



pH-Mediated Potentiation of Aminoglycosides Kills Bacterial Persisters and Eradicates In Vivo Biofilms

David Lebeaux, Ashwini Chauhan, Sylvie Létoffé, Frédéric Fischer, Hilde de Reuse, Christophe Beloin, Jean-Marc Ghigo

► To cite this version:

David Lebeaux, Ashwini Chauhan, Sylvie Létoffé, Frédéric Fischer, Hilde de Reuse, et al.. pH-Mediated Potentiation of Aminoglycosides Kills Bacterial Persisters and Eradicates In Vivo Biofilms. *Journal of Infectious Diseases*, 2014, 210 (9), pp.1357 - 1366. 10.1093/infdis/jiu286 . pasteur-01381819

HAL Id: pasteur-01381819

<https://pasteur.hal.science/pasteur-01381819>

Submitted on 17 Nov 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - ShareAlike 4.0 International License

Major Article

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

David Lebeaux^{1†}, Ashwini Chauhan^{1†}, Sylvie Létoffé¹, Frédéric Fischer², Hilde de Reuse²,
Christophe Beloin^{1*} and Jean-Marc Ghigo^{1*}

[†] *These authors contributed equally to this work*

¹ *Institut Pasteur, Genetics of Biofilms Unit, Department of Microbiology, 28 rue du Dr.
Roux, 75724 Paris cedex 15, France*

² *Institut Pasteur, Helicobacter Pathogenesis Unit, Department of Microbiology, 28 rue du
Dr. Roux, 75724 Paris cedex 15, France*

***Corresponding authors:**

Jean-Marc Ghigo. E-mail address: jmghigo@pasteur.fr

Tel: 33 01 40 61 34 18; Fax: 33 01 45 68 80 07

Christophe Beloin. E-mail address: cbeloin@pasteur.fr

Tel: 33 01 44 38 95 97; Fax: 33 01 45 68 80 07

Running title: pH-mediated eradication of persisters

Word count: Abstract (143 words), text (3116 words)

Conflict of interest statement. The authors do not have any commercial or other association that might pose a conflict of interest.

Funding statement. This work was supported by an Institut Pasteur grant and by the French government's Investissement d'Avenir Program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID). D.L. was supported by a grant from the AXA Research Fund.

Presentation during meetings. This work has been presented in part at the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy 2013, Denver, Colorado, September 10-13

Corresponding authors:

Jean-Marc Ghigo.

Institut Pasteur, Genetics of Biofilms Unit, Department of Microbiology, 28 rue du Dr. Roux, 75724 Paris cedex 15, France

Tel: 33 01 40 61 34 18; Fax: 33 01 45 68 80 07. E-mail address: jmghigo@pasteur.fr

Christophe Beloin.

Institut Pasteur, Genetics of Biofilms Unit, Department of Microbiology, 28 rue du Dr. Roux, 75724 Paris cedex 15, France

Tel: 33 01 44 38 95 97; Fax: 33 01 45 68 80 07. E-mail address: cbeloin@pasteur.fr

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

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

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

100

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_Δ*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 alkalized 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 1A, 1C and 1E). 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 1B, 1D and 1F). 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:CrI) 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 alkalization enhanced the effects of aminoglycosides upon planktonic bacteria [10]. Consistently, we observed that medium alkalization 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 1A 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 1A 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 1B 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 1A 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 2A). Similar results were obtained with *E. coli* (Figure 2B) and *P. aeruginosa* (Figure 2C), 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 3A). 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 3A). 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 3B).

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 4B), 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 4A and Figure 4C). Similar results were obtained for *P. aeruginosa* with amikacin, another clinically relevant aminoglycoside (Supplementary Figure 3A and 3B).

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 5A, 5B and 5C), combined use of gentamicin with non-buffered L-arginine led to complete, long-lasting eradication of *S. aureus* and *E. coli* biofilms (Figure 5D and Figure 6A and 6B). 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 5G and 5H and Figure 6A and 6B). In contrast, gentamicin supplemented with non-buffered L-arginine reduced, but failed to eradicate *in vivo* *P. aeruginosa* biofilms (Figure 6C and 6D).

