, NJ) was used for biofilm production, cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories) was used to determine the minimum inhibitory concentration (MIC) and minimum biofilm-eradication concentration (MBEC) values, and tryptic soy agar (TSA, Difco Laboratories) was used in colony counts to determine the minimum bactericidal concentrations (MBC) and MBEC values and in time-kill curve (TKC) analysis.

2.4. MIC and MBC determinations:

MICs of antibiotics and AMPs were determined with a microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) [3]. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth.

MBCs were determined at the end of the incubation period by removing two 10-µL samples from each well demonstrating no visible growth and plated onto TSA. Resultant colonies were counted after an overnight incubation at 37°C. The MBC was defined as the lowest concentration of antimicrobials that killed at least 99.9% of the initial inoculums [18].

2.5. Biofilm formation:

P. aeruginosa strains were cultured in 5 mL TSB-glucose for 24 h at 37°C with 360° rotation (50 rpm) and diluted 1/50 in fresh TSB-glucose, yielding a final concentration of approximately 1×10^7 cfu/200 µL. This suspension was added to each well of a 96-well tissue culture microtitre plate (Greiner Bio-One, Kremsmuenster, Austria), and incubated at 37°C, 24 h. The negative control was TSB-glucose. After the incubation, the waste media was aspirated gently and the wells of the plates were washed three times with 250 µl physiological buffered saline (PBS) solutions to remove unattached bacteria and air-dried. 200 µl of 99% methanol was added per well for 15 min for fixation and aspirated, and plates were allowed to dry. Wells were stained with 200 µl of 0.1% crystal violet (in water) for 5 min. Excess stain

was gently rinsed off with tap water, and plates were air-dried. Stain was resolubilized in 200 µl of 95% ethanol, shaking in orbital shaker for 30 min. and measured the absorbance at 595 nm [8].

2.6. Biofilm attachment assay:

Biofilm attachment assays were performed using a previously described method with some modifications [19]. The overnight cultures were diluted 1/50 to give 1×10^7 cfu/200 µL in TSB-glucose, and standard and clinical *P. aeruginosa* strains were added to each well of a 96-well tissue culture microtitre plate with 1/10× MIC concentrations of AMPs and antibiotics. The plates were incubated for 1, 2, or 4 h at 37°C. Six wells were used for each AMP and antibiotic condition. The positive controls were *P. aeruginosa* strains in TSB-glucose without peptide or antibiotic. After incubation, the wells were washed twice with phosphate-buffered saline (PBS) solution and measured in PBS at 595 nm.

2.6. Inhibition of biofilm formation:

Standard and clinical *P. aeruginosa* strains (1×10^5 cfu/200 µL) in TSB-glucose were incubated at 37°C, 24 h with AMPs or antibiotics at 1×, 1/10×, and 1/100× MIC concentrations in 96-well tissue culture microtitre plates. Six wells were used for each AMP or antibiotic. The positive controls were *P. aeruginosa* strains in TSB-glucose without peptide or antibiotic. After the incubation, wells were washed twice with PBS solutions and measured at 595 nm.

2.7. Minimum biofilm eradication concentration (MBEC):

Measurements of the antimicrobial susceptibilities of the *P. aeruginosa* biofilms were assessed as previously described MBEC assay with the following modifications [2]. The 24-h biofilms in a 96 well tissue culture microtitre plates were washed three times with 250 µl PBS solutions and air-dried. Serial two fold dilutions ranging from 640 to 0.06 mg/L for AMPs and 5120 to 5 mg/L for antibiotics were prepared in CAMHB. 200 µl of each concentration

was added to each corresponding well and plates were incubated 24 h at 37°C. After the incubation, antibiotics were aspirated gently and plates were washed two times with sterile PBS solutions, wells were scraped thoroughly, with particular attention to well edges. Well contents were removed, placed in 1 ml PBS solution, placed in a sonicating waterbath **(**Bandelin sonopuls HD 2200) for 5 min to disrupt the biofilm, and 100 µl samples were plated on TSA. Colonies were counted after 24 h at 37°C. The MBEC was defined as the lowest concentration of AMP or antibiotic that prevented bacterial regrowth.

