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Major Article

pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates *in vivo* biofilms

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ABSTRACT

Limitations in treatment of biofilm-associated bacterial infections are often due to subpopulation of persistent bacteria (persisters) tolerant to high concentrations of antibiotics. Taking advantage of increased aminoglycoside efficiency under alkaline conditions, we demonstrated that a combination of gentamicin and the clinically compatible basic amino acid L-arginine increases *in vitro* planktonic and biofilm susceptibility to gentamicin, with 99% mortality amongst the clinically relevant pathogens, i.e. *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* persistent bacteria. Moreover, although gentamicin local treatment alone showed poor efficacy in a clinically relevant *in vivo* model of catheter-related infection, gentamicin supplemented with L-arginine led to complete, long-lasting eradication of *S. aureus* and *E. coli* biofilms, when used locally. Given that intravenous administration of L-arginine to human patients is well tolerated, combined use of aminoglycoside and the non-toxic adjuvant L-arginine as catheter lock solution could constitute a new option for the eradication of pathogenic biofilms.

Key words: Biofilm, persister, aminoglycoside, proton motive force, catheter-related infections

INTRODUCTION

Biofilm bacterial communities that develop on the surface of indwelling devices and mucosa play a key role in healthcare-associated infections due to their characteristic high tolerance to antibiotics [1]. Biofilm formation therefore impairs treatment efficacy and requires frequent therapeutic removal of colonized devices, leading to increased morbidity and medical cost [1-3]. Most treatment difficulties are related to biofilm tolerance, defined as the ability of a subset of biofilm cells to survive in the presence of high concentrations of bactericidal antibiotics [3]. Although biofilm tolerance is multifactorial, it is believed to originate mainly from a subpopulation of non-growing persistent bacteria characterized by phenotypically high, but reversible, tolerance to otherwise lethal concentrations of antibiotics [3]. These persisters are not resistant mutants, and may resume growth and repopulate biofilm after partially effective antibiotic treatment, thereby leading to infection recurrence [3]. Several studies identified promising anti-biofilm strategies that inhibit bacterial adhesion or induce dispersal [4, 5]. However, such approaches were associated with risk of massive release of pathogenic bacteria from the infected site into the patient's bloodstream [6]. Hence, alongside identification of non-biocidal anti-biofilm compounds, anti-persister approaches able to kill planktonic and biofilm bacteria are currently being actively investigated [7, 8].

For instance, use of adjuvant molecules such as mannitol or fructose was demonstrated to increase aminoglycoside uptake, thereby enhancing their *in vitro* and *in vivo* efficacy against persisters [9]. More recently, it has been shown that corrupting a target in dormant cells through the activation of a bacterial protease could also increase antibiotic-induced persister cell death [8].

It was previously shown that sensitivity of planktonic bacteria exposed to aminoglycosides was increased in alkaline media and, inversely, reduced under acidic conditions [10-12]. Those results, along with several other reports, raised the possibility that

local, physiologically compatible alkalinization could constitute an alternative approach to improving antibiotic efficiency and eradicating biofilm persisters [10, 13].

Here we show that raising the pH using harmless, clinically compatible basic amino-acid L-arginine leads to an increase in the killing efficiency of gentamicin toward persisters. We demonstrate that L-arginine and pH-mediated potentiation of aminoglycosides is effective against both *in vitro* and *in vivo* biofilms produced by Gram-positive and Gram-negative nosocomial pathogens. These results therefore demonstrate that pH-mediated stimulation of the effects of aminoglycosides could constitute a new curative approach to eradicating localized vascular catheter-associated biofilm infections.

MATERIAL AND METHODS

Bacterial strains and growth media. Luminescent variants of *Staphylococcus aureus*, *Escherichia coli and Pseudomonas aeruginosa* were purchased (*S. aureus* Xen36 from Caliper, Hopkinton, MA) or constructed (*E. coli* EAEC 55989 transformed with stable plasmid pAT881 and *P. aeruginosa* PAK*lux*, a bioluminescent derivative of the PAK clinical strain) [14-16]. *S. aureus* was grown in tryptic soy broth (TSB) supplemented with 0.25% glucose (TSB glucose), and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were grown in Lysogeny Broth (LB) broth. *E. coli* TG1Δ*argA* was obtained by P1vir transduction from MG1655_*argA*::Tn10 to TG1. Unless specified, all chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Basic amino acids were resuspended in LB, TSB glucose or 1 x PBS and then diluted. L-arginine at 0.4% was buffered using MOPS (3-(N-morpholino)propanesulfonic acid) at 0.05M. Media were alkalinized using adjusted 0.1 M AMPSO (N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid) buffer. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was purchased from Interchim (Montluçon, France). CCCP stock solutions were prepared in dimethyl sulfoxide at 5 mM and stored at 4°C.

Growth inhibition assays of planktonic bacteria. Growth curves were performed in TPP® 96-well polystyrene microtiter plates (Sigma-Aldrich) using a Tecan Sunrise™ microplate reader (Tecan group, Männedorf, Switzerland). Each condition was replicated in 3 wells. Minimal inhibitory concentrations (MIC) were determined by broth microdilution. To determine whether the basic amino acid effect was pH-mediated, we first looked for a pH-independent effect of L-arginine buffered with MOPS at 0.05 M in order to reach the pH of growth media alone (buffered L-arginine). It is noteworthy that, using TG1Δ*argA,* an *E. coli* derivative auxotrophic towards L-arginine, we confirmed that the buffering process with MOPS did not

alter L-arginine, since bacterial growth was possible with 5-10 µg/mL of L-arginine ± MOPS. Next, 0.1 M-adjusted AMPSO was used as an alkaline buffer so as to reach the same pH as L-arginine 0.4%.

