Complex interbacterial interactions in mixed biofilms as a key determinant of their antimicrobial treatment efficacy

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Abstract

Biofilms are formed by closely adjacent microorganisms embedded into an extracellular matrix this way providing them with strong protection from antimicrobials, which is often further reinforced in polymicrobial biofilms. Despite of the well-known antagonistic interactions between *S. aureus* and *P. aeruginosa*, the most common pathogens causing various nosocomial infections, they often form mixed consortia characterized by increased pathogenicity and delayed recovery in comparison with single species infections. Here we show that, while *S. aureus* could successfully avoid a number of antimicrobials by embedding into the biofilm matrix of *P. aeruginosa* despite of their antagonism, the very same consortium was characterized by 10–fold higher susceptibility to broad-spectrum antimicrobials compared to monocultures. Moreover, quantitatively similar increase in antimicrobials susceptibility could be achieved when *P. aeruginosa* was introduced into *S. aureus* biofilm, compared to *S. aureus* monoculture. In a reverse experiment, intervention of *S. aureus* into the mature *P. aeruginosa* biofilm significantly increased the efficacy of ciprofloxacin against *P. aeruginosa*. A broader perspective is provided by antagonistic bacteria intervention into already preformed monoculture biofilms leading to the considerable enhancement of their antibiotic susceptibility. We believe that this approach has a strong potential of further development towards innovative treatment of biofilm-associated infections such as transplantation of the skin residential microflora to the wounds and ulcers infected with nosocomial pathogens to speed up their microbial decontamination.

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Author summary

Biofilms formation is one of the key mechanisms providing pathogenic bacteria with extreme resistance to antimicrobials. On the *S. aureus* and *P. aeruginosa* mixed culture model we show explicitly that antimicrobials efficacy against bacteria in mixed biofilms differs considerably from monoculture biofilms. From the one hand, *S. aureus* avoids vancomycin and ampicillin by the rearrangements to the lower layers of the *P. aeruginosa* biofilm matrix. On the other hand, in the same consortium susceptibility to ciprofloxacin and aminoglycosides increases nearly 10–fold compared to monocultures. This finding allowed suggesting that intervention of antagonistic bacteria into already preformed monoculture biofilms could be used as an innovative approach to their treatment by increasing their antibiotic susceptibility. Thus, by introducing *P. aeruginosa* into preformed *S. aureus* biofilm, susceptibility of *S. aureus* to aminoglycosides was increased 4-fold, compared to monoculture. The intervention of *S. aureus* into the mature *P. aeruginosa* biofilm significantly increased the efficacy of ciprofloxacin against *P. aeruginosa*. We believe that this approach has a strong potential of further development towards innovative treatment of biofilm-associated infections such as introduction of the skin residential microflora to the wounds and ulcers infected with nosocomial pathogens to speed up their microbial decontamination.

Introduction

Bacterial fouling is an important factor that strongly affects acute and chronic wounds healing. Recent reports indicate that bacterial biofilms prevent wound scratch closure [1]. Besides the physical obstruction of the cells, pathogenic bacteria produce various virulence factors including toxins and proteases that also affect cytokine production by keratinocytes, induce apoptosis of the host cells and cause inflammation [2-6].

S. aureus and *P. aeruginosa* are one of the most widespread pathogenic agents causing various nosocomial infections, including pneumonia on the cystic fibrosis background, healthcare associated pneumonia and chronic wounds [7-10]. During infection, bacterial cells are embedded into a self-produced extracellular matrix of organic polymers this way forming either mono- or polymicrobial biofilms [11, 12] which drastically reduce their susceptibility to both antimicrobials and the immune system of the host [13, 14]. Accordingly, interspecies interactions between *S. aureus* and *P. aeruginosa* within mixed biofilms attracted major attention in recent years including both *in vitro* [15] and *in vivo* studies [16]. Current data suggests that bacterial pathogenicity is promoted during polymicrobial infections and recovery is delayed in comparison with monoculture infections [15-17]. Recently, *P. aeruginosa* was reported to be the dominant pathogen in *S. aureus*-*P. aeruginosa* mixed infections [16]. *P. aeruginosa* is known as a common dominator in polymicrobial biofilm-associated infections due to multiple mechanisms allowing its rapid adaptation to the specific conditions of the host. In particular, *P. aeruginosa* produces multiple molecules to compete with other microorganisms for space and nutrients. Therefore, it either strongly reduces or even completely outperforms *S. aureus* during co-culture *in vitro* in both planktonic and biofilm forms [18-21].

While it is known that *S. aureus* and *P. aeruginosa* exhibit rather antagonistic relationship [22, 23], several studies reported their mutual association in acute and chronic wounds embedded in a mixed biofilm [8, 24-28], with *S. aureus* typically residing on the wound surface, whereas

P. aeruginosa being rather observed in the deep layers [15, 28-31]. Interestingly, in mixed *P. aeruginosa* - *S. aureus* biofilms from cystic fibrosis patients *S. aureus* was shown to be dominating during childhood, with *P. aeruginosa* prevalence increasing with aging and worsening patient prognosis [32-34].

During the biofilm formation *P. aeruginosa* produces three main exopolysaccharides, namely alginate, Pel, and Psl, which form an extracellular matrix in the biofilm exhibiting both structural and protective functions [35-38]. Under prevalent Pel secretion, loose biofilm structures are formed [39] and thus *S. aureus* is able to penetrate into the biofilm [39]. When growing in consortium with *P. aeruginosa*, *S. aureus* switches to the small colony variants (SCVs), a well-characterized phenotype detected in various diseases, including cystic fibrosis and device-related infections [40-43]. SCVs appear as small, smooth colonies on a culture plate and grow significantly slower compared to wild type colonies. Remarkably, switch to the SCV phenotype improves the survival of *S. aureus* under unfavorable conditions, as it exhibits increased aminoglycoside resistance, biofilm formation, and intracellular survival [40, 43-45]. Prolonged co-culture with *P. aeruginosa* leads to higher proportions of stable *S. aureus* SCVs that is further increased in the presence of aminoglycosides [43]. In has been recently suggested that rare observation of *S. aureus* and *P. aeruginosa* together in diagnostic cultures of sputum of cystic fibrosis patients could be attributed to the existence of *S. aureus* as SCVs that are more difficult to detect due to their small size and fastidious growth requirements [40, 45].

S. aureus is a common opportunistic pathogen responsible for the majority of skin infections resulting in increased morbidity, mortality, and exhibiting increased rise of antibiotic-resistant strains in the last decades. Investigations on alternative treatment options against biofilm-associated infections are largely based upon using specialized agents (such as quaternary ammonium compounds, curcumin or chlorquinaldol) or enzymatic treatment that in combinations with antibiotics provide high local drug concentrations avoiding systemic adverse effects [46-52]. While many approaches to targeting staphylococcal biofilms were reported [50,

117 53-57], only few successive ways of targeting *P. aeruginosa* are known [52, 58-60]. Among
118 various compounds exhibiting anti-biofilm activities, the derivatives of 2(5*H*)-furanone have
119 been reported to inhibit biofilm formation by *Staphylococci* [61-65]. While many of these
120 approaches exhibited promising results against staphylococcal monocultures, their efficiency
121 against polymicrobial biofilms remains questionable.

122 Only few investigations indicated that extracellular polymeric substances forming the
123 biofilm matrix provide protection against antibiotics to all inhabitants of the biofilm, including
124 the non-producers, although the biofilm as a whole is weakened [39, 66], this way proposing that
125 *S. aureus* could potentially survive in the presence of *P. aeruginosa* and even co-exist with it in a
126 polymicrobial biofilm, benefiting from the antimicrobial barrier formed by the *P. aeruginosa*
127 matrix components.

