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1 **Major Article**

2 **pH-mediated potentiation of aminoglycosides kills bacterial**

3 **persisters and eradicates *in vivo* biofilms**

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46

47 **ABSTRACT**

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

61

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

64

65

66 INTRODUCTION

67 Biofilm bacterial communities that develop on the surface of indwelling devices and mucosa
68 play a key role in healthcare-associated infections due to their characteristic high tolerance to
69 antibiotics [1]. Biofilm formation therefore impairs treatment efficacy and requires frequent
70 therapeutic removal of colonized devices, leading to increased morbidity and medical cost [1-
71 3]. Most treatment difficulties are related to biofilm tolerance, defined as the ability of a
72 subset of biofilm cells to survive in the presence of high concentrations of bactericidal
73 antibiotics [3]. Although biofilm tolerance is multifactorial, it is believed to originate mainly
74 from a subpopulation of non-growing persistent bacteria characterized by phenotypically
75 high, but reversible, tolerance to otherwise lethal concentrations of antibiotics [3]. These
76 persisters are not resistant mutants, and may resume growth and repopulate biofilm after
77 partially effective antibiotic treatment, thereby leading to infection recurrence [3]. Several
78 studies identified promising anti-biofilm strategies that inhibit bacterial adhesion or induce
79 dispersal [4, 5]. However, such approaches were associated with risk of massive release of
80 pathogenic bacteria from the infected site into the patient's bloodstream [6]. Hence, alongside
81 identification of non-biocidal anti-biofilm compounds, anti-persister approaches able to kill
82 planktonic and biofilm bacteria are currently being actively investigated [7, 8].

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

88 It was previously shown that sensitivity of planktonic bacteria exposed to
89 aminoglycosides was increased in alkaline media and, inversely, reduced under acidic
90 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

101 **MATERIAL AND METHODS**

102 **Bacterial strains and growth media.** Luminescent variants of *Staphylococcus aureus*,
103 *Escherichia coli* and *Pseudomonas aeruginosa* were purchased (*S. aureus* Xen36 from
104 Caliper, Hopkinton, MA) or constructed (*E. coli* EAEC 55989 transformed with stable
105 plasmid pAT881 and *P. aeruginosa* PAK*lux*, a bioluminescent derivative of the PAK clinical
106 strain) [14-16]. *S. aureus* was grown in tryptic soy broth (TSB) supplemented with 0.25%
107 glucose (TSB glucose), and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were grown
108 in Lysogeny Broth (LB) broth. *E. coli* TG1 Δ *argA* was obtained by P1vir transduction
109 from MG1655_ *argA*::Tn10 to TG1. Unless specified, all chemicals and antibiotics were
110 purchased from Sigma-Aldrich (St. Louis, MO, USA). Basic amino acids were resuspended in
111 LB, TSB glucose or 1 x PBS and then diluted. L-arginine at 0.4% was buffered using MOPS
112 (3-(N-morpholino)propanesulfonic acid) at 0.05M. Media were alkalinized using adjusted 0.1
113 M AMPSO (N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid)
114 buffer. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was purchased from Interchim
115 (Montluçon, France). CCCP stock solutions were prepared in dimethyl sulfoxide at 5 mM and
116 stored at 4°C.

117

118 **Growth inhibition assays of planktonic bacteria.** Growth curves were performed in TPP[®] 96-
119 well polystyrene microtiter plates (Sigma-Aldrich) using a Tecan Sunrise[™] microplate reader
120 (Tecan group, Männedorf, Switzerland). Each condition was replicated in 3 wells. Minimal
121 inhibitory concentrations (MIC) were determined by broth microdilution. To determine
122 whether the basic amino acid effect was pH-mediated, we first looked for a pH-independent
123 effect of L-arginine buffered with MOPS at 0.05 M in order to reach the pH of growth media
124 alone (buffered L-arginine). It is noteworthy that, using TG1 Δ *argA*, an *E. coli* derivative
125 auxotrophic towards L-arginine, we confirmed that the buffering process with MOPS did not

126 alter L-arginine, since bacterial growth was possible with 5-10 $\mu\text{g}/\text{mL}$ of L-arginine \pm MOPS.
127 Next, 0.1 M-adjusted AMPSO was used as an alkaline buffer so as to reach the same pH as L-
128 arginine 0.4%.