2.8. MBECs of antibiotics in the presence of AMP:

When considering the large concentrations of AMPs and antibiotics required to treat biofilm-associated infections, we investigated the in vitro activities of the antibiotics with AMPs at subMBEC values. For this purpose, we added 64 mg/L AMP 1/10×MBEC) to each concentration of antibiotic and then performed the MBEC assay as explained above.

2.9. Time killing curve (TKC) studies:

The modified TKC method, which we previously described, was used to determine the dynamic bactericidal activities of AMPs, alone or in combination with antibiotics against 24-h biofilms [7]. For this purpose, 24-h biofilms were prepared in a 96-well tissue culture microtiter plate, washed three times with 250 µL PBS, and air-dried. AMPs and antibiotics were diluted in CAMHB and added to each corresponding well, yielding a final concentration of 1× MBEC, and the plates were incubated for 0, 2, 4, 7, or 24 h at 37°C. After incubation, the plates were washed two times with sterile PBS, scraped and the contents were incubated in a sonicating water-bath, as described above. Following the disruption, serial 1/10-fold dilutions were made and 100 µL samples were plated on TSA. Colonies were counted 24 h after incubation at 37°C. An antibiotic-free control of each strain was also included.

TKCs were constructed by plotting mean colony counts ($\log_{10}$ cfu/mL) versus time. The lower limit of detection for the time-kill assays was 2 $\log_{10}$ cfu/mL. Bactericidal activity was

defined as a ≥ 3-$\log_{10}$ cfu/mL decrease from the initial inoculum. The results of drug combinations were interpreted by comparing the effects of the various combinations and the effects of the most active agent alone. Synergy and antagonism were defined as a ≥ 2-$\log_{10}$ decrease or increase, respectively, in the colony count after 24 h. If there was no 2-$\log_{10}$ increase or decrease, the effect of combination was assumed as additive [18]. We also calculated the fractional inhibitory concentrations of bliss synergy coefficients to measure the degree of synergy/antagonism. For this purpose, we used the equation below, which mentioned in the model of Prichard et al [22].

$$Z = X + Y(1 - X)$$

Z represents the total inhibition produced by the combination of drugs X and Y and X and Y represent the inhibition produced by drugs X and Y alone, respectively. Any peaks above this plane would be indicative of synergy. Similarly, any depressions below the plane would indicate antagonism.

2.10. Statistical analysis:

All experiments were performed in two independent assays. When the results were different for MIC, MBC, or MBEC determinations, a third test was performed. In biofilm attachment and inhibition of biofilm formation assays, results are presented as the mean ± standard deviations of two independent experiments performed with standard and four clinical strains. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests were used to compare differences between control and antimicrobial-treated biofilms. P values <0.001 were considered statistically significant.

3. Results

3.1. Susceptibility:

The *in vitro* activities of the studied antibiotics and AMPs against *P. aeruginosa* planktonic cells and biofilms are summarized in Table 1. The MIC values of the antibiotics

against *P. aeruginosa* ATCC 27853 were within the CLSI accuracy range throughout the study [4]. As shown in Table 1, there were no major differences between bactericidal and inhibitory concentrations of the antibiotics and AMPs. The MBC values were generally equal to or two-fold greater than those of the MIC values.

When we investigated the *in vitro* activities of the antibiotics that were active against biofilms, with the active AMPs at 1/10×MBEC values against biofilms, the MBEC values of those antibiotics were decreased by up to eight-fold (Table 2).

3.2. Biofilm attachment assay:

When we incubated the 1/10 concentrations of AMPs and antibiotics with standard and clinical *P. aeruginosa* strains for 1, 2, or 4 h at 37°C to assess the adherence to the wells of tissue culture microtitre plates, all of the antimicrobial agents inhibited biofilm attachment in a time-dependent fashion (Figure 1).