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 5E to 5H). Similarly, use of gentamicin in an alkaline buffer led to eradication of *E. coli* biofilm in 2 of the 3 tested rats (Figure 6B).

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 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.

359 **ACKNOWLEDGEMENTS.**

360 We thank Steve Bernier for constructing strain *E. coli* TG1 Δ *argA*.

REFERENCES

1. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **2002**; 15:167-93.
2. Arciola CR, Montanaro L, Costerton JW. New trends in diagnosis and control strategies for implant infections. *The International journal of artificial organs* **2011**; 34:727-36.
3. Lewis K. Multidrug tolerance of biofilms and persister cells. *Current topics in microbiology and immunology* **2008**; 322:107-31.
4. Fey PD. Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? *Curr Opin Microbiol* **2010**; 13:610-5.
5. Yang L, Liu Y, Wu H, et al. Combating biofilms. *FEMS immunology and medical microbiology* **2012**; 65:146-57.
6. O'Toole GA. Microbiology: Jekyll or hide? *Nature* **2004**; 432:680-1.
7. Allison KR, Brynildsen MP, Collins JJ. Heterogeneous bacterial persisters and engineering approaches to eliminate them. *Curr Opin Microbiol* **2011**; 14:593-8.
8. Conlon BP, Nakayasu ES, Fleck LE, et al. Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature* **2013**; 503:365-70.
9. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **2011**; 473:216-20.
10. Sabath LD, Toftegaard I. Rapid microassays for clindamycin and gentamicin when present together and the effect of pH and of each on the antibacterial activity of the other. *Antimicrob Agents Chemother* **1974**; 6:54-9.
11. Schlessinger D. Failure of aminoglycoside antibiotics to kill anaerobic, low-pH, and resistant cultures. *Clin Microbiol Rev* **1988**; 1:54-9.

12. Létoffé S, Audrain B, Bernier S, Delepierre D, Ghigo JM. Aerial exposure to the bacterial volatile compound trimethylamine modifies antibiotic resistance of physically separated bacteria by raising medium pH. *mBio* in press **2013**.
13. Moriarty TF, Elborn JS, Tunney MM. Effect of pH on the antimicrobial susceptibility of planktonic and biofilm-grown clinical *Pseudomonas aeruginosa* isolates. *Br J Biomed Sci* **2007**; 64:101-4.
14. Bernier C, Gounon P, Le Bouguenec C. Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as a sensitive probe for detecting the AAF-encoding operon family. *Infect Immun* **2002**; 70:4302-11.
15. Foucault ML, Thomas L, Goussard S, Branchini BR, Grillot-Courvalin C. *In vivo* bioluminescence imaging for the study of intestinal colonization by *Escherichia coli* in mice. *Appl Environ Microbiol* **2010**; 76:264-74.
16. Ramphal R, Balloy V, Jyot J, Verma A, Si-Tahar M, Chignard M. Control of *Pseudomonas aeruginosa* in the lung requires the recognition of either lipopolysaccharide or flagellin. *J Immunol* **2008**; 181:586-92.
17. Sun M, Wartel M, Cascales E, Shaevitz JW, Mignot T. Motor-driven intracellular transport powers bacterial gliding motility. *Proc Natl Acad Sci U S A* **2011**; 108:7559-64.
18. Chauhan A, Lebeaux D, Decante B, et al. A rat model of central venous catheter to study establishment of long-term bacterial biofilm and related acute and chronic infections. *PLoS One* **2012**; 7:e37281.
19. Van Praagh AD, Li T, Zhang S, et al. Daptomycin antibiotic lock therapy in a rat model of staphylococcal central venous catheter biofilm infections. *Antimicrobial agents and chemotherapy* **2011**; 55:4081-9.