129

130 ***Effects upon stationary phase bacteria and persister cells.*** Since stationary-phase cultures of
131 *E. coli* routinely reach pH9 and were therefore already alkalized, late-stationary-phase
132 cultures of *S. aureus*, *E. coli* or *P. aeruginosa* were washed twice (6000 rpm, 10 min),
133 resuspended in 1X PBS and treated with different concentrations of gentamicin supplemented
134 or not with L-arginine or buffered L-arginine under continuous agitation. This strategy
135 allowed standardizing initial pH while maintaining nutrient limitations and thus impeding
136 persisters' resuscitation. At different time points, a 0.5 mL sample was removed and washed
137 twice in 1X PBS to remove excess antibiotic, and surviving CFU were quantified. In order to
138 eradicate all non-persistent bacteria, we first sought to determine the gentamicin concentration
139 and time of exposure leading to a surviving CFU plateau (Supplementary Figure 1A, 1C and
140 1E). Use of 100-fold the gentamicin MIC for 6 h (*S. aureus* and *E. coli*) or 500-fold the MIC
141 for 6 h (*P. aeruginosa*) led to maximum bacterial mortality. To confirm that this population
142 was hyper-tolerant to bactericidal antibiotics, this 6-h-treated culture was washed twice and
143 resuspended in 1X PBS and then treated with gentamicin or ofloxacin at 10-, 100- or 500-fold
144 the MIC (Supplementary Figure 1B, 1D and 1F). For *E. coli*, L-arginine was used at 0.2%, as
145 higher concentrations led to significant bacterial mortality even without gentamicin. Buffered
146 L-arginine with MOPS at 0.05 M was used as control, as described above.

147

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

151 supplemented or not with L-arginine (0.4%) under continuous agitation. To study the
152 consequences of PMF disruption on the effect of L-arginine, we used CCCP, a proton
153 ionophore that abolishes both $\Delta\psi$ and ΔpH [17]. CCCP was added at a final concentration of
154 20 μM . After 30 min, a 15 μL sample was removed and serially diluted for quantification of
155 surviving CFU.

156

157 ***In vitro* biofilm formation and treatment.** *In vitro* biofilms were grown in triplicate for 24 h
158 (*S. aureus* and *E. coli*) or 48 h (*P. aeruginosa*) on UV-sterilized polyvinyl chloride (PVC) 96-
159 well plates (Thermo Scientific, Rochester, NY, USA). Planktonic bacteria were removed by
160 1X PBS washing and biofilms were treated for 24 h with increasing concentrations of
161 antibiotic (gentamicin or amikacin) +/- L-arginine at 0.4% in TSB glucose or LB. For
162 amikacin, L-arginine at 0.8% was chosen because of better reproducibility compared with L-
163 arginine 0.4%. Buffered L-arginine with MOPS at 0.05 M and pH-modified media with
164 adjusted AMPISO at 0.1 M were used as controls as described above except for L-arginine at
165 0.8% that required MOPS at 0.1 M in order to be buffered. Then, each well was washed twice
166 with 1X PBS to remove planktonic bacteria and excess antibiotics, and surviving CFU were
167 quantified.

168

169 ***In vivo* model and Ethics Statement.** Animals were housed in the Institut Pasteur animal
170 facilities, accredited by the French Ministry of Agriculture for performing experiments on live
171 rodents. Work on animals was performed in compliance with French and European
172 regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law
173 2013-118, February 76th, 2013). The protocols used in this study for animal model, catheter
174 placement, *in vivo* biofilm formation and *in vivo* study of inflammatory responses were
175 approved by the ethic committee "Paris Centre et Sud N°59" under the reference N°2012-

176 0045. We used a previously described rat model of biofilm-related infection (TIVAP) [18].
177 Pediatric TIVAP (cat # 2105ISP, Polysite Micro, Titanium 2000 series, Perouse Medical, Ivry
178 le temple, France) were inserted in CD/SD (IGS:CrI) rats (Charles River) and then left to
179 recover for 4 days [18]. One hundred microliter 1X PBS containing 10^6 cells (for *S. aureus* or
180 *P. aeruginosa*) or 10^4 cells (for *E. coli*) were injected inside the port through the silicone
181 septum using a Huber needle (Perouse Medical) in order to contaminate TIVAP.
182 Bioluminescence was monitored everyday using an IVIS-100 imaging system with a charge-
183 coupled device (CCD) camera (Xenogen Corporation, Alameda, CA). After confirmation of
184 complete catheter colonization (around day 3), rats were treated with systemic antibiotics
185 (daily subcutaneous injection of vancomycin at 50 mg/kg for *S. aureus* or gentamicin at 30
186 mg/kg, for Gram-negative bacteria) and ALT [19, 20]. Vancomycin and gentamicin were
187 chosen as they have been extensively used in animal models with a limited number of
188 injections each day, allowing reducing the number of animal manipulation and thus increasing
189 their comfort. Day 0 corresponds to the beginning of the treatment. ALT consisted of sterile
190 PBS (control), gentamicin or amikacin (5 mg/mL), L-arginine (0.4%), gentamicin (or
191 amikacin) + L-arginine. For amikacin locks, L-arginine was used at 0.8%. All ALT were
192 prepared in sterile water. As controls, we used buffered L-arginine with MOPS or adjusted
193 0.1M AMPSO, as described above. Treatment was carried out for 5 days by replacing the old
194 lock with a new one each day. Seven days after the end of treatment, rats were sacrificed and
195 TIVAP were removed for bioluminescence imaging and CFU count, as described [18].