3.3. Inhibition of biofilm formation:

All of the studied AMPs and antibiotics inhibited standard and clinical *P. aeruginosa* biofilm formation at 24 h in a concentration-dependent manner (Figure 2). Significant biofilm inhibition was observed at all studied antibiotic concentration, while AMPs achieved this inhibition at 1× or 1/10×MIC values.

3.4. TKC studies:

According to the susceptibility test results, we performed the TKC studies with three antibiotics (tobramycin, ciprofloxacin, and colistin) and two AMPs (LL-37, and CAMA) that were active against *P. aeruginosa* biofilms. The results of the TKC analyses show that 3-$\log_{10}$ killing against *P. aeruginosa* biofilms was determined within 24 h using almost all studied antibiotics and AMPs alone. These analyses also demonstrate enhanced killing of biofilms when AMPs were used in combination with all of the examined antibiotics (Figure 3). As shown in Table 2, the synergistic interactions between the AMPs and antibiotics were

observed in most of the studied combinations against biofilms, especially in those that were treated with the CAMA/LL-37 + ciprofloxacin. According to the calculation of bliss synergism, synergistic interactions against *P. aeruginosa* biofilms were increased up to at least three strains with all of the antibiotic + AMP combinations (Data not shown). No antagonism was observed with any of the combinations.

4. Discussion

Biofilms are social communities of bacteria that involve several interactions, such as communication, cooperation, assistance, engagement, balance, and sharing. Generally, biofilm-associated bacteria causes chronic infections that can persist for decades, whereas planktonic forms cause acute infections. Bacteria on biofilm structures are protected from environmental conditions, antimicrobial agents, and host immune responses, and they also exhibit up to 1000-fold-increased antibiotic resistance to a wide range of antimicrobial agents [13].

In this study, we investigated the *in vitro* activities of six clinically used antibiotics and five AMPs against biofilms of standard and clinical *P. aeruginosa* strains. When we examined the MIC values of antibiotics against planktonic *P. aeruginosa* cells, they ranged between 0.25 - 128 mg/L. Only one strain showed resistance to tobramycin and piperacillin, while all of the other strains were susceptible or moderately susceptible to the studied antibiotics. On the other hand, the MIC values of AMPs were within the 8 - >128 mg/L range, and three cationic peptides (LL-37, CAMA, and melittin) were active against standard and clinical *P. aeruginosa* strains. When we considered the anti-biofilm activities of these antibiotics and AMPs, MBEC values ranged between 80 - >5120 mg/L and 640 - >640 mg/L, respectively. According to these results, three antibiotics (tobramycin, ciprofloxacin, and colistin) and two AMPs (LL-37 and CAMA) were active against *P. aeruginosa* biofilms. The MBEC/MIC ratios of these active antibiotics and AMPs were found to be between 320 - 2560 and 5 - 80

245 fold, respectively. These results are similar to our previous studies and other researchers'
246 results [7,17,24,26].

247 Novel biofilm control strategies can be carried out in three main ways: first, reduction of
248 the planktonic cells before biofilm formation; second, initial prevention of cell adhesion to
249 surfaces; and third, removal or inhibition of mature biofilms. Because biofilm-associated
250 bacteria are not affected by therapeutically achievable concentrations of antimicrobial agents,
251 anti-biofilm therapies have generally focused on the inhibition of biofilm formation [14]. In
252 some reports, peptides or antibiotics were able to inhibit the initial biofilm attachment (58-
253 62%) at subMIC values. That was suggesting a potential interaction of these molecules with
254 bacterial adhesins that mediates the intercellular adhesion of bacteria to the surfaces [5, 25].
255 For this purpose, we investigated the inhibition of bacterial attachment to the surfaces, as well
256 as the inhibition of biofilm production by MIC or subMIC values of antibiotics and AMPs.
257 Almost all of the antibiotics and AMPs were able to inhibit the attachment of bacteria at
258 1/10×MIC in 1-4 h ($p<0.001$). Ciprofloxacin and LL-37 significantly decreased the
259 attachment (up to 70%) of *P. aeruginosa* to the surface in 1 h. We were also able to determine
260 that antibiotics and AMPs significantly inhibited 24-h biofilm formation up to 80%, especially
261 at 1× or 1/10×MIC ($p<0.001$). These results suggested that all of the studied antibiotics and
262 AMPs were effective for inhibiting pre-formed biofilms, even if they were not active against
263 formed biofilms. Even though inhibiting mature biofilm is very difficult, inhibition of biofilm
264 formation in early critical stages seems to be more applicable.