128 Here we demonstrate explicitly that *S. aureus* successfully incorporates into the
129 *P. aeruginosa* biofilm matrix under conditions of staphylococcus-specific treatment and survives
130 there in presumably SCV-like form. In contrast, the efficiency of broad-spectrum antimicrobials
131 like ciprofloxacine and aminoglycosides active against both bacterial species in mixed biofilms
132 increased nearly 10-fold in comparison with corresponding monocultures. These data suggest
133 that interspecies interactions appear a key determinant that strongly governs the antibiotic
134 susceptibility in mixed biofilms, the fact that should be taken in account when considering an
135 optimized strategy of polymicrobial infections treatment.
136

Results

Modeling the *S. aureus – P. aeruginosa* mixed biofilm

Despite of known antagonistic interactions between *S. aureus* and *P. aeruginosa* [23], they are still the most common pathogens evoking wound infections and forming mixed biofilms on their surfaces [25, 26, 28]. We have simulated *in vitro* different situations where either *S. aureus* in a fresh broth was added to the preformed 24-h old biofilm of *P. aeruginosa* or, vice versa, *P. aeruginosa* was added to the preformed 24-h old biofilm of *S. aureus*, with cultivation continued for the next 24 h. As a control, both strains were inoculated simultaneously and grown for 48 h with the broth exchange after 24 h of cultivation. Both *S. aureus* and *P. aeruginosa* were able to penetrate into the preformed biofilm of the other bacterium (Fig 1). Irrespective of which bacterium initially preformed the biofilm and which one was added later, the ratio of their CFUs in the biofilm after 24 h cultivation remained around 1:10 with the prevalence of the first biofilm former (Fig 1 A and B), and was 1:1 when both bacteria were inoculated simultaneously (Fig 1 C). Therefore in the following experiments simultaneous inoculation of both bacteria was used to obtain their mixed biofilm.

Next to analyze the biofilm structure and cells distribution in the matrix, the *S. aureus* - *P. aeruginosa* mixed biofilm was grown in imaging cover slips, stained with ViaGram^{TM} Red^{+} to differentiate between *S. aureus* and *P. aeruginosa* followed by their analysis with confocal laser scanning microscopy. To estimate the viability of the cells, SYTO9/propidium iodide staining was also performed, as the ViaGram^{TM} Red^{+} staining requires buffer change that disturbs the biofilm structure. Both *S. aureus* and *P. aeruginosa* formed 20-25 μm-thick biofilms when growing as monocultures (Fig 2A, B). While the mixed biofilm was of similar thickness, it appeared more rigid in comparison with monoculture ones (Fig 2C). Interestingly, in the mixed biofilm, *S. aureus* was distributed unevenly and appeared as cell clumps, apparently as so-called small colony variants (SCV) embedded in the biofilm matrix (see white arrow in Fig 2C). By

using differential staining of *S. aureus* and *P. aeruginosa* (Fig 3) we have also analyzed the distributions of *S. aureus* (red-stained) and *P. aeruginosa* (blue-stained) over the biofilm layers and evaluated their relative fractions in each layer. In agreement with earlier data, *S. aureus* tended to distribute in the upper layers of the biofilm, while *P. aeruginosa* dominated in its lower layers (see Fig 3A, C). The fraction of non-viable cells in the mixed biofilm was just slightly higher than in corresponding monoculture biofilms (compare Fig 2 A, B and C and Fig S1), suggesting stability of *S. aureus* - *P. aeruginosa* consortium under the conditions used.

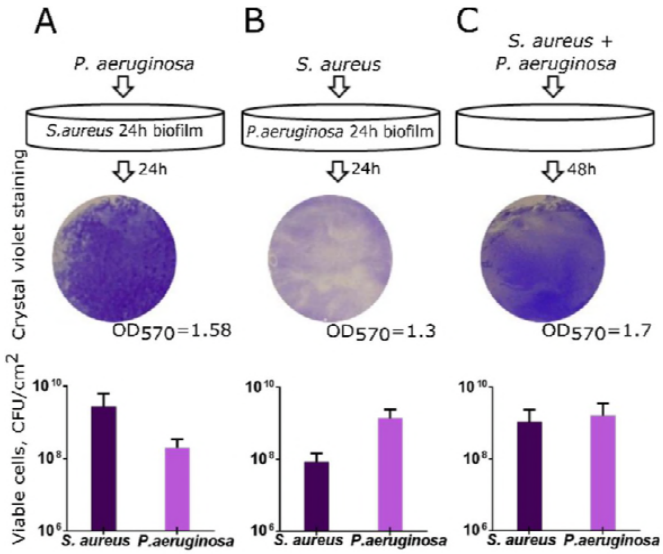


Fig 1. *In vitro* simulation of the *S. aureus-P. aeruginosa* mixed biofilm formation.

(A) *P. aeruginosa* suspension in a fresh broth was added to the preformed 24-h old biofilm of *S. aureus* or (B) *S. aureus* was added to the preformed 24-h old biofilm of *P. aeruginosa* and cultivation was continued for the next 24 h. (C) As a control, both strains were inoculated simultaneously and were grown for 48 h with the broth exchange after 24 h of cultivation. The biofilm formation was assessed by crystal violet staining; the number of viable biofilm-embedded cells was counted by drop plate assay.

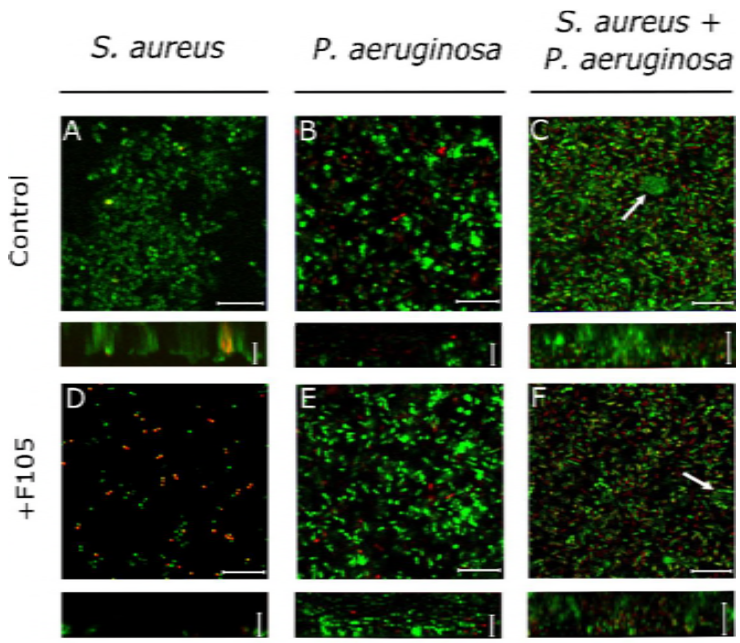


Fig 2. Mono- and polymicrobial biofilms formed by *S. aureus* and *P. aeruginosa*.

Cells were grown without any antimicrobial (A-C) or in presence of 2(5*H*)-furanone derivative (F105, D-F) specifically inhibiting the biofilm formation by *S. aureus* and exhibiting no effects on *P. aeruginosa*. The 48-h old biofilms were stained by Syto9/PI and assessed by CLSM. The images show a plan view on a basal biofilm layer and a cross section through the biofilm. The scale bars indicate 10 µm. *S. aureus* cell clumps in a mixed biofilm are shown by arrows.