20. Zuluaga AF, Agudelo M, Cardeno JJ, Rodriguez CA, Vesga O. Determination of therapeutic equivalence of generic products of gentamicin in the neutropenic mouse thigh infection model. *PloS one* **2010**; 5:e10744.
21. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **2004**; 230:13-8.
22. Taber HW, Mueller JP, Miller PF, Arrow AS. Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* **1987**; 51:439-57.
23. Spoering AL, Lewis K. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **2001**; 183:6746-51.
24. Chauhan A, Lebeaux D, Ghigo JM, Beloin C. Full and broad-spectrum in vivo eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy. *Antimicrobial agents and chemotherapy* **2012**; 56:6310-8.
25. Mermel LA, Allon M, Bouza E, et al. Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. *Clin Infect Dis* **2009**; 49:1-45.
26. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**; 284:1318-22.
27. Mates SM, Eisenberg ES, Mandel LJ, Patel L, Kaback HR, Miller MH. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America* **1982**; 79:6693-7.
28. Bernier SP, Ha DG, Khan W, Merritt JH, O'Toole GA. Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Res Microbiol* **2011**; 162:680-8.

29. Zhu Y, Weiss EC, Otto M, Fey PD, Smeltzer MS, Somerville GA. *Staphylococcus aureus* biofilm metabolism and the influence of arginine on polysaccharide intercellular adhesin synthesis, biofilm formation, and pathogenesis. *Infect Immun* **2007**; 75:4219-26.
30. Borriello G, Richards L, Ehrlich GD, Stewart PS. Arginine or nitrate enhances antibiotic susceptibility of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* **2006**; 50:382-4.
31. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. *Science* **2010**; 328:627-9.
32. Banning AP, Prendergast B. Intravenous L-arginine reduces VE/VCO₂ slope acutely in patients with severe chronic heart failure. *Eur J Heart Fail* **1999**; 1:187-90.
33. Miller HI, Dascalu A, Rassin TA, Wollman Y, Chernichowsky T, Iaina A. Effects of an acute dose of L-arginine during coronary angiography in patients with chronic renal failure: a randomized, parallel, double-blind clinical trial. *Am J Nephrol* **2003**; 23:91-5.
34. Adler A, Yaniv I, Steinberg R, et al. Infectious complications of implantable ports and Hickman catheters in paediatric haematology-oncology patients. *J Hosp Infect* **2006**; 62:358-65.
35. Lebeaux D, Larroque B, Gellen-Dautremer J, et al. Clinical outcome after a totally implantable venous access port-related infection in cancer patients: a prospective study and review of the literature. *Medicine* **2012**; 91:309-18.
36. Fernandez-Hidalgo N, Almirante B, Calleja R, et al. Antibiotic-lock therapy for long-term intravascular catheter-related bacteraemia: results of an open, non-comparative study. *J Antimicrob Chemother* **2006**; 57:1172-80.
37. Lebeaux D, Fernandez-Hidalgo N, Chauhan A, et al. Management of infections related to totally implantable venous-access ports: challenges and perspectives. *The Lancet infectious diseases* **2014**; 14:146-59.

38. Erdogan-Yildirim Z, Burian A, Manafi M, Zeitlinger M. Impact of pH on bacterial growth and activity of recent fluoroquinolones in pooled urine. *Research in microbiology* **2011**; 162:249-52.
39. Irwin NJ, McCoy CP, Carson L. Effect of pH on the in vitro susceptibility of planktonic and biofilm-grown *Proteus mirabilis* to the quinolone antimicrobials. *Journal of applied microbiology* **2013**; 115:382-9.
40. Hoiby N. Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. *BMC Med* **2011**; 9:32.

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 \pm 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) Enterococcal *E. coli* 55989 and (C) *P. aeruginosa* PAKlux 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 \pm 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 μ M. Data are means \pm 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 AMPPO 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) Enterococcal *E. coli* 55989 and (C) *P. aeruginosa* PAKlux *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 AMPPO at 0.1 M. Data represented are means \pm 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 AMPPO 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* PAKlux (*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