Effects upon stationary phase bacteria and persister cells. Since stationary-phase cultures of *E. coli* routinely reach pH9 and were therefore already alkalinized, late-stationary-phase cultures of *S. aureus*, *E. coli* or *P. aeruginosa* were washed twice (6000 rpm, 10 min), resuspended in 1X PBS and treated with different concentrations of gentamicin supplemented or not with L-arginine or buffered L-arginine under continuous agitation. This strategy allowed standardizing initial pH while maintaining nutrient limitations and thus impeding persisters' resuscitation. At different time points, a 0.5 mL sample was removed and washed twice in 1X PBS to remove excess antibiotic, and surviving CFU were quantified. In order to eradicate all non-persistent bacteria, we first sought to determine the gentamicin concentration and time of exposure leading to a surviving CFU plateau (Supplementary Figure 1*A,* 1*C* and 1*E*). Use of 100-fold the gentamicin MIC for 6 h (*S. aureus* and *E. coli*) or 500-fold the MIC for 6 h (*P. aeruginosa*) led to maximum bacterial mortality. To confirm that this population was hyper-tolerant to bactericidal antibiotics, this 6-h-treated culture was washed twice and resuspended in 1X PBS and then treated with gentamicin or ofloxacin at 10-, 100- or 500-fold the MIC (Supplementary Figure 1*B*, 1*D* and 1*F*). For *E. coli*, L-arginine was used at 0.2%, as higher concentrations led to significant bacterial mortality even without gentamicin. Buffered L-arginine with MOPS at 0.05 M was used as control, as described above.

Effect upon exponential phase bacteria and disruption of the proton motive force (PMF). Exponential phase cultures of *E. coli* 55989 (OD 0.3-0.6) were washed twice (6000 rpm, 10 min), resuspended in 1X PBS and treated with 10 x MIC gentamicin (80 µg/mL)

supplemented or not with L-arginine (0.4%) under continuous agitation. To study the consequences of PMF disruption on the effect of L-arginine, we used CCCP, a proton ionophore that abolishes both $\Delta\psi$ and ΔpH [17]. CCCP was added at a final concentration of 20 µM. After 30 min, a 15 µL sample was removed and serially diluted for quantification of surviving CFU.

***In vitro* biofilm formation and treatment.** *In vitro* biofilms were grown in triplicate for 24 h (*S. aureus* and *E. coli*) or 48 h (*P. aeruginosa*) on UV-sterilized polyvinyl chloride (PVC) 96-well plates (Thermo Scientific, Rochester, NY, USA). Planktonic bacteria were removed by 1X PBS washing and biofilms were treated for 24 h with increasing concentrations of antibiotic (gentamicin or amikacin) +/- L-arginine at 0.4% in TSB glucose or LB. For amikacin, L-arginine at 0.8% was chosen because of better reproducibility compared with L-arginine 0.4%. Buffered L-arginine with MOPS at 0.05 M and pH-modified media with adjusted AMPSO at 0.1 M were used as controls as described above except for L-arginine at 0.8% that required MOPS at 0.1 M in order to be buffered. Then, each well was washed twice with 1X PBS to remove planktonic bacteria and excess antibiotics, and surviving CFU were quantified.

In vivo* model and *Ethics Statement. Animals were housed in the Institut Pasteur animal facilities, accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 76th, 2013). The protocols used in this study for animal model, catheter placement, *in vivo* biofilm formation and *in vivo* study of inflammatory responses were approved by the ethic committee "*Paris Centre et Sud N°59*" under the reference N°2012-

0045. We used a previously described rat model of biofilm-related infection (TIVAP) [18]. Pediatric TIVAP (cat # 2105ISP, Polysite Micro, Titanium 2000 series, Perouse Medical, Ivry le temple, France) were inserted in CD/SD (IGS:Crl) rats (Charles River) and then left to recover for 4 days [18]. One hundred microliter 1X PBS containing 10^6 cells (for *S. aureus* or *P. aeruginosa*) or 10^4 cells (for *E. coli)* were injected inside the port through the silicone septum using a Huber needle (Perouse Medical) in order to contaminate TIVAP. Bioluminescence was monitored everyday using an IVIS-100 imaging system with a charge-coupled device (CCD) camera (Xenogen Corporation, Alameda, CA). After confirmation of complete catheter colonization (around day 3), rats were treated with systemic antibiotics (daily subcutaneous injection of vancomycin at 50 mg/kg for *S. aureus* or gentamicin at 30 mg/kg, for Gram-negative bacteria) and ALT [19, 20]. Vancomycin and gentamicin were chosen as they have been extensively used in animal models with a limited number of injections each day, allowing reducing the number of animal manipulation and thus increasing their comfort. Day 0 corresponds to the beginning of the treatment. ALT consisted of sterile PBS (control), gentamicin or amikacin (5 mg/mL), L-arginine (0.4%), gentamicin (or amikacin) + L-arginine. For amikacin locks, L-arginine was used at 0.8%. All ALT were prepared in sterile water. As controls, we used buffered L-arginine with MOPS or adjusted 0.1M AMPSO, as described above. Treatment was carried out for 5 days by replacing the old lock with a new one each day. Seven days after the end of treatment, rats were sacrificed and TIVAP were removed for bioluminescence imaging and CFU count, as described [18].

Statistical analysis. Each experiment was performed at least 3 times. The Wilcoxon Mann-Whitney test (included in Graphpad Prism Version 5.04) was used to compare continuous variables. Treatment groups were considered statistically different if p-values were lower than 0.05.

RESULTS

Basic amino acids increase the susceptibility of planktonic bacteria to gentamicin.

Previous reports showed that medium alkalinization enhanced the effects of aminoglycosides upon planktonic bacteria [10]. Consistently, we observed that medium alkalinization with 0.1 M AMPSO buffer adjusted to pH~8.5 decreased the gentamicin minimal inhibitory concentration (MIC) of Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria (Figure 1*A* and Supplementary Figure 2) [10]. To increase pH using physiologically tolerated compounds, we supplemented TSB and LB broth with the non-buffered basic amino acids L-arginine, L-histidine and L-lysine. Use of increasing of non-buffered L-arginine or L-lysine enabled attaining a wide range of pH values in supplemented TSB or LB media (Supplementary Figure 2), and enhanced bacterial susceptibility to gentamicin (Figure 1*A* and Supplementary Figure 2). Moreover, growth curves performed with increasing concentrations of L-arginine without antibiotic showed no significant inhibition at concentrations that would otherwise have led to increased susceptibility to gentamicin for *S. aureus*, *E. coli* and *P. aeruginosa* (Figure 1*B* and Supplementary Figure 2). To confirm that the effect of L-arginine on gentamicin susceptibility was pH-dependent, we demonstrated that gentamicin MIC was not affected by buffered L-arginine (Figure 1*A* and Supplementary Figure 2).

Non-buffered L-arginine reduces planktonic persister tolerance to gentamicin.