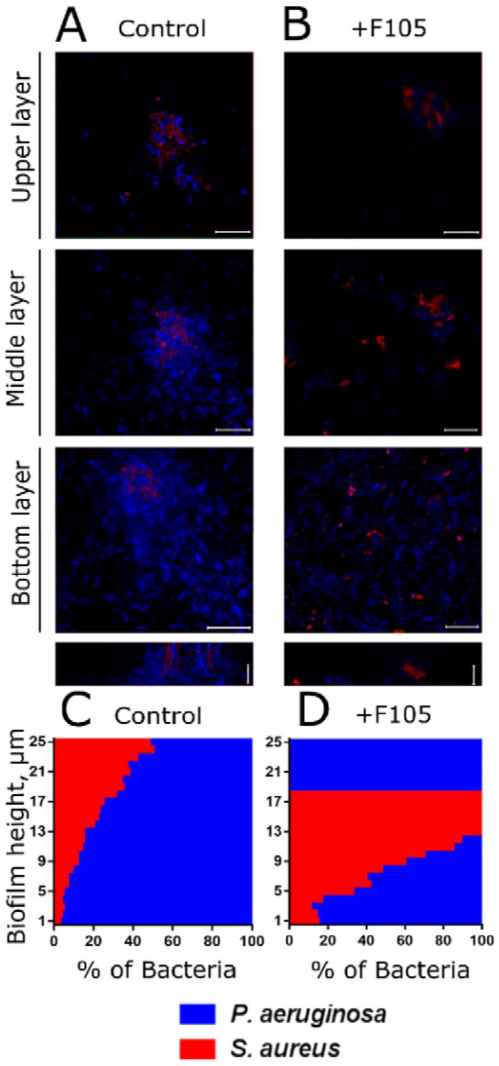


Fig 3. The distribution of *S. aureus* and *P. aeruginosa* in the mixed biofilm.

Cells were grown without any antimicrobial (A, C) or in presence of F105 specifically inhibiting the biofilm formation by *S. aureus* cells (B, D). The 48-h old biofilms were stained by ViaGram^{TM} Red^{+} to differentiate *S. aureus* (stained in red) and *P. aeruginosa* (stained in blue) and assessed by CLSM. The CLSM images show a plan view on an upper, middle or bottom biofilm layer and a cross section through the biofilm. The scale bars indicate 10 µm. (C, D) The distribution of *S. aureus* and *P. aeruginosa* in the biofilm layers expressed as their relative fractions.

In the last decades different approaches to inhibit the biofilm formation by various bacteria were developed [47, 48, 50], appearing nowadays more successful in prevention of *S. aureus* biofilm formation [53, 54, 56]. Therefore we simulated the *S. aureus* - *P. aeruginosa* mixed biofilm formation under the conditions of biofilm-preventing treatment. For that, bacteria were cultivated in the presence of a derivative of 2(5*H*)-furanone denoted as F105, identified recently study as an efficient inhibitor of growth and biofilm formation by *S. aureus* [65, 67], while exhibiting no significant effect against *P. aeruginosa* (Table 1).

When *S. aureus* was grown in the presence of 2.5 μg/ml of F105, no biofilm was formed, while most of the cells remained viable (Fig 2 D). As expected, no significant effect of F105 on cell viability of *P. aeruginosa* could be observed (Fig 2 E, Table 1). Moreover, the biofilm formation was slightly increased as determined by crystal violet staining (Fig S2) and CLSM (compare Fig 2 B and E). Therefore, we next used F105 to obtain a model of *S. aureus* - *P. aeruginosa* mixed biofilm where the biofilm formation by *S. aureus* is repressed and the matrix is produced predominantly by *P. aeruginosa*.

Table 1. ECOFF, MIC and MBC values in μg/mL of various antibiotics against *S. aureus* and *P. aeruginosa*. MIC and MBC were assessed by the broth microdilution.

	S. aureus			*P. aeruginosa*		
	ECOFF	MIC	MBC	ECOFF	MIC	MBC
F105	ND	2.5	5	ND	ND	ND
Van	2.0	4	32	ND	ND	ND
Tet	1.0	0.25	128	ND	16	ND
Cef	8.0	8	128	ND	32	ND
Amp	ND	0.5	16	ND	ND	ND
Ami	8.0	2	64	16	1	64
Gen	2.0	4	32	8.0	8	64
Cip	1.0	1	64	0.5	4	64

*ND – not determined

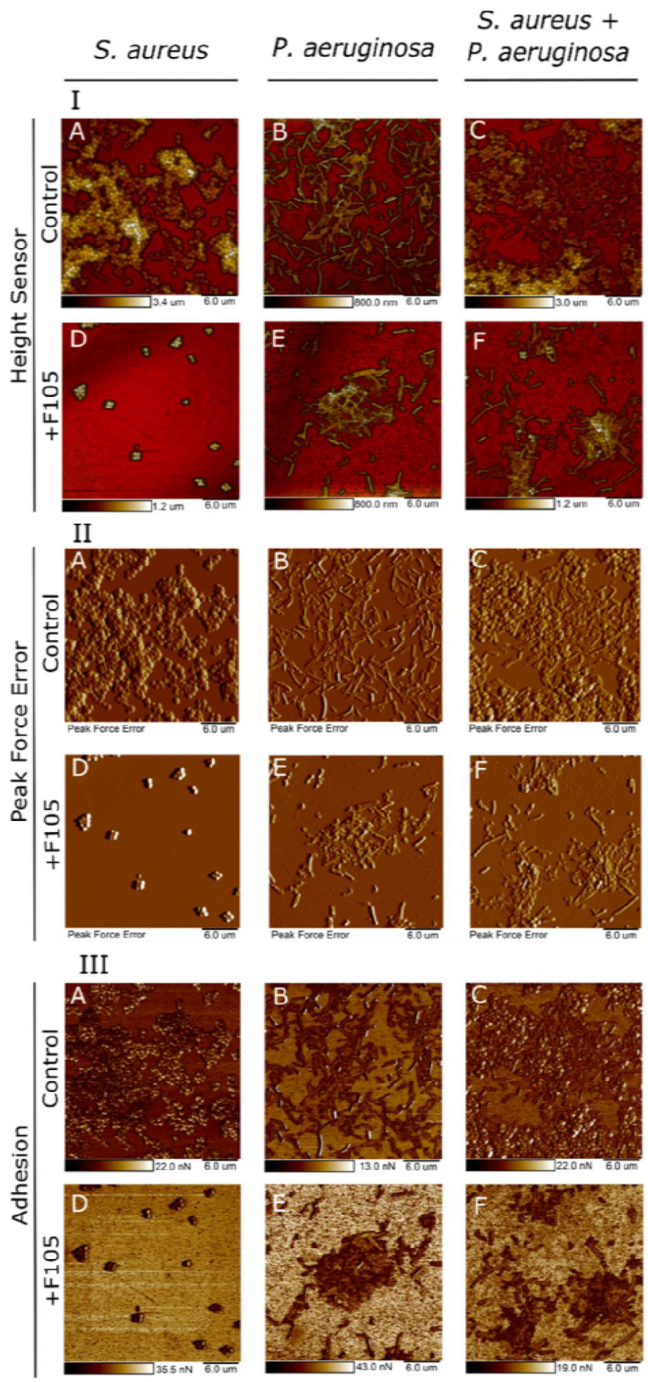
When *S. aureus* and *P. aeruginosa* were grown together in the presence of F105, *S. aureus* clumps were also observed, similarly to the control (compare Fig 2 C and F), suggesting that *S. aureus* cells are able to form clusters inside the biofilm of *P. aeruginosa*, despite of its antagonistic pressure (see white arrows on Fig 2F). In marked contrast to the control, the cells were observed only in the bottom layers of the biofilm (compare Fig 3 C and D) suggesting that under conditions of anti-biofilm pressure *S. aureus* is apparently able to hide in the biofilm formed by *P. aeruginosa* and survive there.

The microscopic data were further validated by direct CFU counting in the biofilm; by using mannitol salt agar plates and cetrimide agar plates the bacterial species were differentiated and their CFUs were counted separately (Fig S3). In the presence of F105 the amount of adherent viable *S. aureus* cells decreased by 6 orders of magnitude in monoculture, suggesting

231 complete inhibition of the biofilm formation, while no significant differences in CFUs of
232 *P. aeruginosa* could be observed (Fig S3). In a mixed biofilm, the *S. aureus* to *P. aeruginosa*
233 ratio remained unchanged in the control, while the fraction of viable *S. aureus* cells decreased
234 slightly in the presence of F105, this way confirming CLSM data and supporting the hypothesis
235 that *S. aureus* is able to survive in the *P. aeruginosa* biofilm when its own biofilm formation is
236 repressed.
237

238 **Atomic force microscopy**

239 The atomic force microscopy of both monocultures and mixed biofilms of *S. aureus* -
240 *P. aeruginosa* confirmed the CLSM data. Thus, in control wells the biofilms of monocultures of
241 both strains formed a typical confluent multilayer biofilm (Fig 4, A, B), in mixed biofilm
242 *S. aureus* was prevalently distributed in the upper layers (Fig 4 C). Interestingly, the adhesion
243 force of the mixed biofilm was 3-fold lower compared to *S. aureus* monoculture biofilm and 2-
244 fold lower compared to *P. aeruginosa* monoculture biofilm (Table 2), suggesting more irregular
245 structure of the mixed biofilm [39]. When growing with F105, only *P. aeruginosa* could be
246 observed on the biofilm surface in the mixed culture, suggesting that *S. aureus* was hidden into
247 the lower biofilm layers. Since the adhesion force of the mixed biofilm in the presence of F105
248 was similar to that one in the monoculture *P. aeruginosa* (Table 2, Fig 7 F), we assumed that the
249 biofilm matrix under these conditions was presumably formed by *P. aeruginosa*.