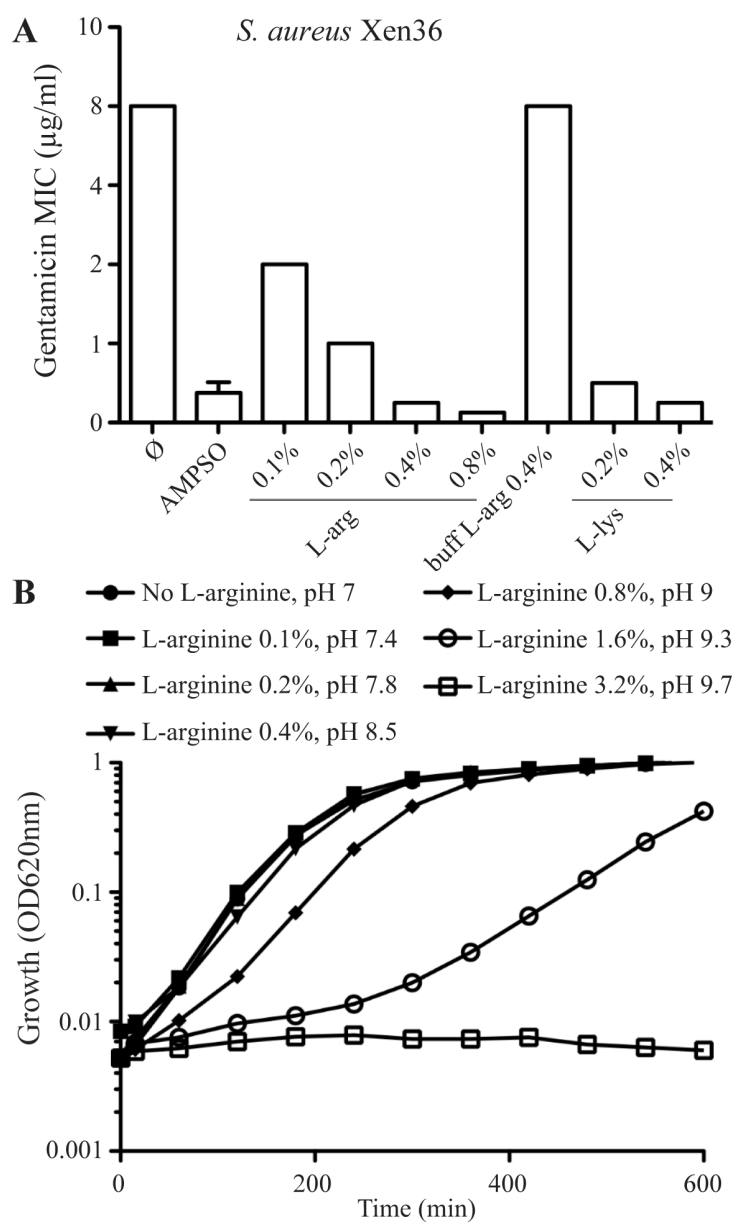


Figure 1.