265 AMPs are one of the most promising groups of natural products for overcoming
266 resistance problems in antimicrobial chemotherapy. Most AMPs exhibit broad spectrum
267 antimicrobial activity against planktonic cells, independent from the antibiotic resistance
268 patterns of bacteria, by pore-forming or non-pore-forming models. They also allow the entry
269 of other substances like antibiotics inside the cell and serve as anti-resistance compounds of

classical antibiotics. However, the anti-biofilm activities of AMPs are not completely understood, a small number of studies have posited possible explanations including matrix disruption; binding of DNA; and altering the expression of biofilm-related genes, such as the production of pili and rhamnolipid, quorum sensing systems, and flagella assembly, and their dual capacity to act on both the cytoplasmic membrane and intracellular targets [1,12,14,28]. Therefore, we investigated the in vitro activities of biofilm active antibiotics with AMPs at subMBEC values that can be tolerated by humans, and we found that the MBEC values of those antibiotics were decreased up to eight-fold. These results suggested that AMPs could be used to treat serious biofilm-related infections in combination with antibiotics.

Although the MIC and MBEC values and inhibition of attachment or biofilm production demonstrated the efficacy of studied antibiotics and AMPs against *P. aeruginosa* biofilms, these techniques do not provide any information about the time course of antimicrobial activities. This limitation can be overcome with the use of TKC studies. The results of the TKC studies revealed that neither antibiotics nor AMPs showed at least 2 $\log_{10}$ (99 %) killing in 3 or 6 h, and 3 $\log_{10}$ (99.9 %) killing was achieved in 24 h at MBEC concentrations as expected.

The synergistic effect achieved by using combinations of antimicrobial agents can be useful to rapidly enhance anti-biofilm activity and to help prevent or delay the emergence of resistance. TKC studies also indicated synergistic interactions against *P. aeruginosa* biofilms for at least two strains with all of the antibiotic + AMP combinations. When we calculated the results with bliss synergy method, the number of strains that showed synergism between antibiotic + AMP combinations, were increased. These results showed that the strains affected by this synergism were the same strains that expressed decreased MBEC values when AMP was applied at subMBEC concentrations. Especially ciprofloxacin + CAMA/LL-37 combinations were synergistic against almost all *P. aeruginosa* biofilms.

Consequently, treatment of biofilm-related infections have become an important part of antimicrobial chemotherapy because biofilms are not affected by therapeutic concentrations of antibiotics. Using a combination of antimicrobial agents including AMPs or inhibiting pre-formed biofilms by blocking the attachment of bacteria to surfaces might be effective alternatives to combating biofilm-associated infections.

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393 **Table 1.** In vitro antibacterial and anti-biofilm activities of antibiotics and AMPs against
394 standard and clinical *P. aeruginosa* strains.

Antimicrobial agents	MIC range (mg/L)	MBC range (mg/L)	MBEC range (mg/L)
Antibiotics			
Ceftazidime	1-4	1-4	2560->5120
Tobramycin	0.25-16	0.25-32	160-2560
Ciprofloxacin	0.25-2	0.25-8	80-1280
Doripenem	0.25-4	0.5-8	>5120
Piperacillin	4-128	4-256	>5120
Colistin	0.25-2	0.5-2	160-2560
Cationic peptides			
LL-37	32-128	64-≥128	640
CAMA	8-64	16-128	640
Melittin	64->128	>128	≥640
Defensin	>128	>128	>640
Magainin II	>128	>128	>640

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401 **Table 2.** In vitro anti-biofilm activities of antibiotic and AMP combinations against biofilms
402 of standard and clinical *P. aeruginosa* strains.