196

197 **Statistical analysis.** Each experiment was performed at least 3 times. The Wilcoxon Mann-
198 Whitney test (included in Graphpad Prism Version 5.04) was used to compare continuous
199 variables. Treatment groups were considered statistically different if p-values were lower than
200 0.05.

201

202 RESULTS

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

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

220

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

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

227 6-h gentamicin treatment displayed high tolerance to bactericidal antibiotics characteristically
228 described for persisters (Supplementary Figure 1). Supplementation with L-arginine 0.4% led
229 to a 100-fold reduction in the tolerance of *S. aureus* stationary phase cultures to gentamicin,
230 while use of buffered L-arginine had no significant effect (Figure 2A). Similar results were
231 obtained with *E. coli* (Figure 2B) and *P. aeruginosa* (Figure 2C), with surviving bacteria
232 below the limit of detection in the latter case when gentamicin was supplemented with non-
233 buffered L-arginine.

234

235 **L-arginine increases the efficiency of gentamicin by modifying the membrane pH** 236 **gradient**

237 Gentamicin efficacy was previously shown to depend on the proton motive force (PMF)
238 composed of both membrane electrical potential ($\Delta\psi$) and the transmembrane difference in
239 the H^+ concentration (ΔpH) [11, 22]. We investigated the consequences of PMF disruption on
240 the effect of L-arginine using CCCP, a proton ionophore that abolishes both $\Delta\psi$ and ΔpH
241 [17]. As stationary phase bacteria displayed high mortality when exposed to CCCP associated
242 with L-arginine, we used exponential phase culture bacteria. We first established that L-
243 arginine also mediates the potentiation of the effect of gentamicin against *E. coli* exponential
244 phase cultures, as demonstrated by a significantly higher bacterial mortality (Figure 3A).
245 Next, we showed that the addition of CCCP abolished gentamicin antibacterial activity,
246 confirming that gentamicin efficacy is dependent on the PMF. Strikingly, the addition of L-
247 arginine in the presence of CCCP partially restored gentamicin activity (Figure 3A). This
248 suggested that the pH effect mediated by non-buffered L-arginine generates a CCCP-
249 insensitive pH gradient, consistent with similar results obtained using alkaline buffer instead
250 of L-arginine (Figure 3B).

251

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

253 Although persisters are produced under both planktonic and biofilm culture conditions [21,
254 23], they are considered to be the major determinants of biofilm-associated antibiotic
255 tolerance [3]. The enhanced gentamicin efficacy in bacteria subjected to an L-arginine-
256 mediated pH increase prompted us to test whether this effect is also found in biofilm bacteria.
257 We first observed that, as expected for biofilm bacteria, *S. aureus*, *E. coli* and *P. aeruginosa*
258 biofilms displayed between 0.1 and 2% survival at gentamicin concentrations as high as
259 200xMIC (Figure 4). Strikingly, while L-arginine alone had no effect on biofilm bacteria,
260 non-buffered L-arginine increased the effect of gentamicin by 2 to 3-logs (Figure 4).
261 Interestingly, although buffered L-arginine combined with gentamicin had no significant
262 effect upon *E. coli* biofilms (Figure 4B), it led to a 2-log decrease in the bacterial viability of
263 *S. aureus* and *P. aeruginosa* biofilms, suggesting that L-arginine might also have a pH-
264 independent effect upon these bacteria (Figure 4A and Figure 4C). Similar results were
265 obtained for *P. aeruginosa* with amikacin, another clinically relevant aminoglycoside
266 (Supplementary Figure 3A and 3B).