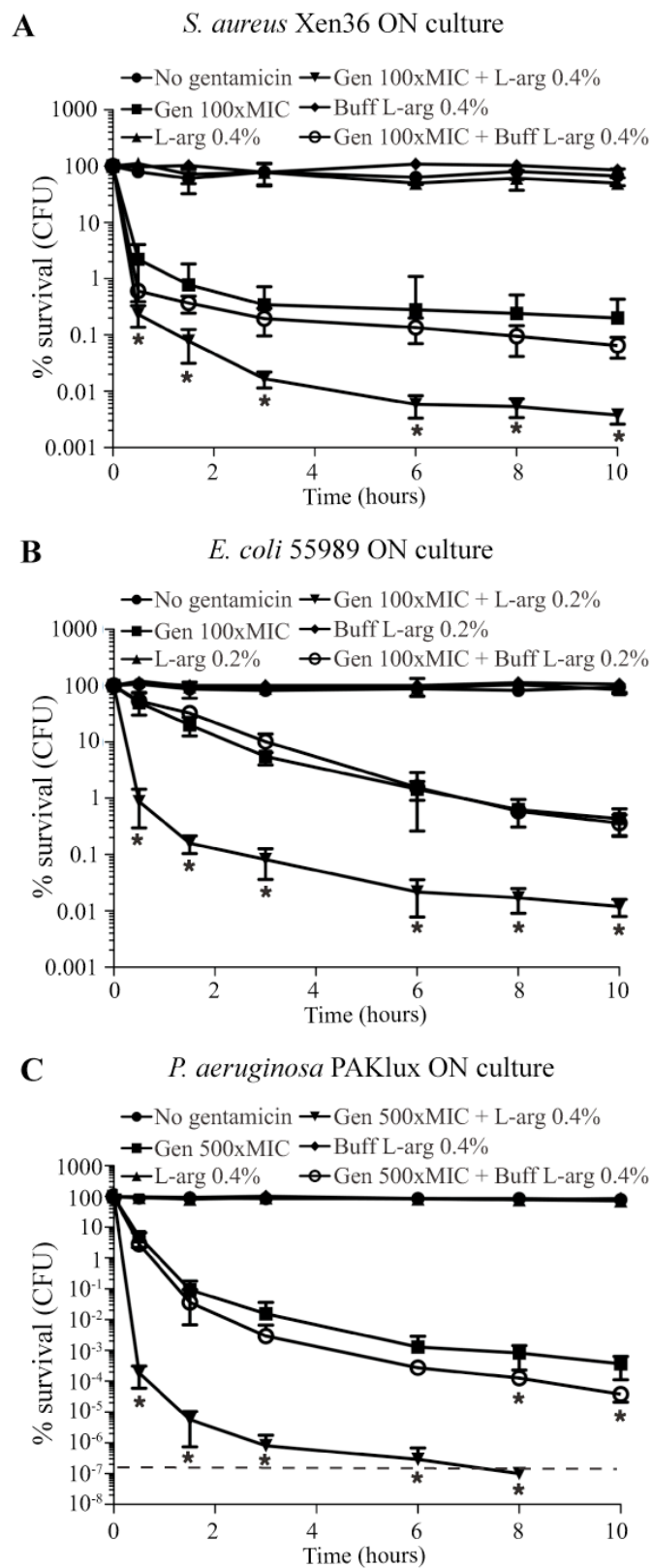


Figure 2.