Stationary phase bacterial cultures display higher antibiotic tolerance due to an increased level of persistent bacteria compared to exponential phase cultures [21]. To test whether combining basic amino acid L-arginine and gentamicin could reduce this tolerance, we treated overnight PBS-washed *S. aureus*, *E. coli* and *P. aeruginosa* cultures for 6 h with high concentrations of gentamicin to kill all non-persistent bacteria. Indeed, we showed that bacteria surviving this

6-h gentamicin treatment displayed high tolerance to bactericidal antibiotics characteristically described for persisters (Supplementary Figure 1). Supplementation with L-arginine 0.4% led to a 100-fold reduction in the tolerance of *S. aureus* stationary phase cultures to gentamicin, while use of buffered L-arginine had no significant effect (Figure 2*A*). Similar results were obtained with *E. coli* (Figure 2*B*) and *P. aeruginosa* (Figure 2*C*), with surviving bacteria below the limit of detection in the latter case when gentamicin was supplemented with non-buffered L-arginine.

L-arginine increases the efficiency of gentamicin by modifying the membrane pH gradient

Gentamicin efficacy was previously shown to depend on the proton motive force (PMF) composed of both membrane electrical potential ($\Delta\psi$) and the transmembrane difference in the H^+ concentration (ΔpH) [11, 22]. We investigated the consequences of PMF disruption on the effect of L-arginine using CCCP, a proton ionophore that abolishes both $\Delta\psi$ and ΔpH [17]. As stationary phase bacteria displayed high mortality when exposed to CCCP associated with L-arginine, we used exponential phase culture bacteria. We first established that L-arginine also mediates the potentiation of the effect of gentamicin against *E. coli* exponential phase cultures, as demonstrated by a significantly higher bacterial mortality (Figure 3*A*). Next, we showed that the addition of CCCP abolished gentamicin antibacterial activity, confirming that gentamicin efficacy is dependent on the PMF. Strikingly, the addition of L-arginine in the presence of CCCP partially restored gentamicin activity (Figure 3*A*). This suggested that the pH effect mediated by non-buffered L-arginine generates a CCCP-insensitive pH gradient, consistent with similar results obtained using alkaline buffer instead of L-arginine (Figure 3*B*).

L-arginine and alkaline pH reduce *in vitro* biofilm tolerance to aminoglycosides

Although persisters are produced under both planktonic and biofilm culture conditions [21, 23], they are considered to be the major determinants of biofilm-associated antibiotic tolerance [3]. The enhanced gentamicin efficacy in bacteria subjected to an L-arginine-mediated pH increase prompted us to test whether this effect is also found in biofilm bacteria. We first observed that, as expected for biofilm bacteria, *S. aureus*, *E. coli* and *P. aeruginosa* biofilms displayed between 0.1 and 2% survival at gentamicin concentrations as high as 200xMIC (Figure 4). Strikingly, while L-arginine alone had no effect on biofilm bacteria, non-buffered L-arginine increased the effect of gentamicin by 2 to 3-logs (Figure 4). Interestingly, although buffered L-arginine combined with gentamicin had no significant effect upon *E. coli* biofilms (Figure 4*B*), it led to a 2-log decrease in the bacterial viability of *S. aureus* and *P. aeruginosa* biofilms, suggesting that L-arginine might also have a pH-independent effect upon these bacteria (Figure 4*A* and Figure 4*C*). Similar results were obtained for *P. aeruginosa* with amikacin, another clinically relevant aminoglycoside (Supplementary Figure 3*A* and 3*B*).

Gentamicin combined with L-arginine leads to *in vivo* biofilm eradication

Our *in vitro* results suggested that non-buffered L-arginine constitutes a new anti-biofilm adjuvant for use in combination with aminoglycosides in case of local biofilm-associated infections. We tested this hypothesis using an *in vivo* rat model of a totally implantable venous access port (TIVAP) amenable to antibiotic lock therapy (ALT) [18, 24]. ALT corresponds to instillation of a high concentration of antibiotics dwelling in the lumen of a catheter, designed to cure resilient biofilms on a central venous catheter [25]. We performed controlled inoculation of a rat-implanted TIVAP using bioluminescent *S. aureus*, *E. coli* or *P.*

aeruginosa strains, and monitored the evolution of biofilm formation in the device, which was fully colonized after 3 days (Day 0 on Figure 5).

While ALT solutions corresponding to PBS (control), L-arginine or gentamicin (625xMIC) alone had little or no effect on biofilm formed in TIVAP (Figure 5*A,* 5*B* and 5*C*), combined use of gentamicin with non-buffered L-arginine led to complete, long-lasting eradication of *S. aureus* and *E. coli* biofilms (Figure 5*D* and Figure 6*A* and 6*B*). This was demonstrated by the absence of bioluminescence and the disappearance of surviving CFU, even at 7 days after the end of combined L-arginine-gentamicin ALT (Figure 5*G* and 5*H* and Figure 6*A* and 6*B*). In contrast, gentamicin supplemented with non-buffered L-arginine reduced, but failed to eradicate *in vivo P. aeruginosa* biofilms (Figure 6*C* and 6*D*).

Interestingly, although gentamicin combined with alkaline buffer did not eradicate biofilms in any of the tested rats, use of gentamicin and buffered L-arginine eradicated *S. aureus* biofilms in 4 out of 5 rats (Figure 5*E* to 5*H*). Similarly, use of gentamicin in an alkaline buffer led to eradication of *E. coli* biofilm in 2 of the 3 tested rats (Figure 6*B*).

Taken together, these results demonstrate that gentamicin supplemented with L-arginine enables eradication of *in vivo* biofilm formed by *S. aureus* and *E. coli* via both a pH-mediated and a pH-independent effect.

DISCUSSION

The existence of subpopulations of persistent bacteria highly tolerant to antibiotics constitutes a major cause of therapeutic inefficacy against biofilms that colonize indwelling devices in medical settings [3, 26]. Here we show that use of non-buffered L-arginine as a non-toxic basic compound leads to enhancement of aminoglycoside effectiveness and eradication of persisters formed by several nosocomial pathogens in *in vitro* and *in vivo* biofilms.

The effect of gentamicin is dependent on the proton motive force, which is composed of $\Delta\psi$ (the electrical potential across the membrane) and ΔpH (the transmembrane difference in the H^+ concentration) [11, 22, 27]. While use of mannitol or fructose leads to NADH production that stimulates PMF through an increase in membrane electrical potential [9], our results demonstrate that the pH-mediated L-arginine effect probably relies on an effect upon the ΔpH-dependent transmembrane difference in the H^+ ion concentration rather than on membrane electrical potential. The mortality of stationary phase bacteria exposed to CCCP and L-arginine was not expected and was not observed using exponential phase cultures. Very likely, stationary phase cultures are less able to cope with high pH when CCCP is added. In stationary phase culture, proton motive force is probably more important for survival at high pH.