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251

252 **Fig 4. Atomic force microscopy (Peak Force Tapping mode) of mono- and polymicrobial**

253 **biofilms formed by *S. aureus* and *P. aeruginosa*.** Cells were grown without any antimicrobials

254 (A-C) or in presence of F105 specifically inhibiting the biofilm formation by *S. aureus* cells (D-

255 F) for 48 hours, then the plates were washed, fixed with glutardialdehyde and analyzed with

256 AFM. While (I) shows sensor height (topography), (II) shows 3D reconstruction of height
257 channel image and (III) indicates adhesion.
258
259 **Table 2. The adhesion force of *S. aureus* and *P. aeruginosa* monoculture and mixed**
260 **biofilms.** To repress the biofilm formation F105 was added up to 2.5 μg/ml.

Biofilm former	F105 concentration, μg/ml	Adhesion, nN
S. aureus	0	17.4 ± 4.84
S. aureus	2.5	12.1 ± 3.50
P. aeruginosa	0	11.3 ± 3.40
P. aeruginosa	2.5	10.4 ± 5.70
S. aureus + *P. aeruginosa*	0	6.1 ± 0.34
S. aureus + *P. aeruginosa*	2.5	10.3 ± 0.47

261

262 ### *S. aureus* and *P. aeruginosa* susceptibility to antibiotics in mixed
263 ### biofilms

264 Our data suggest that *S. aureus* under anti-biofilm treatment conditions is able to form
265 cell clumps in the biofilm of *P. aeruginosa*, thereby apparently changing their tolerance to
266 antimicrobials. To further verify this assumption, the effect of various conventional antibiotics
267 on preformed mono- and polymicrobial biofilms was studied. The 48-h old monoculture and
268 mixed biofilms were prepared in 24-well adhesive plates in either absence or presence of F105 to
269 repress the biofilm formation by *S. aureus* itself. Then the biofilms were washed with sterile
270 0.9% NaCl and wells were loaded with fresh broth supplemented with antibiotics at wide range
271 of final concentrations to fill the range of their 1-16 fold MBCs (see Table 1 for MBC values).
272 After 24h incubation the amount of CFUs of both *S. aureus* and *P. aeruginosa* in the biofilm was

273 determined by the drop plate assay and the distribution of cells in the mixed biofilm was
274 assessed by CLSM.

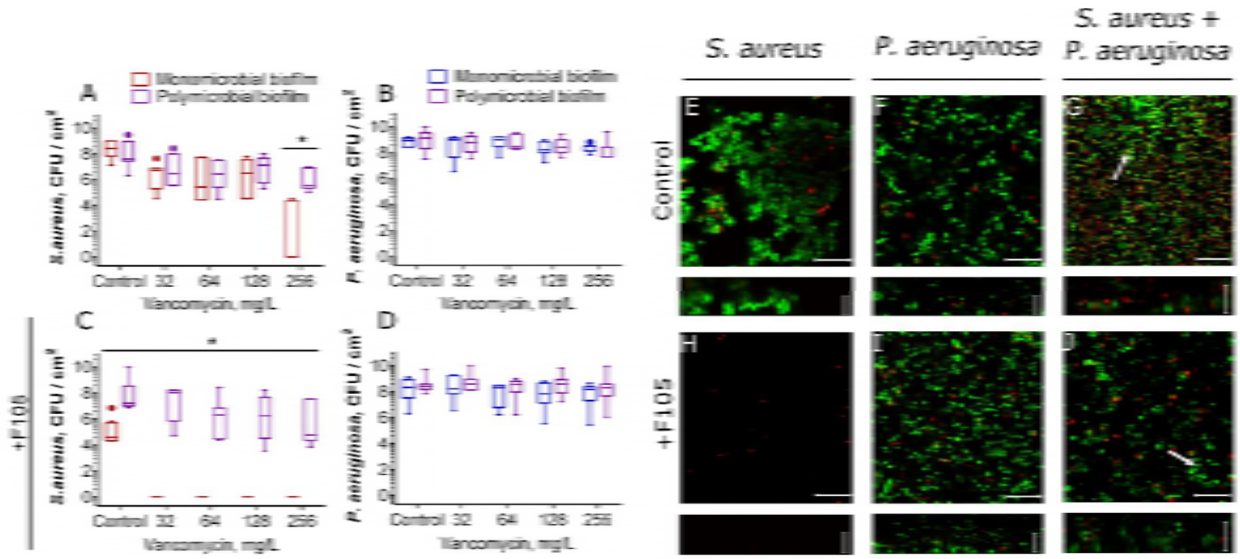
275 First, the biofilm-eradicating activity was investigated for the antibiotics conventionally
276 used for *S. aureus* treatment but typically inefficient against *P. aeruginosa* including
277 vancomycin, tetracycline, ampicillin and ceftriaxone (Fig 5, S4). In monoculture, vancomycin
278 reduced the amount of viable *S. aureus* cells in the biofilm by 3 orders of magnitude at 16×MBC
279 (Fig 5 A). Expectedly, when *S. aureus* cells were grown in the presence of F105 (2.5 μg/ml) and
280 therefore no biofilm could be formed, bacteria were found completely dead after 24-h exposition
281 to the antibiotic at 1-2×MBC (Fig 5 C). Irrespective of either presence or absence of F105
282 *P. aeruginosa* remained resistant to the antibiotic (Fig 5 B, D).

283 In a mixed culture, irrespective of the *S. aureus* biofilm formation repression by F105,
284 viable *S. aureus* cells were identified within the biofilm and the efficiency of antibiotics reduced
285 drastically (Fig 5 A, C, compare reds and violets). Statistical significance of this discrepancy was
286 confirmed by the Kruskal-Wallis statistical test at $p < 0.05$.

287 For a deeper understanding of localization and viability of bacteria in mixed biofilms
288 under vancomycin treatment also CLSM analysis was performed. In the presence of F105 no
289 biofilm of *S. aureus* could be observed resulting in significant decrease of viable cells fraction
290 after vancomycin treatment, in contrast to the biofilm-embedded cells (compare Fig 5 E and H).
291 In the mixed biofilm coccal cell clusters were formed in the biofilm matrix similarly to the
292 control (compare Fig 2 C, F and 5, G, J), suggesting that *staphylococci* are able to escape the
293 antimicrobials and survive by embedding itself into the polymicrobial biofilm.