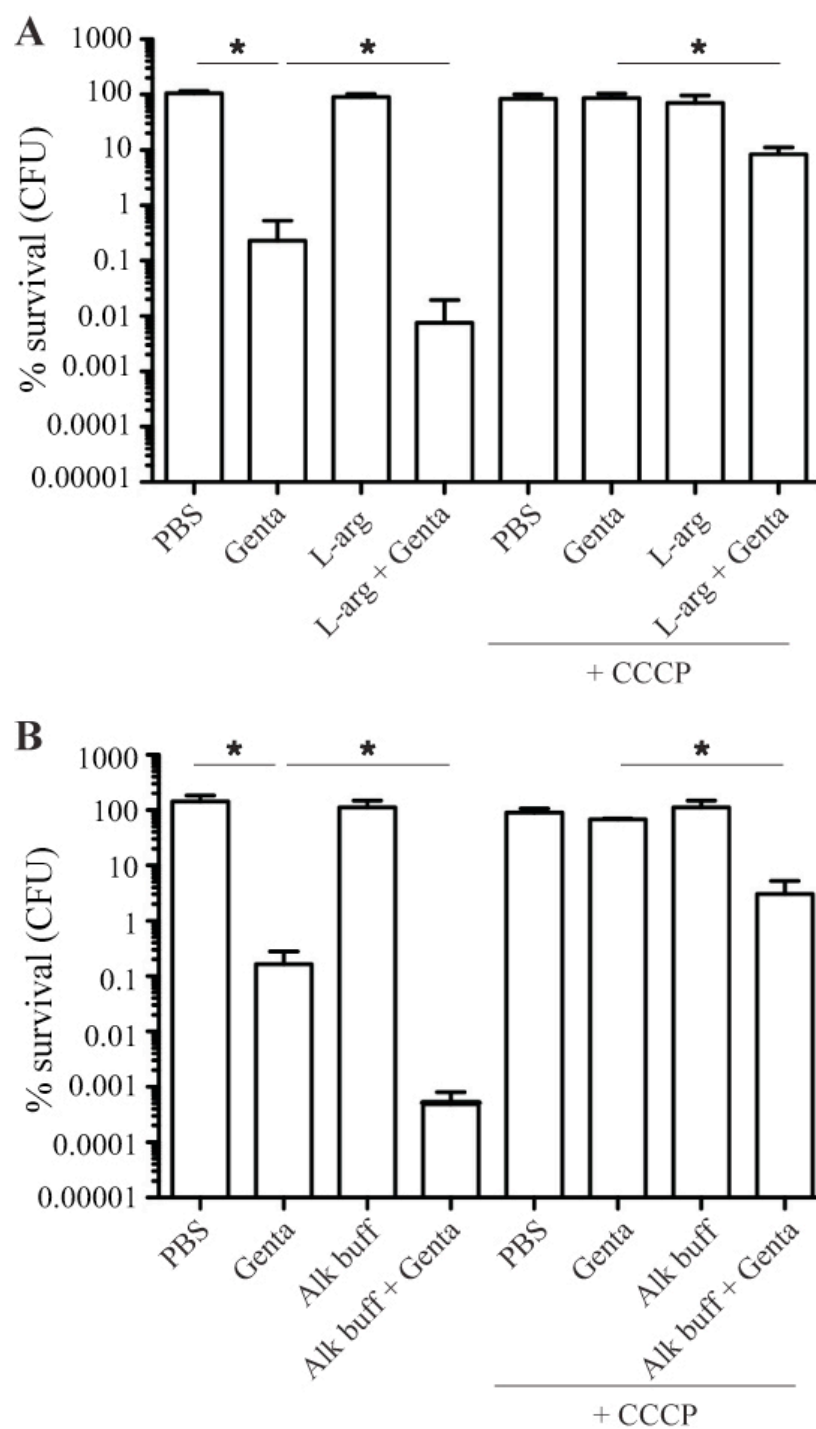


Figure 3.