	Decrease in MBEC					Interactions in TKC				
	CI1	CI2	CI3	CI4	Std	CI1	CI2	CI3	CI4	Std
Tobramycin										
+LL-37	1×	2×	1×	4×	4×	A	A	A	S	S
+CAMA	1×	1×	2×	4×	2×	A	A	S	S	A
Ciprofloxacin										
+LL-37	4×	1×	2×	4×	4×	S	A	S	S	S
+CAMA	4×	2×	8×	4×	8×	S	S	S	S	S
Colistin										
+LL-37	2×	1×	2×	2×	2×	S	A	S	A	A
+CAMA	1×	1×	2×	2×	2×	A	A	S	S	A

403 CI: Clinical isolate, Std: Standard, A: additive, S: synergist, 1x: no significant decrease, 2-8x:
404 2-8 fold decrease in MBEC. Synergist or additive effects were defined as a ≥2-$\log_{10}$ decrease,
405 or no 2-Log_{10} increase/decrease, respectively, in the colony count after 24 h.
406

406 **Highlights**

407

408 • MBEC values of all antibiotics&AMPs were 80->5120 and 640->640 mg/L,
409 respectively.

410 • Tobramycin, ciprofloxacin, colistin, LL-37 and CAMA were active against biofilms.

411 • All studied antibiotics and AMPs were effective for inhibiting pre-formed biofilms.

412 • The MBEC values of antibiotics, with AMPs at subMBEC, were decreased up to 8-
413 fold.

414 • Especially ciprofloxacin+CAMA/LL-37 combinations were synergistic against
415 biofilms.