294 The distribution of bacteria in the mixed biofilm layers was also assessed by differential
295 staining of *S. aureus* and *P. aeruginosa* by the ViaGram^{TM} Red^{+} (Fig 6). In marked contrast to
296 the control where *S. aureus* was mostly located in the top layers of the biofilm, under
297 vancomycin treatment most of the cells appeared in the lower and middle layers of the biofilm

298 (compare Fig 3 and Fig 6) suggesting that vancomycin-resistant *P. aeruginosa* cells in the upper
299 layers of the biofilm apparently prevented the penetration of the antibiotic into the matrix this
300 way reducing the susceptibility of *S. aureus* to antibiotics considerably. Of note, *S. aureus* cells
301 remained presumably viable in bottom layers apparently because of protection by *P. aeruginosa*
302 cells (Fig S5).
303



304

305 **Fig 5. The effect of vancomycin on viability of *S. aureus* and *P. aeruginosa* embedded into**
306 **their mono- and polymicrobial biofilms.**
307 Antimicrobial was added to 48 hours-old biofilms grown in absence (A-B) or presence (C-D) of
308 F105 to inhibit the biofilm formation by *S. aureus*. After 24 h incubation, the biofilms were
309 analyzed by CFUs counting or Syto9/PI staining followed by CLSM. The images show a plan
310 view on a basal biofilm layer and a cross section through the biofilm. The scale bars indicate 10
311 µm. Asterisk shows significant difference between CFUs number between monoculture and
312 mixed biofilms.
313
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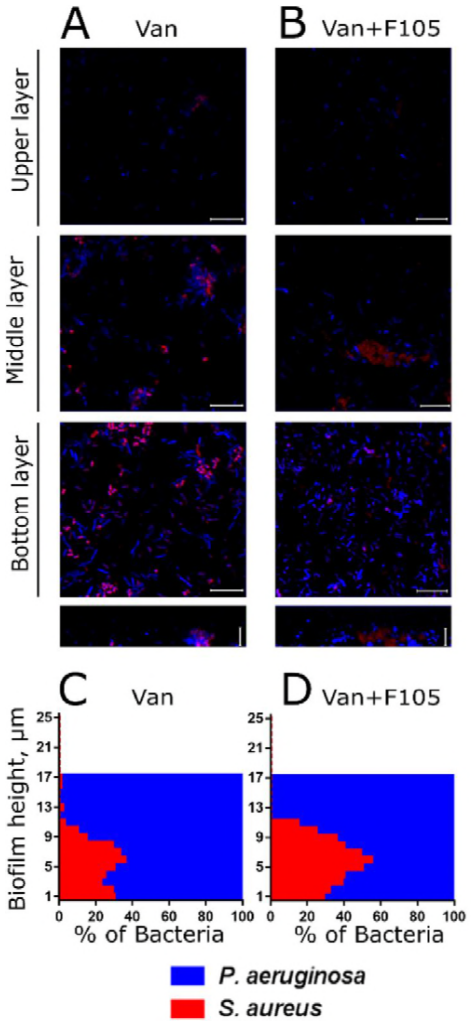

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317 **Fig. 6. The effect of vancomycin on *S. aureus* and *P. aeruginosa* distribution in mixed**
318 **biofilms grown either in absence (A, C) or in presence of F105 specifically inhibiting the**
319 **biofilm formation by *S. aureus* cells (B, D).**

320 Vancomycin (256 µg/mL corresponding to 8×MBC for *S. aureus*) was added to 48 hours-old
321 biofilms. After 24 h incubation, the biofilms were stained by ViaGram^{TM} Red^{+} to differentiate
322 *S. aureus* (stained in red) and *P. aeruginosa* (stained in blue) and assessed by CLSM. The
323 images show a plan view on an upper, middle or bottom biofilm layer and a cross section
324 through the biofilm. The scale bars indicate 10 µm. (C, D) The distribution of *S. aureus* and
325 *P. aeruginosa* in the biofilm layers are given by their relative fractions.

326

Similarly to vancomycin, treatment by ampicillin, tetracycline and ceftriaxone was almost inefficient against biofilm-embedded *S. aureus*, while under conditions of biofilm formation repression by F105, the 1-2×MBC of antimicrobials led to the complete death of cells in 24 h (Fig S4). Again, in the mixed culture, despite of the *S. aureus* biofilm formation repression, viable *S. aureus* cells could be identified within the biofilm. CLSM analysis indicated considerable redistribution of *S. aureus* from upper to the bottom layers of the biofilm (Fig S6, cells distribution patterns) leading to reduced antibiotic efficacy. Under double treatment by F105 and antimicrobials, the prevalence of *P. aeruginosa* in the biofilm was observed in agreement with the CFU count data (Fig S4 and S6). These data suggest that under anti-biofilm or antimicrobial treatment conditions *S. aureus* changes its preferred topical localizations by hiding in the lower layers of mixed biofilm formed by another bacterium like *P. aeruginosa* insensitive to most antimicrobials thereby increasing its resistance to the treatment.

Next, we investigated the effect of broad-spectrum antimicrobials such as ciprofloxacin, amikacin and gentamycin which are active against both *S. aureus* and *P. aeruginosa* (see Table 1). In contrast to the previous group of antimicrobials, in monoculture high concentrations of ciprofloxacin efficiently eradicated even the biofilm-embedded *P. aeruginosa* (Fig 7 B). Interestingly, when the mixed biofilm was treated, nearly 10-fold lower concentration of antimicrobial was required to obtain similar reduction of *P. aeruginosa* CFUs in the biofilm. Moreover, in the mixed biofilm complete death of both *P. aeruginosa* and *S. aureus* could be observed at 8×MBC of ciprofloxacin, in marked contrast to monocultures. Similarly, 1-2×MBC of aminoglycosides (amikacin or gentamicin) led to the complete death of both *P. aeruginosa* and *S. aureus* in mixed biofilm (Fig 8) while reducing their CFUs in monocultures only by 2-3 orders of magnitude at 8×MBCs.

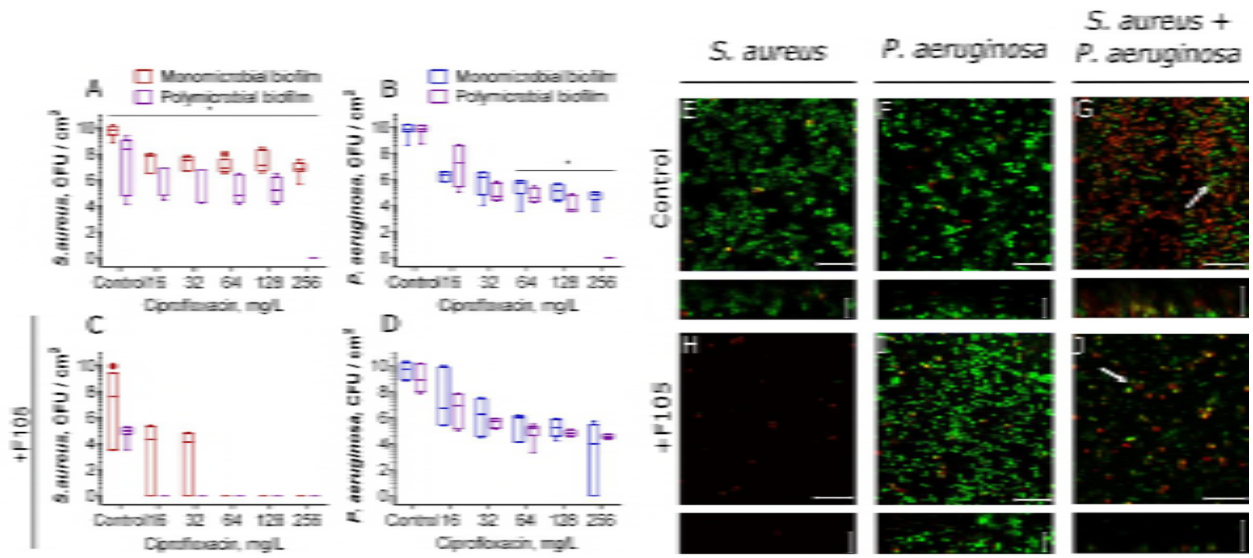


Fig 7. The effect of ciprofloxacin on viability of *S. aureus* and *P. aeruginosa* embedded into their mono- and polymicrobial biofilms. Antimicrobial was added to 48 hours-old biofilms grown in absence (A-B) or presence (C-D) of F105 to inhibit the biofilm formation by *S. aureus*. After 24 h incubation, the biofilms were analyzed by CFUs counting or Syto9/PI staining followed by CLSM. The images show a plan view on a basal biofilm layer and a cross section through the biofilm. The scale bars indicate 10 µm. Asterisks show significant difference between CFUs number between monoculture and mixed biofilms.