Although L-arginine-mediated effects were found to be exclusively pH-mediated against *S. aureus*, *E. coli* and *P. aeruginosa* planktonic bacteria and against *E. coli* biofilm, we observed that medium supplementation with buffered L-arginine significantly reduced *S. aureus* and *P. aeruginosa in vitro* biofilm tolerance towards aminoglycosides. Although mechanistic aspects of this effect are still currently under investigation, this pH-independent activity could be due to known metabolic stimulation of *S. aureus* and *P. aeruginosa* biofilm bacteria exposed to L-arginine [28, 29]. Indeed, supplementation of another aminoglycoside

(tobramycin) with 0.4% L-arginine was shown to reduce *P. aeruginosa* biofilm antibiotic tolerance *in vitro*, possibly via stimulation of anaerobic metabolism in deep layers of *P. aeruginosa* biofilm [30]. However, whether or not this L-arginine solution was buffered was not mentioned in the study. In this paper, the effect was more potent in anaerobic conditions than in aerobic conditions. It has been has been previously shown that anaerobic conditions could prevail in deep layers of biofilms {Stewart, 2008 #194}. Thus, we believe that some observations made in anaerobic conditions {Borriello, 2006 #26} can be translated in aerobic conditions. Furthermore, experimental studies with D-arginine could be interesting considering the potential effects of D-amino acids on biofilm disruption [31].

While clinical use of alkaline buffers may be potentially toxic, our choice of L-arginine as an *in vivo* alkalinization adjuvant to aminoglycoside-based therapies is justified by numerous reports of the lack of toxicity of intravenous administration of L-arginine to patients in various clinical situations [32, 33]. We tested the clinical relevance of our results using an appropriate rat model of catheter-associated biofilm infection amenable to controlled ALT. We showed that increasing the pH of highly concentrated yet partially ineffective gentamicin solutions in the lumen of a TIVAP eradicated the representative Gram-positive and Gram-negative bacterial pathogens frequently responsible for catheter-related infections [34, 35].

Since pH-mediated stimulation of antibiotic activity is likely to be effective against a wide range of bacteria, our results suggest that the observed rapid (one day for *S. aureus* and *E. coli*) and long-lasting eradication of *in vivo* bacterial biofilms could constitute a clinically relevant therapeutic alternative to the current standard catheter removal approach used in common biofilm infections [25, 36, 37]. Moreover, while the L-arginine effect is not restricted to gentamicin aminoglycoside, but also increases amikacin activity in bacterial biofilms, the existence of both pH-dependent and pH-independent stimulation, by L-arginine,

of aminoglycoside effects upon biofilm bacteria suggests that L-arginine might be a good adjuvant candidate for reducing biofilm persister tolerance towards gentamicin in clinical situations, in which pH might be locally modified at the site of biofilm-associated infections. In addition, other classes of antibiotics may have a pH-dependent modified spectrum of action, like fluoroquinolones, the effects of which could be increased under alkaline conditions in clinical settings such as that of urinary tract infections [38, 39]. Lastly, as some amino acids can be inhaled, the association of L-arginine and aminoglycosides could be studied in the treatment of *P. aeruginosa* biofilm-associated pneumonia in cystic fibrosis patients [40].

In conclusion, these results point to an original and alternative anti-biofilm approach to potentiating the effects of aminoglycosides, eliminating persisters and achieving biofilm eradication of different types of clinical and industrial biofilm contamination.

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FIGURE LEGENDS

Figure 1. **Basic amino acids increase *S. aureus* susceptibility to gentamicin under planktonic conditions.** (*A*) Gentamicin minimal inhibitory concentrations (MIC) of *S. aureus* Xen36 in TSB glucose medium supplemented with different basic amino acids. Data represented are the means ± SEM of at least three independent experiments. (*B*) *S. aureus* Xen36 growth curves in TSB glucose without antibiotics with increasing concentrations of L-arginine. L-arginine was buffered using MOPS (3-(N-morpholino)propanesulfonic acid) at 0.05M. AMPSO: N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; L-arg: L-arginine; L-lys: L-lysine, TSBg: TSB + glucose at 0.25%.

Figure 2. L-arginine decreases the tolerance of *S. aureus*, *E. coli* and *P. aeruginosa* stationary phase cultures towards gentamicin. (*A*) Survival curve of *S. aureus* Xen36; (*B*) Enteroaggregative *E. coli* 55989 and (*C*) *P. aeruginosa* PAK*lux* overnight (ON) cultures treated in 1X PBS with 100 or 500xMIC of gentamicin supplemented with or without L-arginine or buffered L-arginine. Data represented are the means ± SEM of at least three independent experiments. * ($p<0.05$). Values for gentamicin + L-arginine or gentamicin + buffered L-arginine were compared with the group treated with gentamicin alone at 100 (*S. aureus* and *E. coli*) or 500 x MIC (*P. aeruginosa*) (dark squares). Dotted line represents the limit of detection.

Figure 3. The L-arginine and pH-dependent decrease in *E. coli* persister tolerance towards gentamicin is effective despite disruption of the proton motive force. *A*. Survival of exponential phase *E. coli* 55989 cultures after 30 min treatment in 1 x PBS with gentamicin (10 x MIC), L-arginine (0.4%) or both, with or without CCCP at 20 µM. Data are means ± SEM of at least three independent experiments. * ($p<0.05$). CCCP: carbonyl cyanide m-

chlorophenyl hydrazone. *B*. Same experiment but using alkaline buffer instead of L-arginine. Alkaline buffer was 0.1M adjusted AMPSO in 1X PBS.

Figure 4. L-arginine reduces *in vitro* biofilm tolerance of Gram-positive and Gram-negative bacteria towards gentamicin. (*A*) *S. aureus* Xen36, (*B*) Enteroaggregative *E. coli* 55989 and (*C*) *P. aeruginosa* PAK*lux in vitro* biofilms were treated with increasing concentrations of gentamicin +/- L-arginine, buffered L-arginine with MOPS 0.05 M or media buffered with adjusted AMPSO at 0.1 M. Data represented are means ± SEM of at least three independent experiments. * ($p<0.05$), ** ($p<0.001$) and *** ($p<0.0001$). Values were compared with the control (dark bar) at the same antibiotic concentration.