267

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

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

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

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

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

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

293

294

295

296 **DISCUSSION**

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

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

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

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

330 While clinical use of alkaline buffers may be potentially toxic, our choice of L-
331 arginine as an *in vivo* alkalinization adjuvant to aminoglycoside-based therapies is justified by
332 numerous reports of the lack of toxicity of intravenous administration of L-arginine to
333 patients in various clinical situations [32, 33]. We tested the clinical relevance of our results
334 using an appropriate rat model of catheter-associated biofilm infection amenable to controlled
335 ALT. We showed that increasing the pH of highly concentrated yet partially ineffective
336 gentamicin solutions in the lumen of a TIVAP eradicated the representative Gram-positive
337 and Gram-negative bacterial pathogens frequently responsible for catheter-related infections
338 [34, 35].

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

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

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

358

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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. Létouffé 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) Enteroaggregative *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 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* 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 AMPSO 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 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* 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

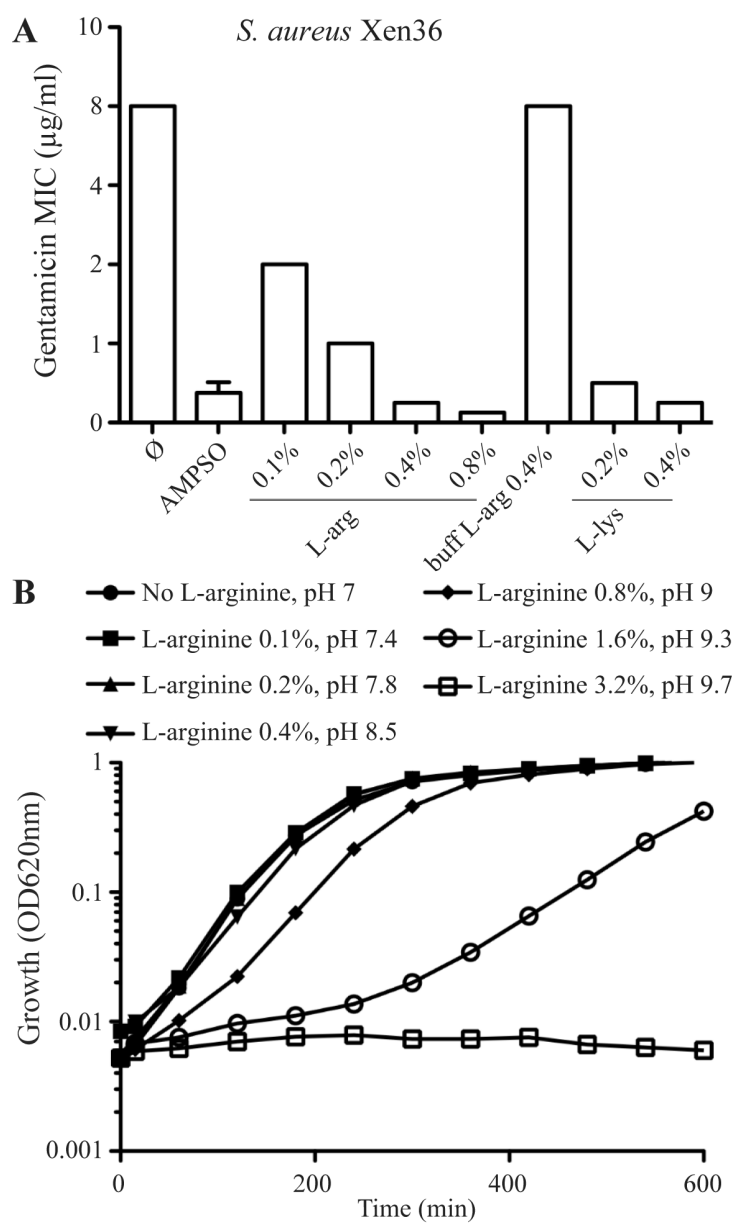


Figure 1.