416

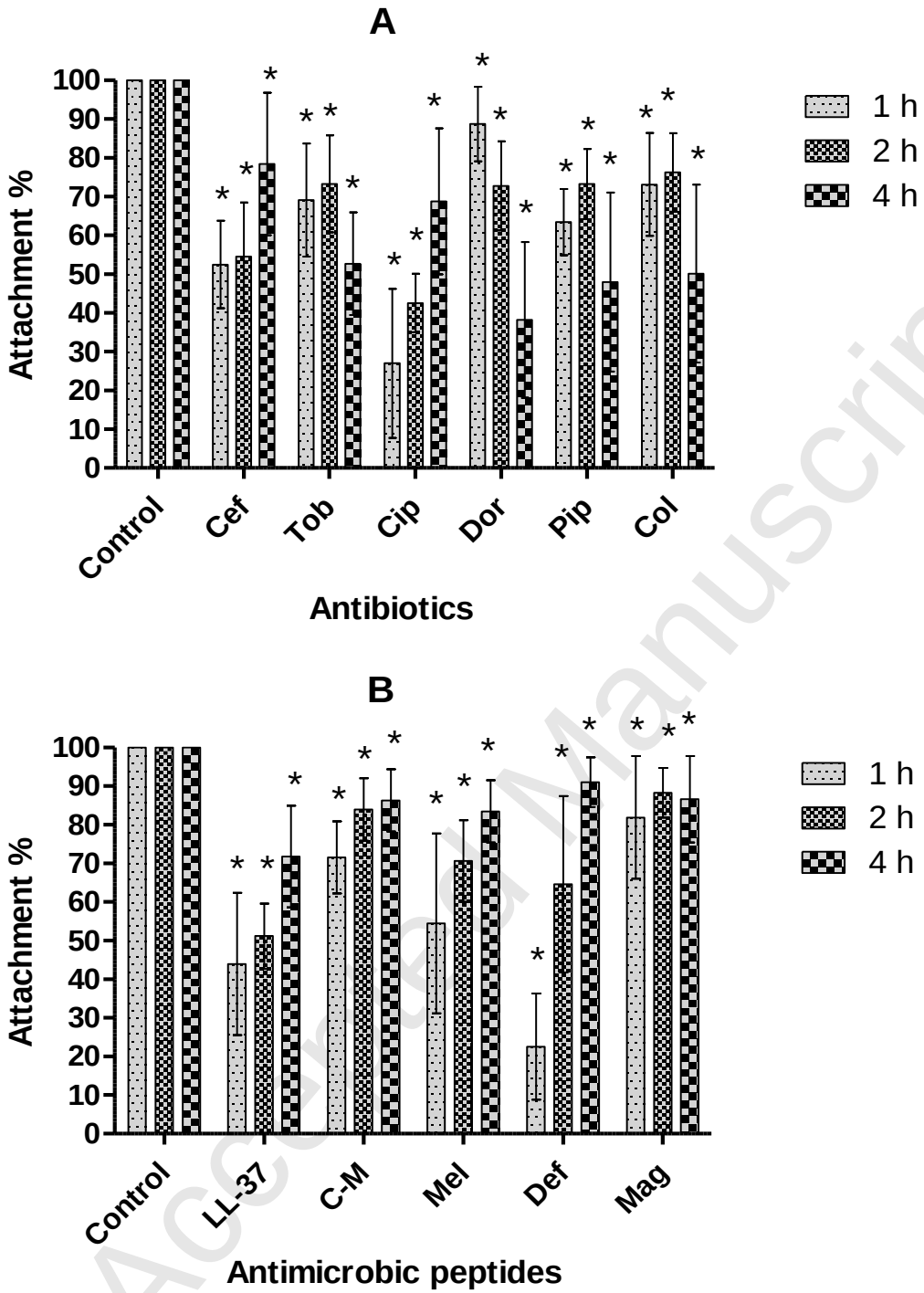

Figure 1. Inhibition of *P. aeruginosa* surface attachment in wells containing (A) antibiotics and (B) AMPs. Each well in the 96-well plates contained 1/10 the MIC of antibiotics or AMPs and an inoculum of 1×10^7 cfu/200 µl *P. aeruginosa* in TSB-glucose. The plates were incubated for 1, 2, or 4 h at 37°C. Six wells were used for each antibiotic and AMP. Each experiment is representative of at least two independent tests with standard and four clinical isolates of *P. aeruginosa*; the error bars indicate the standard deviations between strains. Control bars indicate *P. aeruginosa* biofilms without any antimicrobial, set as 100%. Results were expressed as percentage of biofilm formed with respect to control. *P < 0.001 vs control, ANOVA test followed by Bonferroni's multiple comparison test.