Figure 5. *In vivo* eradication of *S. aureus* biofilm with gentamicin supplemented with L-arginine. ALT was instilled in TIVAP (day 0) and was associated with systemic vancomycin to treat 3-day-old *S. aureus* Xen36 biofilm (number (n) of rats used for each treatment, n=5). ALT was renewed every 24 h for 5 days and its efficacy was monitored as photon emissions. (*A*) 1X PBS ALT (control). (*B*) L-arginine ALT (0.4%). (*C*) Gentamicin ALT (5 mg/mL). (*D*) Combined gentamicin (5 mg/mL) and L-arginine (0.4%) ALT. (*E*) Combined gentamicin (5 mg/mL) and buffered L-arginine (0.4%) with MOPS 0.05 M. (*F*) Gentamicin (5 mg/mL) in adjusted AMPSO 0.1 M. (*A-F*) Results from a representative animal. (*G*) Seven days after administration of the last ALT, rats were sacrificed; TIVAP was harvested and monitored for photon emissions. (*H*) Bacterial cells from TIVAP were harvested, serially diluted and plated on TSB agar for CFU counting. Results for each rat are represented for each treatment group. Mean CFU and standard deviation are also presented as horizontal lines and whiskers. P value<0.05 was considered significant; * ($p<0.05$); ** ($p<0.001$) and ns: non-significant.

Figure 6. *In vivo* eradication of Gram-negative bacteria biofilm using L-arginine as an adjuvant to gentamicin. ALT was instilled in TIVAP and associated with systemic gentamicin to treat 3-day old *E. coli* 55989 (*A* and *B*) or *P. aeruginosa* PAK*lux* (*C* and *D*) biofilms (number (n) of rats used for each treatment, n=3). Seven days after the administration of the last ALT, rats were sacrificed; TIVAP was harvested and monitored for photon emissions (*A* and *C*). Bacterial cells from TIVAP were harvested, serially diluted and plated on LB agar for CFU counting (*B* and *D*). Results for each individual rat is represented for each treatment group. Mean CFU and standard deviation are also presented as horizontal line and whisker. L-arginine was buffered using MOPS (3-(N-morpholino)propanesulfonic acid)) at 0.05M. AMPSO: N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid.

FIGURES

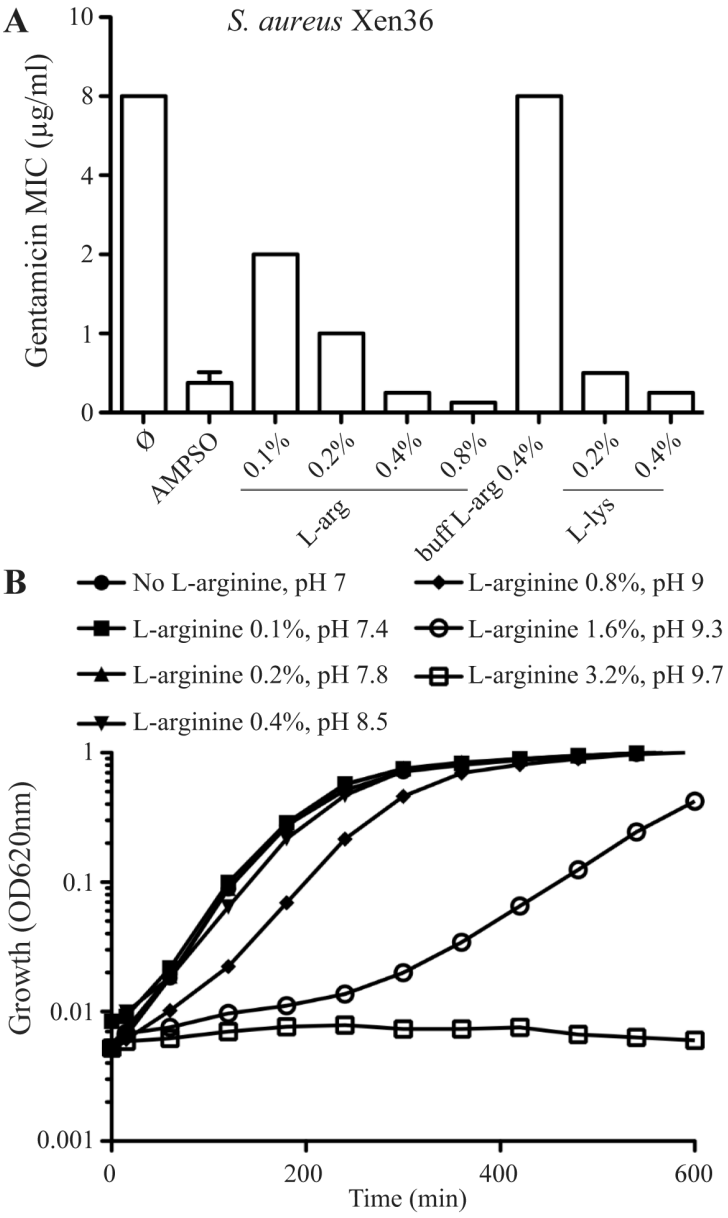


Figure 1.