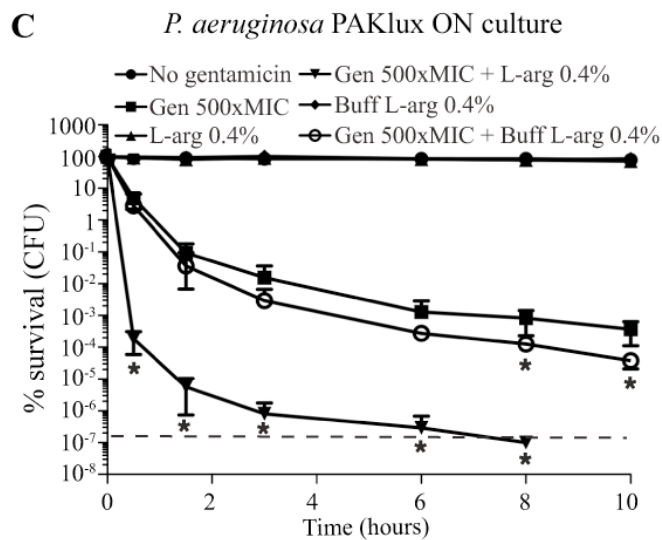
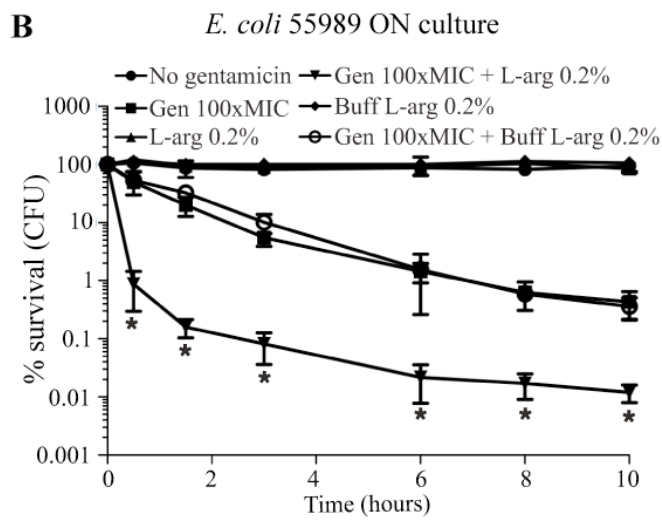
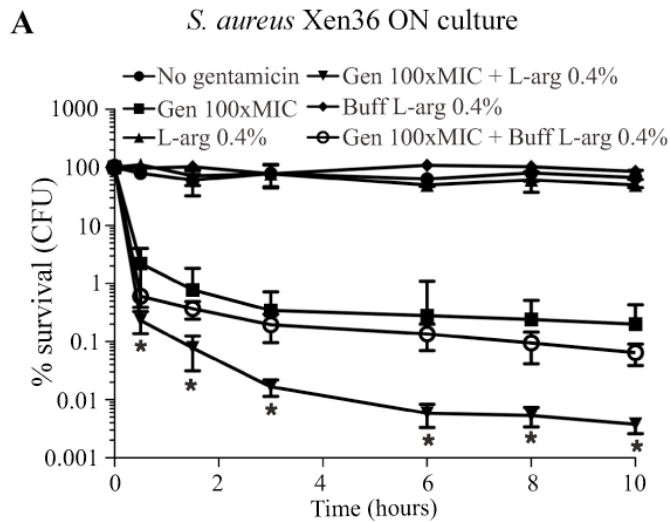


Figure 2.