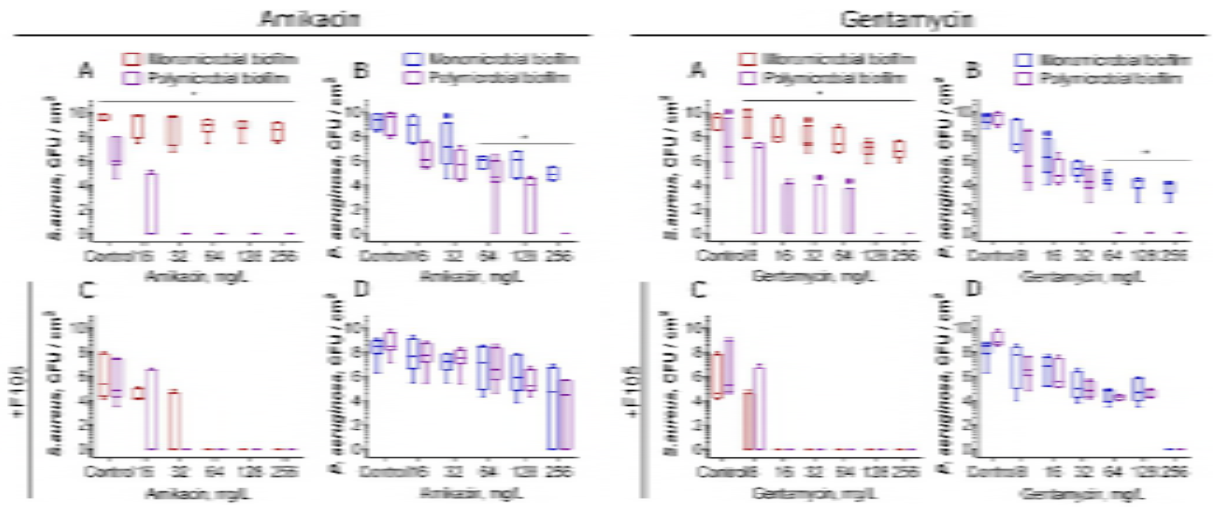


Fig 8. The effect of amikacin and gentamicin on viability of *S. aureus* and *P. aeruginosa* in mono- and polymicrobial biofilms.

Antimicrobials were added to 48 hours-old biofilms grown in absence (A-B) or presence (C-D) of F105 to inhibit the biofilm formation by *S. aureus*. After 24 h incubation, the biofilms were analyzed by CFUs counting. Asterisks show significant difference between CFUs number between monoculture and mixed biofilms.

In the presence of F105, just 1×MBC of any tested antimicrobial was already sufficient for the complete eradication of *S. aureus* biofilm (see Fig 7 C and Fig 8 C), similarly to the previous group of antibiotics such as vancomycin, tetracycline, ampicillin and ceftriaxone (see Fig 5 and Fig S4). The presence of F105 did not affect the susceptibility of monoculture *P. aeruginosa* biofilm to antibiotics. In contrast, in mixed biofilms inhibition of *S. aureus* by F105 restored the susceptibility of *P. aeruginosa* back to the monoculture level, suppressing the observed high efficiency of antimicrobials against this bacterium in the mixed biofilm (compare Fig 7 B and D, Fig 8 B and D). In contrast to *S. aureus*-specific antibiotics, the efficiency of ciprofloxacin and aminoglycosides against *S. aureus* in mixed biofilm in the presence of F105 was similar to the monoculture level.

The CLSM analysis of *S. aureus* and *P. aeruginosa* monoculture and mixed biofilms treated with Ciprofloxacin confirmed the CFUs counting data. In particular, while 8×MBC did not affect either *S. aureus* or *P. aeruginosa* cells in monoculture biofilms (Fig 7E, F), in the mixed biofilm *P. aeruginosa* was identified as non-viable, although *S. aureus* remained partially alive (Fig 7 G). In marked contrast, repression of the *S. aureus* biofilm production by F105 led to a reversal with most *P. aeruginosa* cells green-stained while *S. aureus* identified as non-viable in mixed culture (Fig 7 J).

The distribution of bacteria in the mixed biofilm layers under treatment with ciprofloxacin was also assessed by differential staining of *S. aureus* and *P. aeruginosa* using ViaGram^{TM} Red^{+} (Fig 9). In contrast to vancomycin treatment, here *S. aureus* dominated in the upper layers of the mixed biofilm (compare Fig 6 and 9 A and C) and remain alive, while *P. aeruginosa* were presumably dead (See Fig S5, S7, S8) suggesting no reversal protection of *P. aeruginosa* by *S. aureus* biofilm. On the other hand, double treatment by ciprofloxacin combined with F105 resulted in hiding of *S. aureus* in the bottom layers of the biofilm and increased resistance of *P. aeruginosa*. Treatment by amikacin and gentamycin led to considerably different distributions of bacteria over the biofilm layers with the prevalence of *S. aureus* in the bottom layers irrespective of its biofilm repression by F105 (Fig S8, cells distribution patterns) but qualitatively similar bacterial survival patterns (see Fig S6). Moreover, under single antibiotic treatment *P. aeruginosa* were presumably dead, while *S. aureus* remained viable (Fig S8). In the presence of F105 *P. aeruginosa* remained alive and much less *S. aureus* cells could be observed in the biofilm, as almost all of them were identified as non-viable.

Taken together these data suggest complex interspecies interactions between *S. aureus* and *P. aeruginosa* in mixed biofilm under treatment by antimicrobials with different specificity.

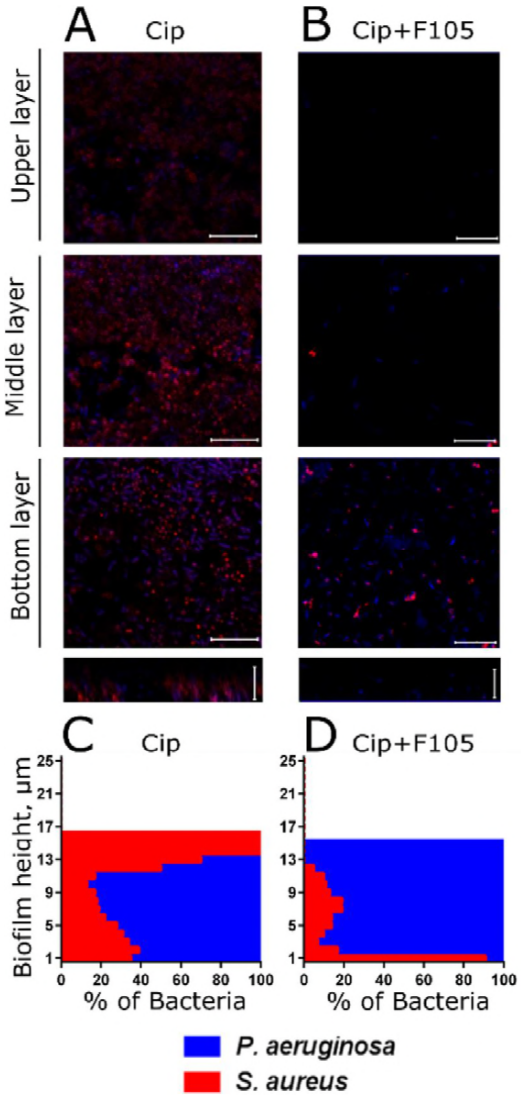


Fig 9. The effect of ciprofloxacin on *S. aureus* and *P. aeruginosa* distribution in mixed biofilms grown normally (A, C) or in presence of F105 specifically inhibiting the biofilm formation by *S. aureus* cells (B, D).