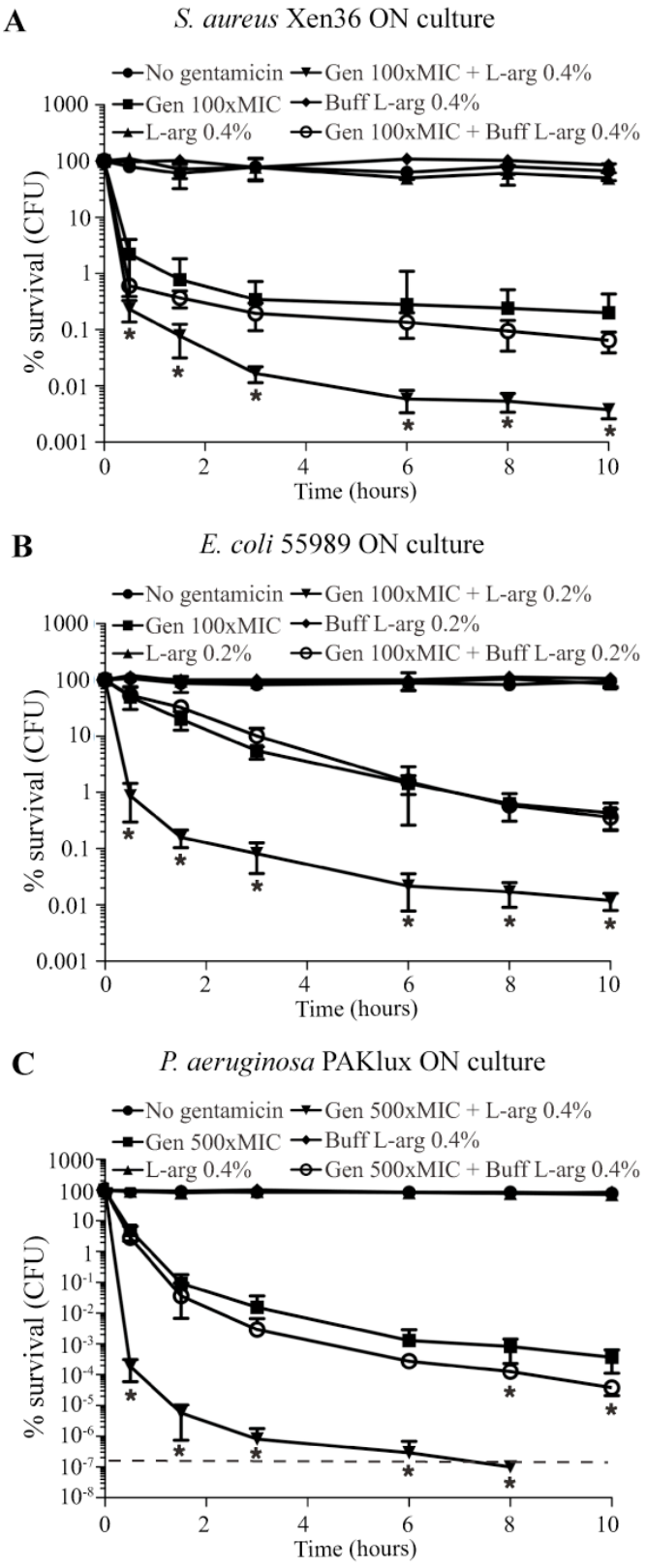

A
S. aureus Xen36 ON culture
No gentamicin
Gen 100xMIC
L-arg 0.4%
Gen 100xMIC + L-arg 0.4%
Buff L-arg 0.4%
Gen 100xMIC + Buff L-arg 0.4%
% survival (CFU)
Time (hours)
B
E. coli 55989 ON culture
No gentamicin
Gen 100xMIC
L-arg 0.2%
Gen 100xMIC + L-arg 0.2%
Buff L-arg 0.2%
Gen 100xMIC + Buff L-arg 0.2%
% survival (CFU)
Time (hours)
C
P. aeruginosa PAKlux ON culture
No gentamicin
Gen 500xMIC
L-arg 0.4%
Gen 500xMIC + L-arg 0.4%
Buff L-arg 0.4%
Gen 500xMIC + Buff L-arg 0.4%
% survival (CFU)
Time (hours)

Figure 2.

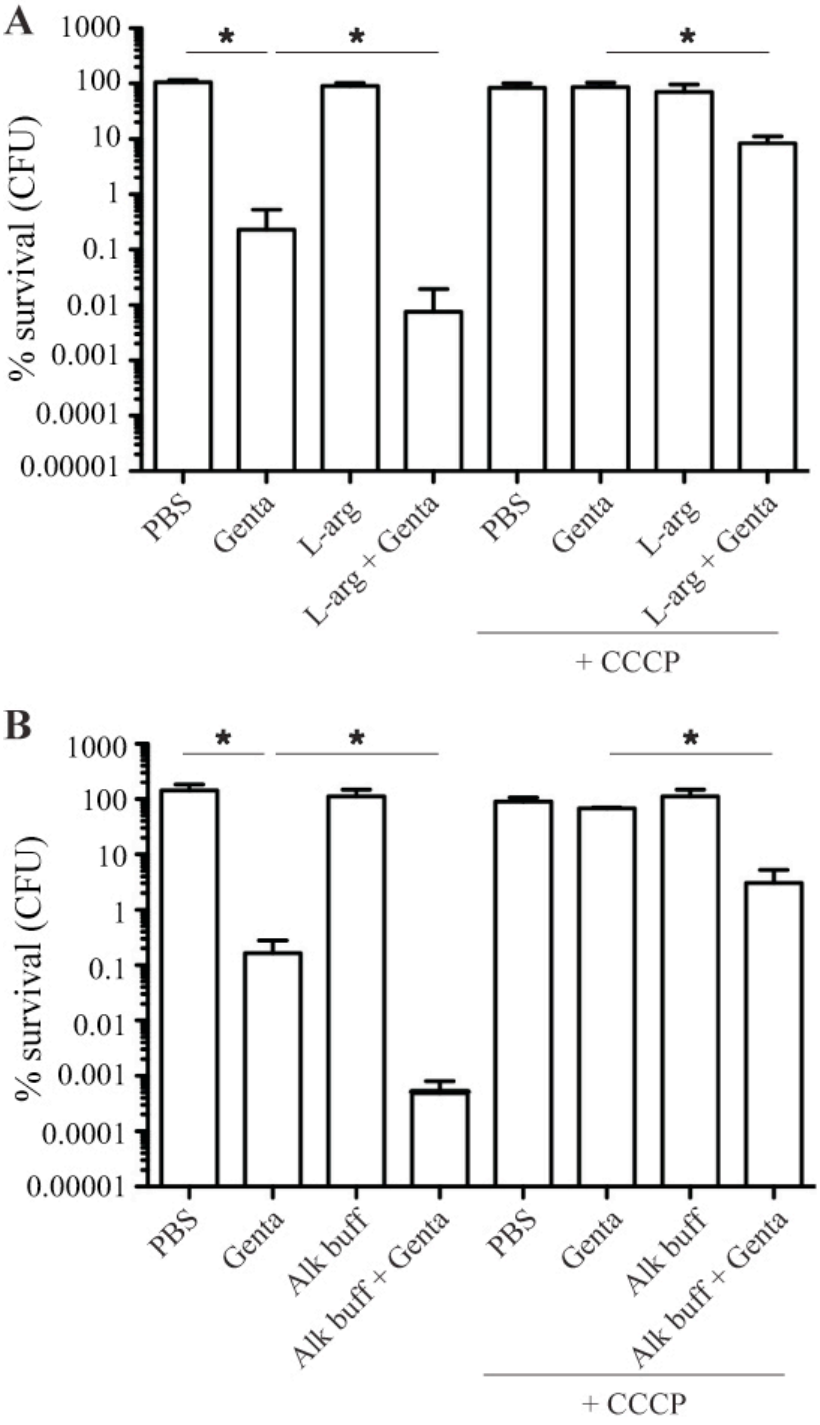

A
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
% survival (CFU)
*
*
*
PBS
Genta
L-arg
L-arg + Genta
PBS
Genta
L-arg
L-arg + Genta
+ CCCP
B
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
% survival (CFU)
*
*
*
PBS
Genta
Alk buff
Alk buff + Genta
PBS
Genta
Alk buff
Alk buff + Genta
+ CCCP

Figure 3.