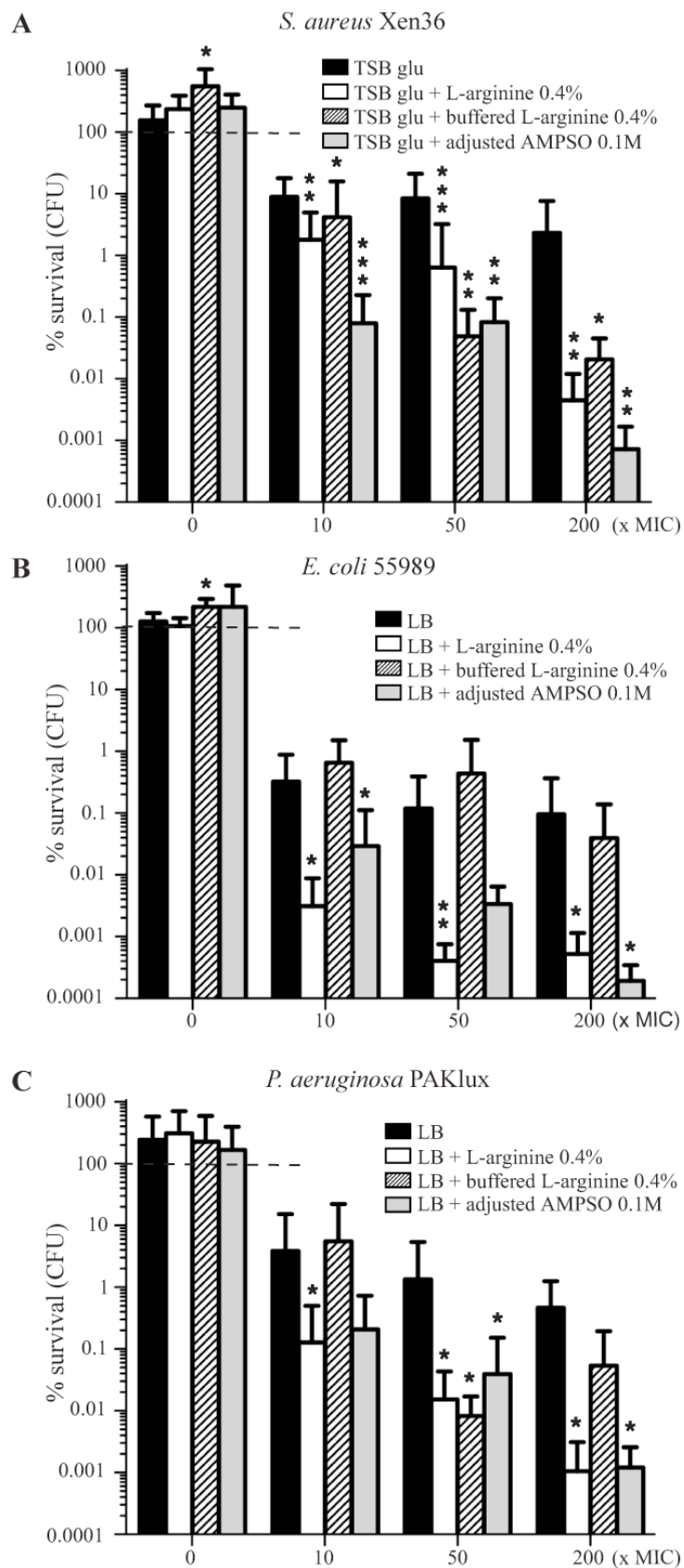


Figure 4.

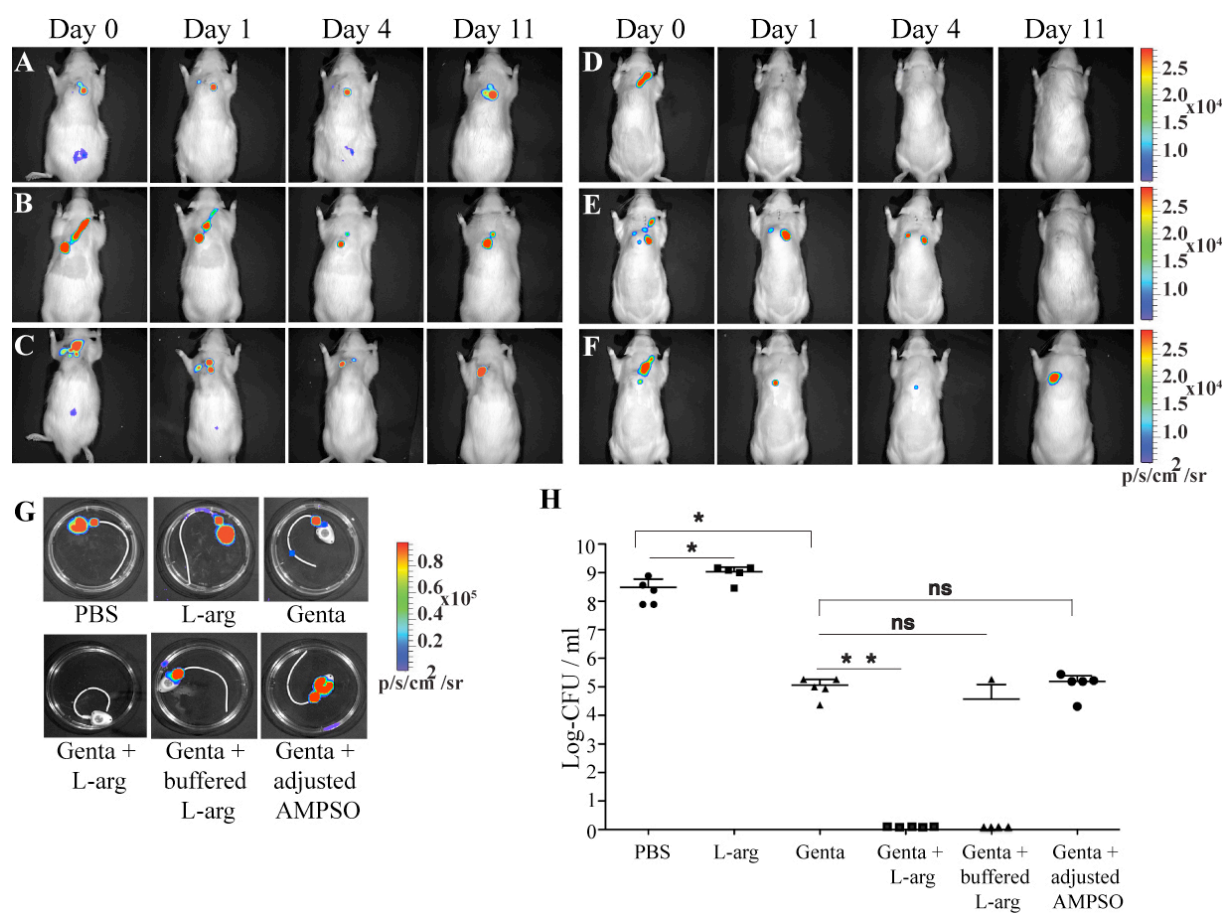


Figure 5.