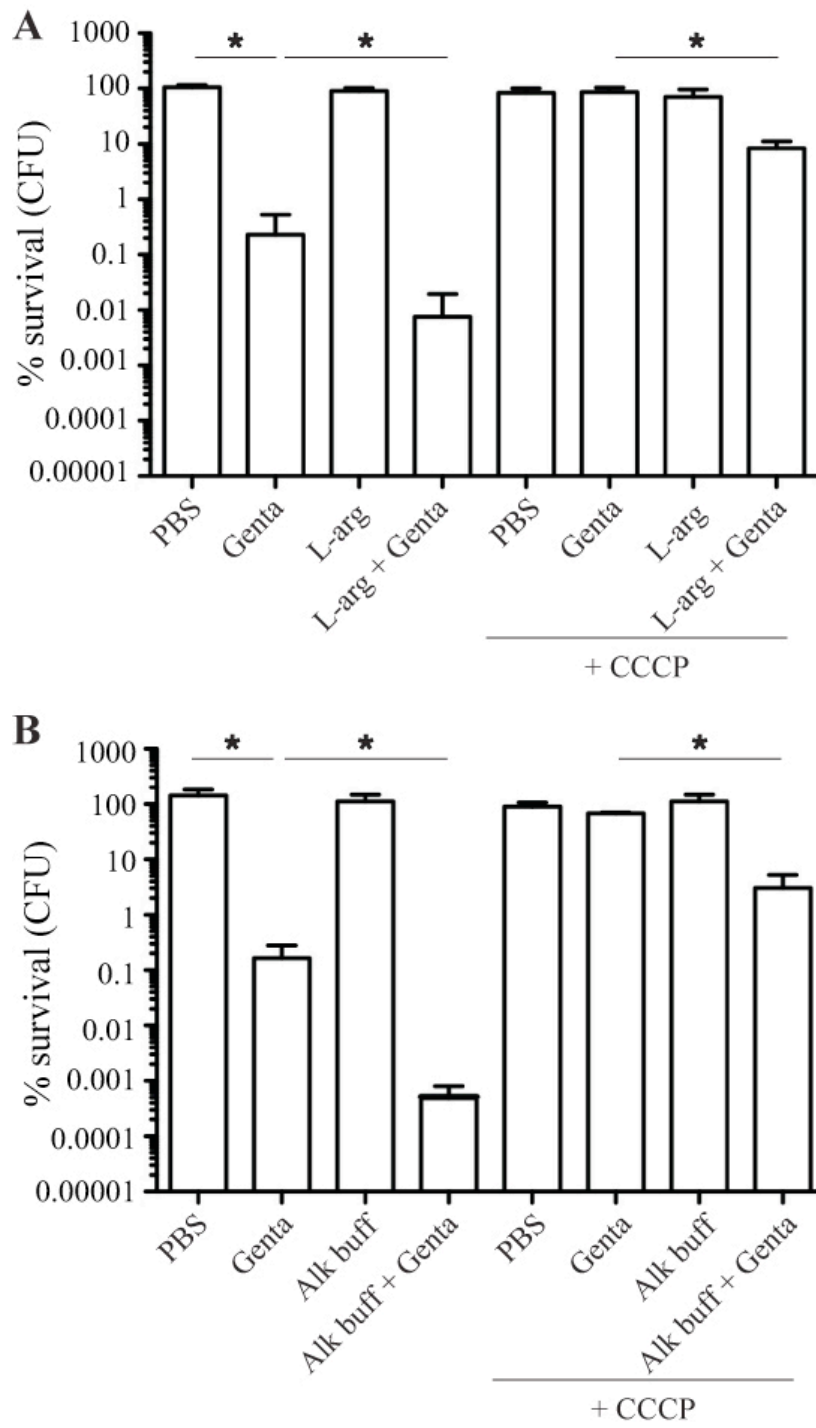


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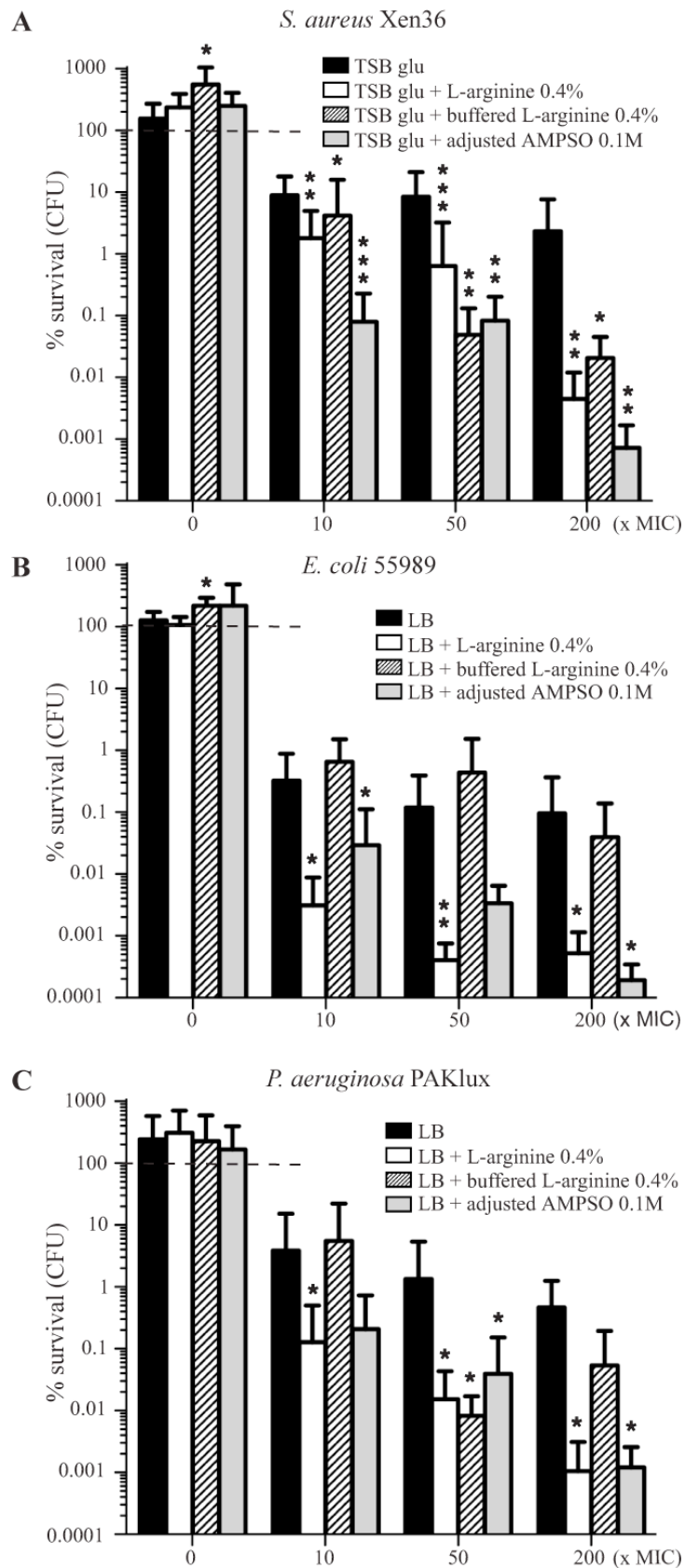