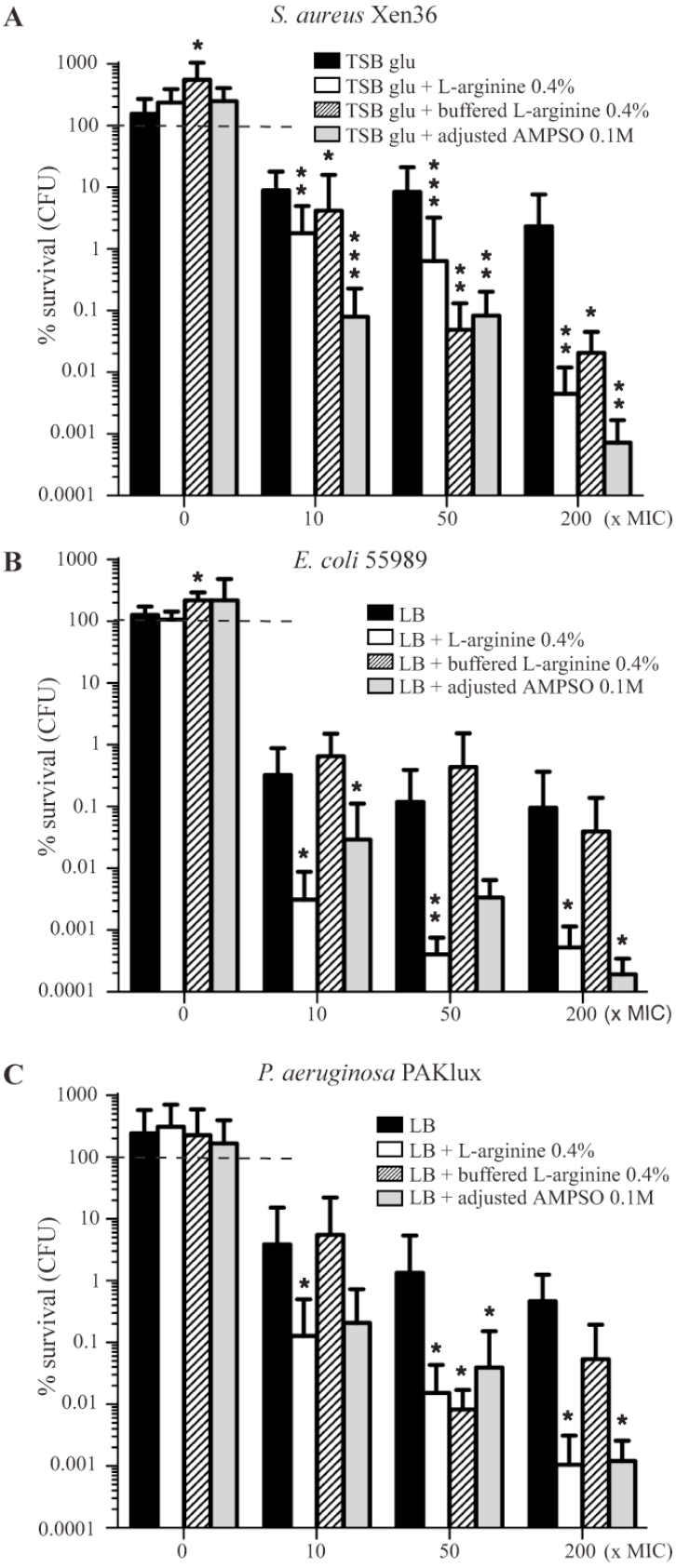

A
S. aureus Xen36
TSB glu
TSB glu + L-arginine 0.4%
TSB glu + buffered L-arginine 0.4%
TSB glu + adjusted AMPSO 0.1M
% survival (CFU)
1000
100
10
1
0.1
0.01
0.001
0.0001
0
10
50
200 (x MIC)
B
E. coli 55989
LB
LB + L-arginine 0.4%
LB + buffered L-arginine 0.4%
LB + adjusted AMPSO 0.1M
% survival (CFU)
0
10
50
200 (x MIC)
C
P. aeruginosa PAKlux
LB
LB + L-arginine 0.4%
LB + buffered L-arginine 0.4%
LB + adjusted AMPSO 0.1M
% survival (CFU)
0
10
50
200 (x MIC)

Figure 4.