Ciprofloxacin (512 µg/mL corresponding to 8×MBC for *S. aureus*) was added to 48 hours-old biofilms. After 24 h incubation, the biofilms were stained by ViaGram^{TM} Red^{+} to differentiate between *S. aureus* (red-stained) and *P. aeruginosa* (blue-stained) and assessed by CLSM. The images show a plan view on an upper, middle or bottom biofilm layer (indicated by arrows) and a cross section through the biofilm. The scale bars indicate 10 µm. (C, D). *S. aureus* and *P. aeruginosa* distributions over the biofilm layers are expressed by their relative fractions.

413

414 **Intervention of *P. aeruginosa* into *S. aureus* biofilm and vice versa as a**
415 **possible way to enhance antimicrobial susceptibility**

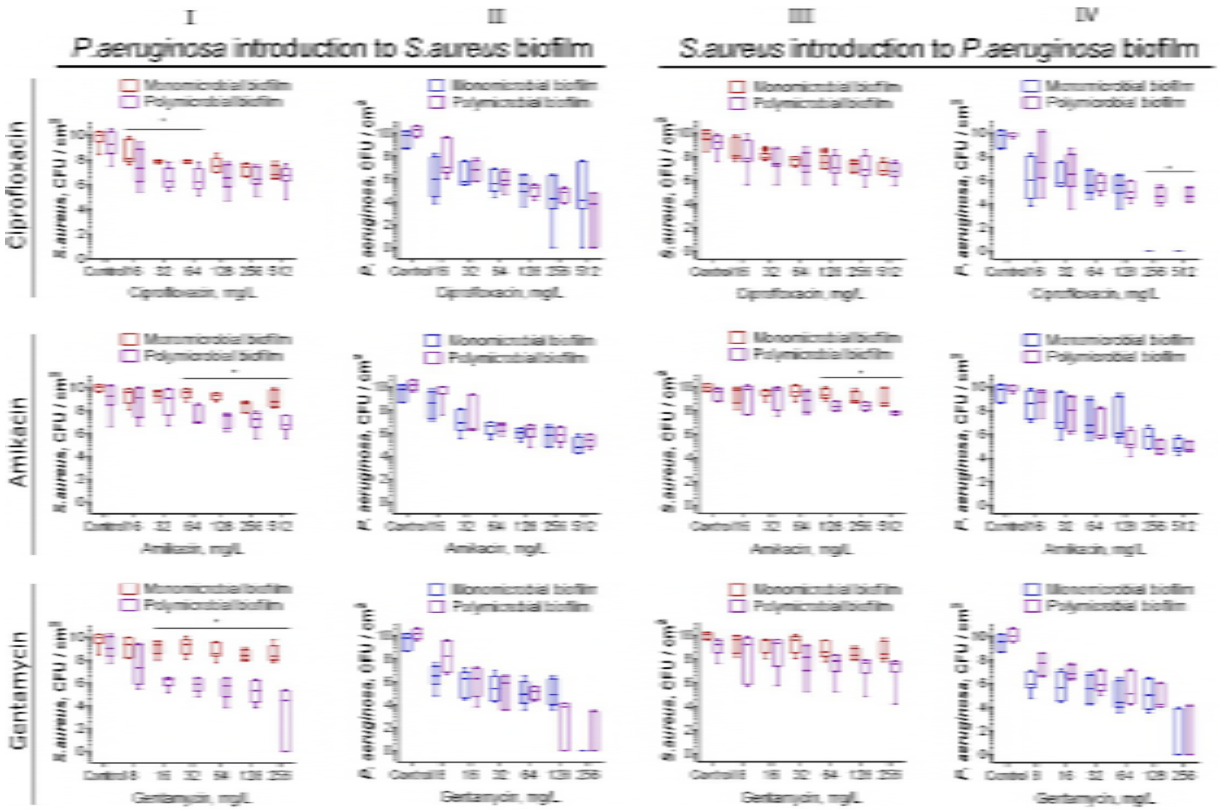
416 Our results indicate that under appropriate conditions both *S. aureus* and *P. aeruginosa*
417 due to their antagonistic interactions appear more susceptible to broad-spectrum antimicrobials
418 in polymicrobial biofilms, compared to their monoculture counterparts. Based on these data, we
419 have suggested that also the susceptibility of monoculture biofilms could be increased by
420 deliberate intervention of *P. aeruginosa* into preformed *S. aureus* biofilm, and vice versa.

421 To verify the efficacy of this approach, *P. aeruginosa* suspension (10^6 CFU/mL) was
422 added to the 24 h-old *S. aureus* biofilm and bacteria were incubated for the next 24 h. Then the
423 biofilm was washed by sterile saline and fresh broth containing different antimicrobials was
424 added into the wells. After 24 h the number of *P. aeruginosa* and *S. aureus* CFUs was counted
425 by using differential media.

426 The introduction of *P. aeruginosa* into *S. aureus* biofilm did not change the efficacy of
427 any antibiotic against *P. aeruginosa* itself (Fig 10, lane II). In contrast, 1×MBC of ciprofloxacin
428 led to the reduction of viable *S. aureus* in biofilm by 3 orders of magnitude, while in the
429 monoculture 4-8×MBC was required to achieve the same effect (Fig 10, lane I, compare reds and
430 violets). Amikacin and gentamycin, being almost inefficient against *S. aureus* monoculture
431 biofilm up to 8×MBC, were able to decrease the *S. aureus* CFUs in biofilm by 3 orders of
432 magnitude already at 1-2×MBC after introduction of *P. aeruginosa* with the most pronounced
433 effect observed for gentamycin.

434 In the reverse experiment, when *S. aureus* was added to the *P. aeruginosa* biofilm, a
435 remarkable increase of ciprofloxacin efficacy against *P. aeruginosa* could be observed (Fig 10,
436 lane IV, compare blues and violets), while the susceptibility of *S. aureus* itself did not change

437 significantly. The efficacy of aminoglycosides has increased only against *S. aureus*, while not
438 against *P. aeruginosa*.
439
440



441

442 **Fig 10. The susceptibility of *P. aeruginosa* and *S. aureus* after introduction of the antagonist**
443 **into monoculture biofilms.**

444 (I-II) *P. aeruginosa* suspension in a fresh broth was added to the preformed 24-h old biofilm of
445 *S. aureus* or (III-IV) *S. aureus* was added to the preformed 24-h old biofilm of *P. aeruginosa* and
446 cultivation was continued for the next 24 h. Then antimicrobials were added and after 24 h
447 incubation the biofilms were analyzed by CFUs counting. Asterisks show significant difference
448 between CFUs number between monoculture and mixed biofilms.
449
450

Discussion

Biofilm formation represents an important virulence factor of many bacteria, as the extracellular matrix drastically reduces their susceptibility to antimicrobials resulting in up to 1000-fold higher tolerance to antibiotics of biofilm-embedded cells compared to their planktonic forms [14, 68, 69]. In contrast, polymicrobial communities are often characterized by concurrent interspecies interactions that likely overwhelm the potential benefits from biofilm protection. Here we have shown that the antagonistic interactions between *S. aureus* and *P. aeruginosa*, the most common pathogenic agents causing various nosocomial infections [7-9], drastically affect their susceptibility to antibiotics making them significantly more or less vulnerable to treatment than in monoculture biofilms depending on both conditions and chosen antimicrobial agents.

Despite of the antagonistic relationship between *S. aureus* and *P. aeruginosa* described in multiple studies [22, 23], these bacteria can be found in close association in acute and chronic wounds being embedded into mixed biofilms [8, 24-28]. Our *in vitro* data show that the inoculation of *S. aureus* to the mature *P. aeruginosa* biofilm or *vice versa* leads to the formation of mixed biofilm, although with the prevalence of the first biofilm former (Fig 1). The co-cultivation of both bacteria results in the formation of a more rigid biofilm, where *S. aureus* is located mainly in the upper layers, while *P. aeruginosa* can be found mostly in the lower layers of the biofilm (Fig 3), in agreement with earlier data [15, 28-31].