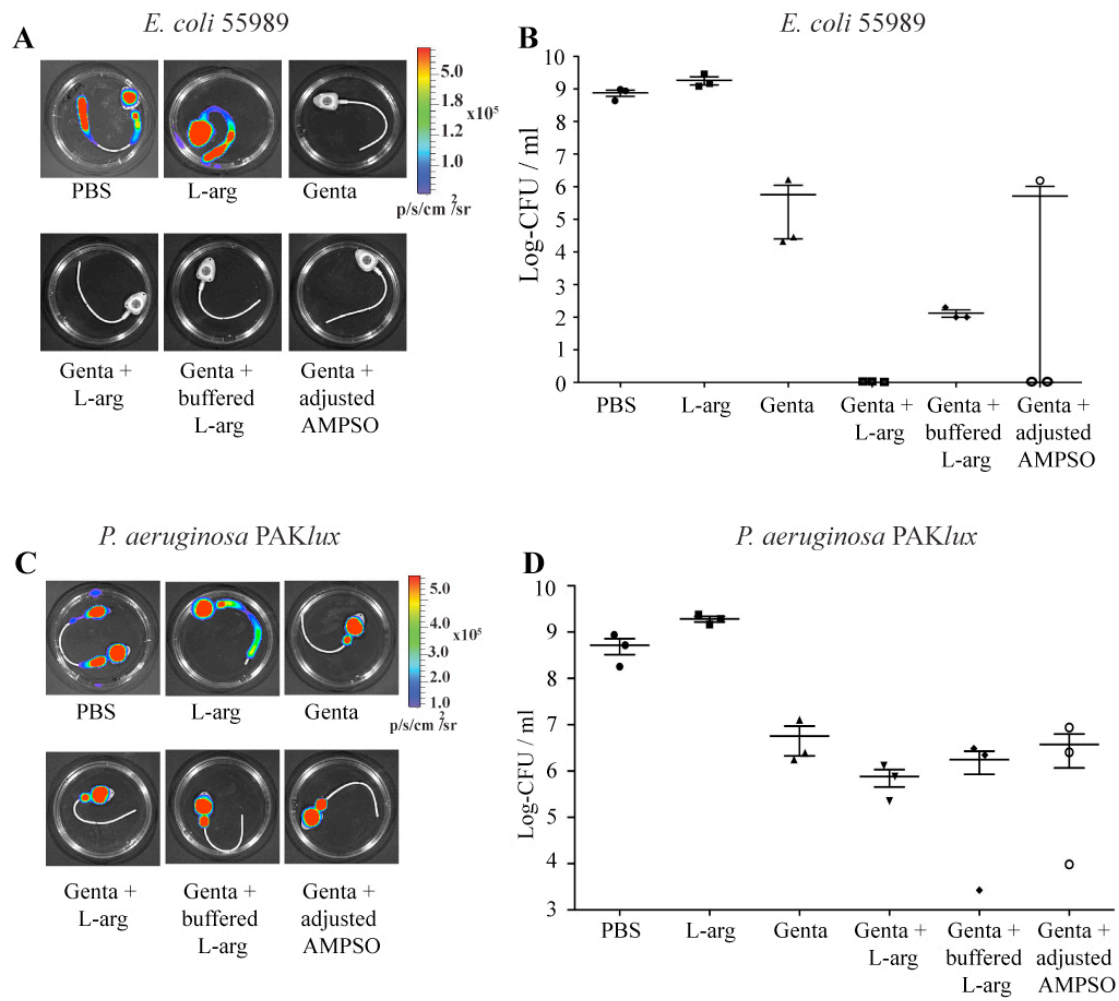


Figure 6.