Figure 4.

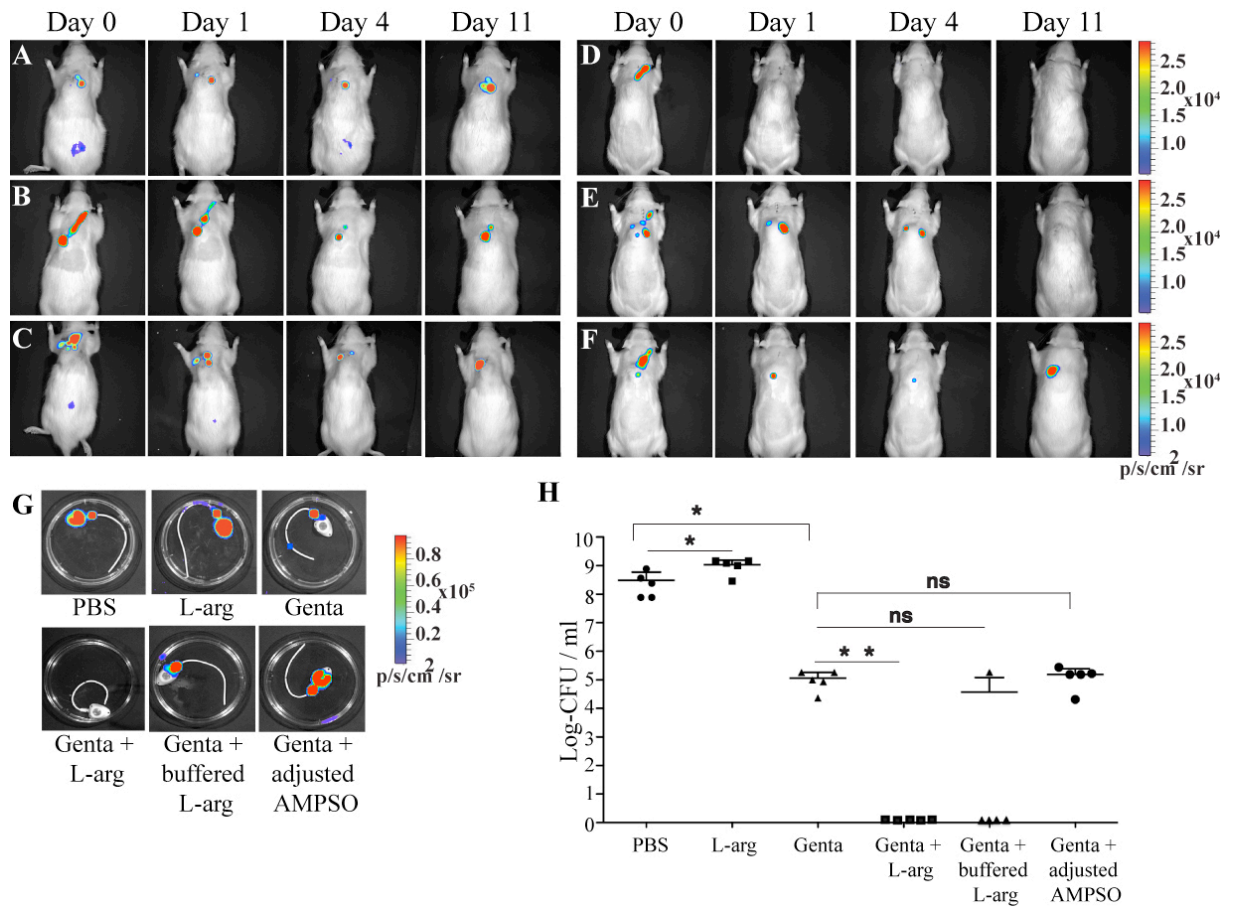