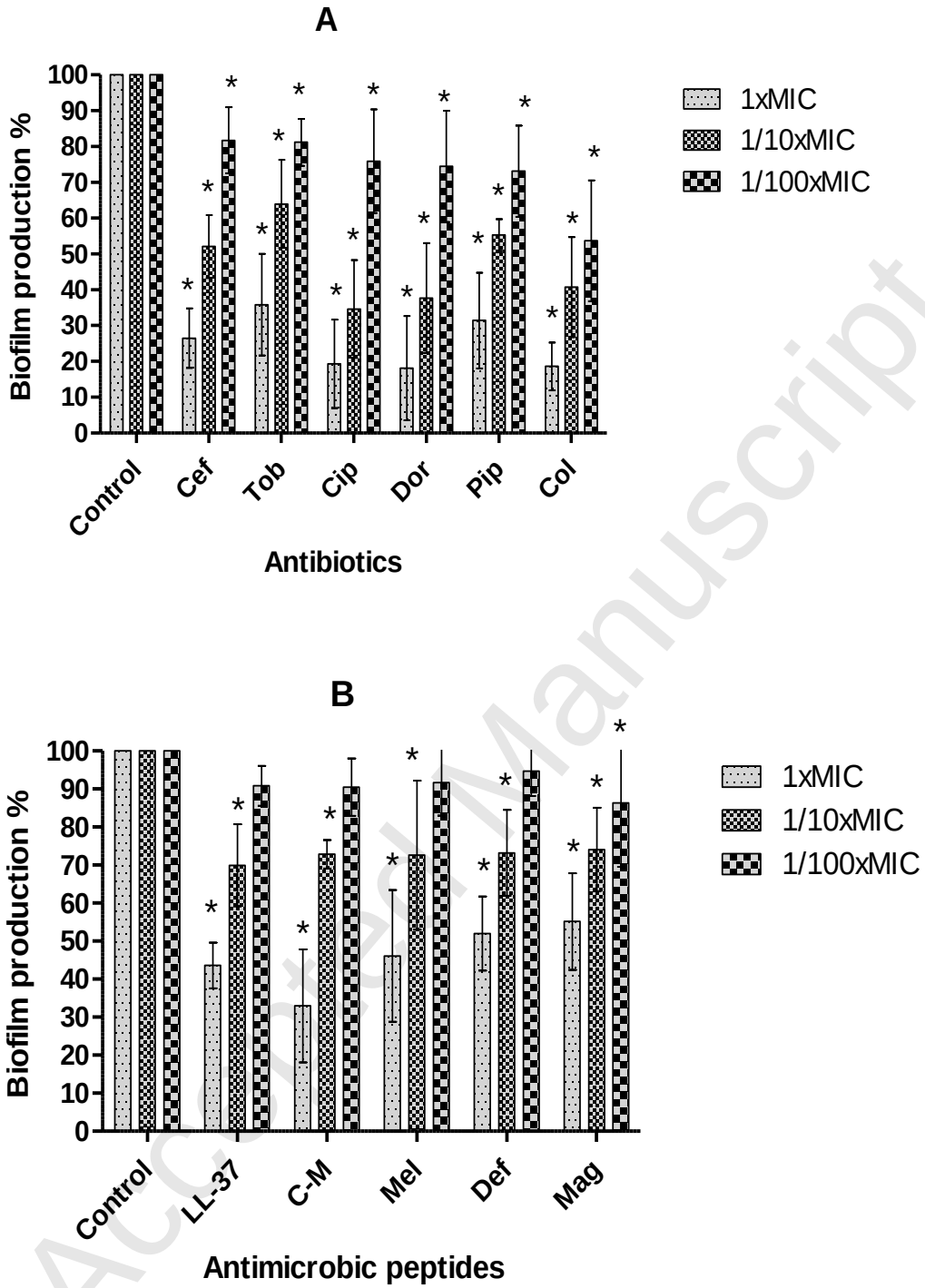

Figure 2. Inhibition of *P. aeruginosa* biofilm formation by (A) antibiotics and (B) AMPs. Each well of the 96-well plates contained 1×, 1/10×, or 1/100×MIC of antibiotic or AMP, and an inoculum of 5×10^5 cfu/200 µl *P. aeruginosa* in TSB-glucose. The plates were incubated for 24 h at 37°C. Six wells were used for each antibiotic and AMP condition. Each experiment is representative of at least two independent tests with standard and four clinical isolates of *P. aeruginosa*; the error bars indicate the standard deviations between strains. Control bars indicate *P. aeruginosa* biofilms without any antimicrobial, set as 100%. Results were expressed as percentage of biofilm formed with respect to control. *P < 0.001 vs control, ANOVA test followed by Bonferroni's multiple comparison tests.