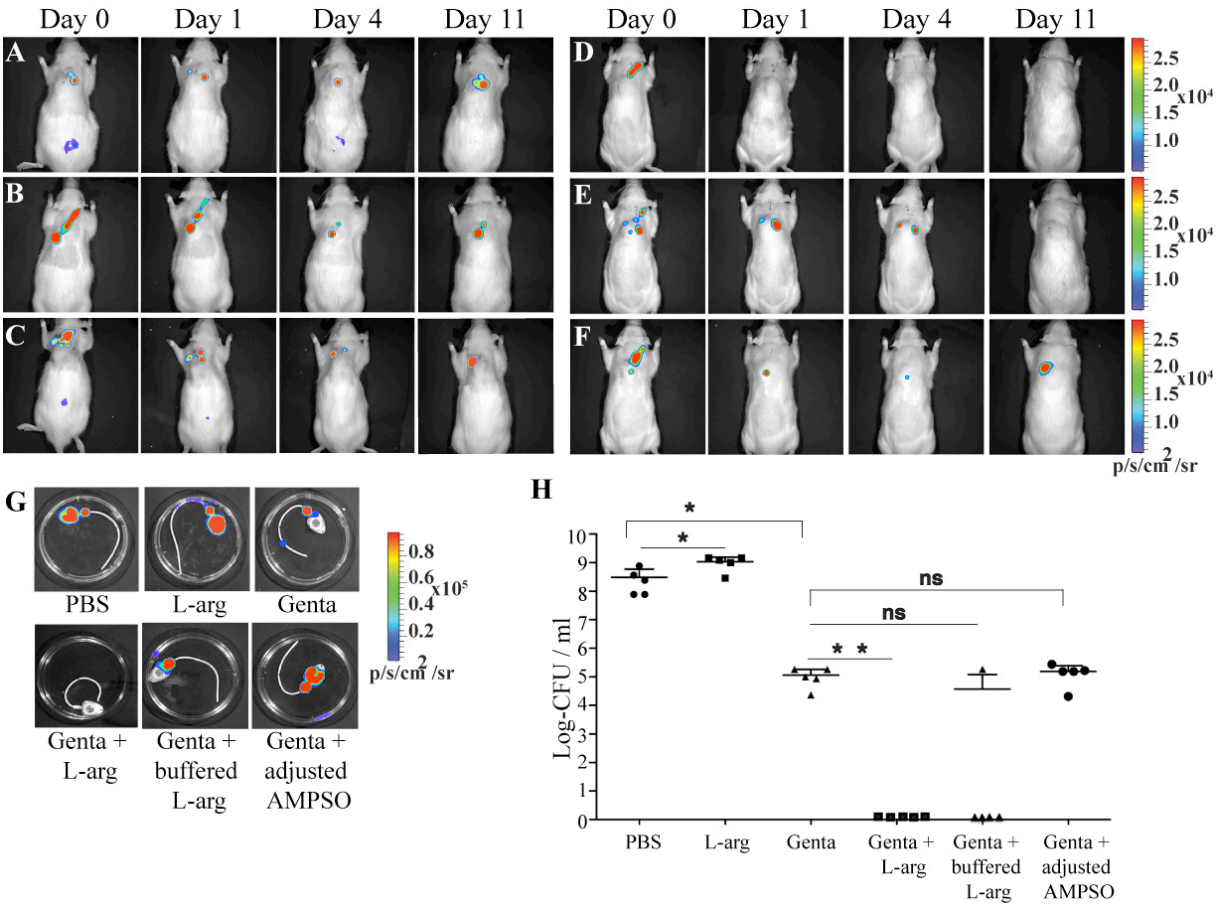

Day 0
Day 1
Day 4
Day 11
A
B
C
D
E
F
2.5
2.0
1.5
1.0
x10⁴
p/s/cm²/sr
G
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO
0.8
0.6
0.4
0.2
x10⁵
p/s/cm²/sr
H
Log-CFU / ml
ns
ns
*
*
* *
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO

Figure 5.

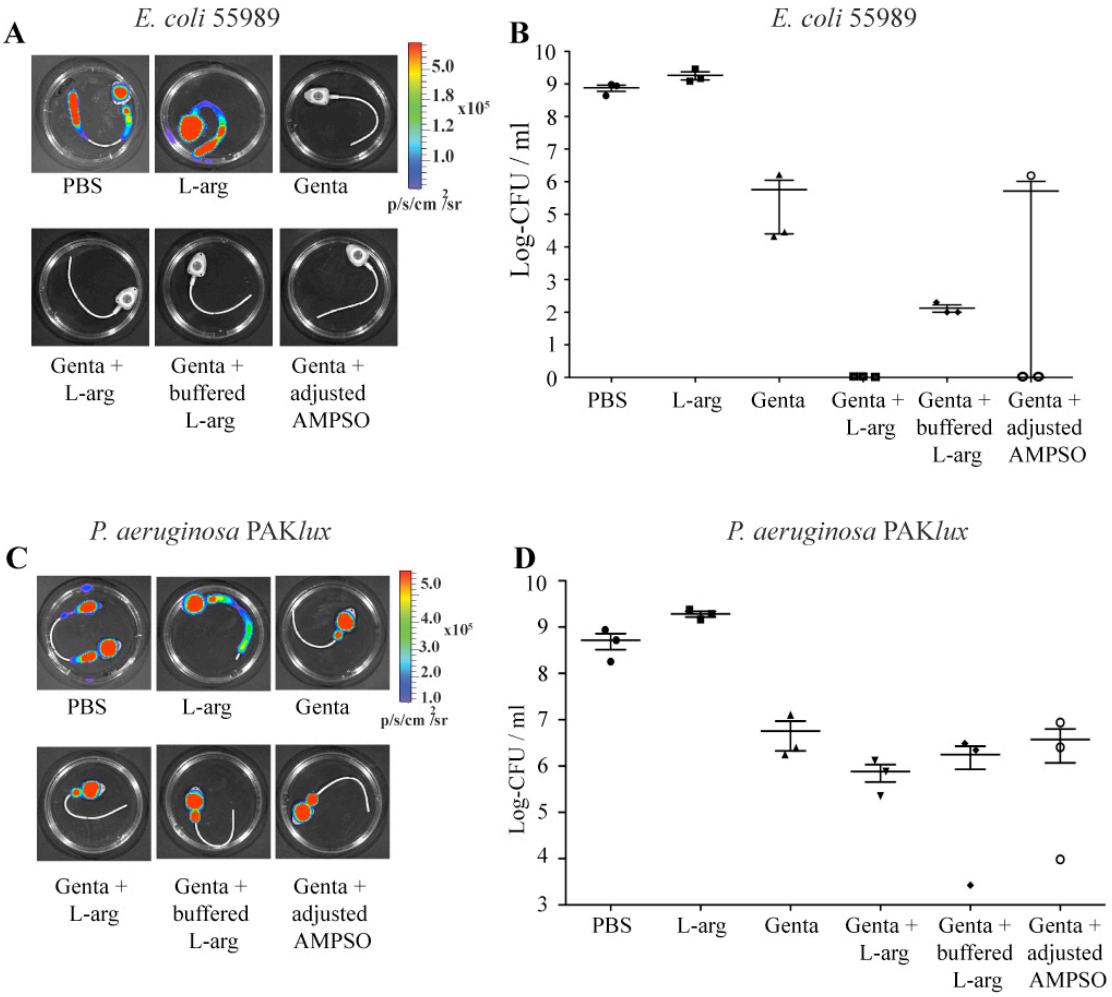

A
E. coli 55989
5.0
1.8
1.2
1.0
x10⁵
p/s/cm²/sr
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO
B
E. coli 55989
Log-CFU / ml
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO
C
P. aeruginosa PAKlux
5.0
4.0
3.0
2.0
1.0
x10⁵
p/s/cm²/sr
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO
D
P. aeruginosa PAKlux
Log-CFU / ml
PBS
L-arg
Genta
Genta + L-arg
Genta + buffered L-arg
Genta + adjusted AMPSO

Figure 6.