Figure 5.

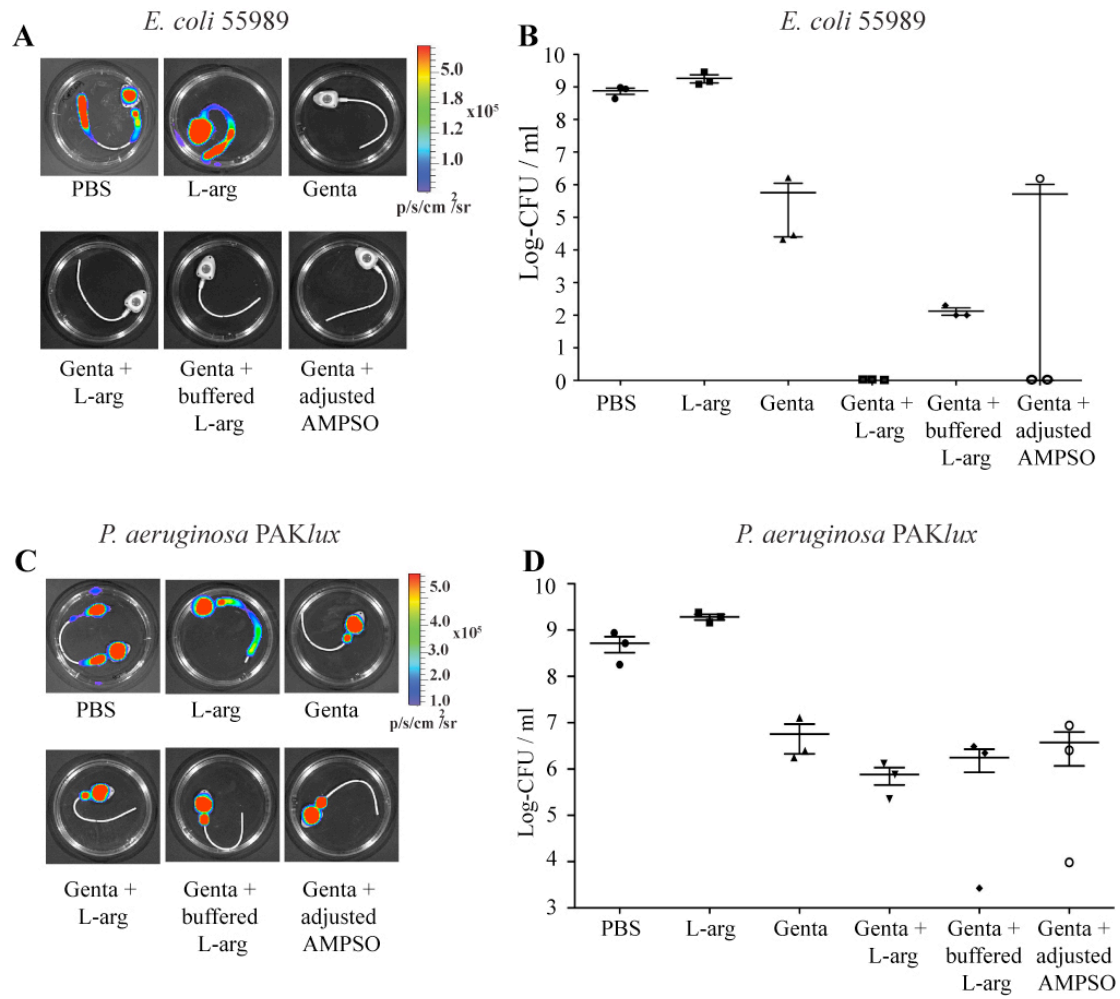


Figure 6.