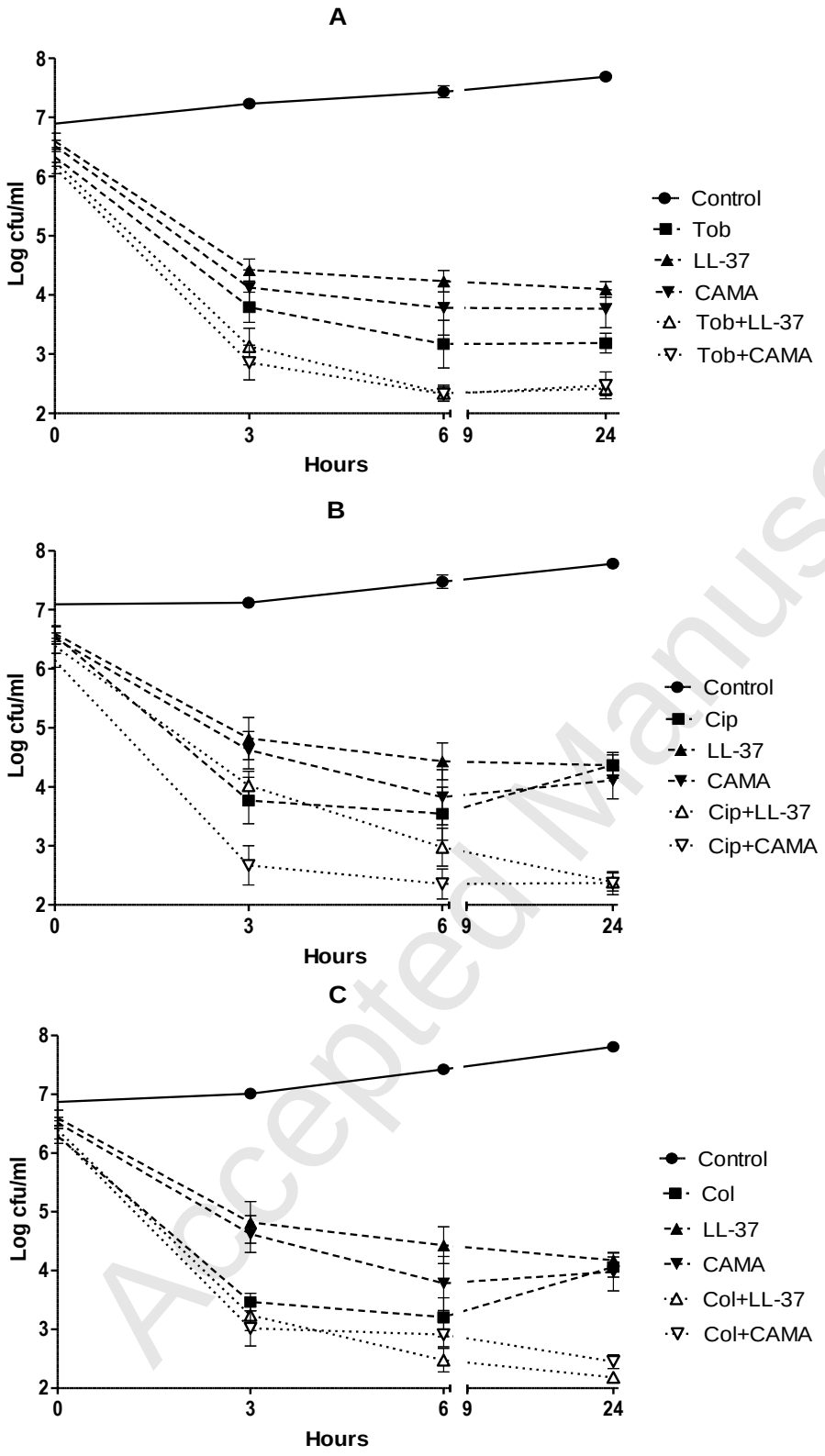

Figure 3. (A) Tob: tobramycin, (B) Cip: ciprofloxacin, (C) and Col: colistin + LL-37 or CAMA combinations observed by time-kill determinations against biofilms of standard and clinical *P. aeruginosa* strains at 1× MBEC. The X- and Y-axis represents time, and logarithmic *P. aeruginosa* survival in biofilm, respectively. Error bars indicate the standard deviations between strains. cfu: colony-forming unit, Control: *P. aeruginosa* biofilms without any antimicrobial treatment.