Next, we investigated the effect of two groups of antimicrobials on bacterial viability in mixed biofilms. The first group contained vancomycin, tetracycline, ampicillin and ceftriaxone that are known to exhibit specific activity against *S. aureus* while leaving *P. aeruginosa* nearly unaffected. The second group included broad-spectrum antibiotics such as Ciprofloxacin, Gentamicin and Amikacin that exhibited comparable MBC values against both studied bacteria (see Table 1). Additionally, we also simulated the biofilm-preventing treatment with earlier described compound F105, specifically affecting only *S. aureus* biofilm formation[65]. In

476 control experiments with *S. aureus* monoculture biofilms, none of the antimicrobials exhibited
477 any bactericidal effect at their 8-16×MBCs, while 1×MBC was already sufficient for the
478 complete eradication of all adherent cells under biofilm repression conditions with F105
479 (compare Figs 5, 7, 8, S4, reds on panels A and C). In addition, ciprofloxacin, gentamicin and
480 amikacin at 8×MBCs significantly reduced the number of CFUs of biofilm-embedded
481 *P. aeruginosa* (Figs 5, 7, 8, S4, blues on panels B and D).

482 In mixed biofilms, treatment with antimicrobials active specifically against *S. aureus*
483 such as vancomycin, tetracycline, ampicillin and ceftriaxone as well as by biofilm repressing
484 agent F105, *S. aureus* could successfully escape from the treatment by re-localization to the
485 middle and lower layers of the biofilm. Irrespective of the *S. aureus* biofilm formation
486 repression, *S. aureus* cells remained viable under these conditions being embedded into the
487 matrix of *P. aeruginosa* biofilm and were insensitive to antimicrobials (see Figs 6, S6)
488 suggesting that *staphylococci* are able to escape the antimicrobials by embedding into the biofilm
489 matrix of *P. aeruginosa* and survive there, despite of their antagonistic interactions. Notably, in
490 mixed biofilms *S. aureus* formed cell clumps in the biofilm matrix (compare Fig 2 C, F and 5, C,
491 F) presumably in the form of small colonies.

492 Remarkably, when the *S. aureus* – *P. aeruginosa* mixed biofilms were treated with any of
493 the broad-spectrum antimicrobials such as ciprofloxacin, gentamicin or amikacin, nearly 10–fold
494 lower concentrations were sufficient to achieve the same reduction in the CFUs number of both
495 bacteria, in comparison with monoculture treatment (Figs 7 and 8, compare violets with reds or
496 blues on panels A and B). This effect was more pronounced for aminoglycosides, which at
497 already 1–2×MBC led to the complete eradication of the mixed biofilm, while in monocultures
498 8×MBC was required to reduce the number of CFUs by 3–5 orders of magnitude (Fig 8).
499 Moreover, tetracycline and ceftriaxone, while being inefficient against *P. aeruginosa*, at high
500 concentrations significantly reduced the CFUs of this bacterium in the mixed biofilms (Fig S4).

Interestingly, under repression of the *S. aureus* biofilm formation by F105, the efficiency of antimicrobials against *S. aureus* did not change significantly, while the sensitivity of *P. aeruginosa* was restored to the level characteristic for its monoculture biofilm (Figs 7 and 8, compare violets with reds or blues on panels C and D). This effect could be attributed to the redistribution of the *S. aureus* cells to the bottom levels of the mixed biofilm and significant reduction of their fraction (see Figs 9, cells distributions). On the other hand, the observed reinstatement of the *P. aeruginosa* sensitivity to antimicrobials could originate from the repression of the antagonistic factors production by *S. aureus* due to complex changes in its cell metabolism in the presence of F105. Nevertheless, the molecular basis of these complex interbacterial interactions that under certain conditions lead to a clear reversal in the antimicrobials susceptibility requires further investigations.

Taken together, our data clearly indicate that efficient treatment of biofilm-associated mixed infections requires antimicrobials which would be active against dominant pathogens. As we have shown for the *S. aureus* and *P. aeruginosa* mixed culture model, in this case the interbacterial antagonism under certain conditions assists antimicrobial treatment. In contrast, treatment by antibiotics with different efficacy against various consortia members leads to the survival of sensitive cells in the matrix formed by the resistant ones.

Finally, we have shown that *S. aureus* and *P. aeruginosa* are able to penetrate into each other's mature biofilms (see Fig 1 A and B) and by this intervention significantly affect the susceptibility of the mixed biofilm to antimicrobials (Fig 10). When *P. aeruginosa* was introduced into *S. aureus* biofilm, all antimicrobials reduced the amount of CFUs of both bacteria in the biofilm by 3 orders of magnitude at 1–2×MBC with more pronounced effect observed for gentamicin. In the reverse experiment, the inoculation of *S. aureus* to the mature *P. aeruginosa* biofilm significantly increased the efficacy of ciprofloxacin against *P. aeruginosa*.

From a broader perspective, we believe that artificial intervention of antagonistic bacteria into already preformed monoculture biofilms could be used to enhance their

527 antimicrobial treatment efficacy. We suggest that this approach has a strong potential of further
528 development towards innovative treatment of biofilm-associated infections such as introduction
529 of the skin residential microflora to the wounds and ulcers infected with nosocomial pathogens
530 to speed up their microbial decontamination. While in this work we demonstrated the synergy of
531 interbacterial antagonism with antimicrobials using the well-studied *S. aureus* - *P. aeruginosa*
532 model system, we believe that many other bacteria of normal body microflora are available to
533 antagonize with nosocomial pathogens and thus can be used for the enhancement of microbial
534 infections treatment by using microbial transplantation.
535

Materials and methods

Derivate of 2(5H)-furanone designed as F105 (3-Chloro-5(S)-[(1R,2S,5R)-2-isopropyl-5-methylcyclohexyloxy]-4-[4-methylphenylsulfonyl]-2(5H)-furanone) was described previously [67] and synthesized at the department of Organic Chemistry, A.M. Butlerov Chemical Institute, Kazan Federal University.

Bacterial strains and growth conditions

Staphylococcus aureus subsp. aureus (ATCC® 29213™) and *Pseudomonas aeruginosa* (ATCC® 27853™) were used in this assay. The bacterial strains were stored in 10 % (V/V) glycerol stocks at –80 °C and freshly streaked on blood agar plates (BD Diagnostics) followed by their overnight growth at 35°C before use. Fresh colony material was used to adjust an optical density to 0.5 McFarland (equivalent to 10^8 cells/mL) in 0.9 % NaCl solution that was used as a working suspension. For the biofilm assay the previously developed BM broth (glucose 5g, peptone 7g, $MgSO_4 \times 7H_2O$ 2.0g and $CaCl_2 \times 2H_2O$ 0.05g in 1.0 liter tap water) [49, 63, 70] where both *S. aureus* and *P. aeruginosa* formed rigid biofilms in 2 days was used. The mannitol salt agar (peptones 10g, meat extract 1g, NaCl 75g, D-mannitol 10g, agar-agar 12g in 1.0 liter tap water, Oxoid) and cetrimide agar (Sigma) were used to distinguish *S. aureus* and *P. aeruginosa*, respectively, in mixed cultures. Bacteria were grown under static conditions at 35°C for 24–72 hours as indicated.

Biofilm assays

Biofilm formation was assessed in 24-well polystirol plates (Eppendorf) by staining with crystal violet as described earlier in [71] with modifications. Bacteria with an initial density of 3×10^7 CFU/ml were seeded in 2 ml BM at 37°C and cultivated for 48 h under static conditions. Then the culture liquid was removed and the plates were washed once with

phosphate-buffered saline (PBS) pH=7.4 and dried for 20 min. Then, 1 ml of a 0.5% crystal violet solution (Sigma-Aldrich) in 96% ethanol was added per well followed by incubation for 20 min. The unbounded dye was washed off with PBS. The bound dye was eluted in 1 ml of 96% ethanol, and the absorbance at 570 nm was measured on a Tecan Infinite 200 Pro microplate reader (Switzerland). Cell-free wells subjected to all staining manipulations were used as control.

The biofilms were additionally analyzed by confocal laser scanning microscopy (CLSM) on Carl Zeiss LSM 780 confocal microscope. Both mono- and mixed cultures of *S. aureus* and *P. aeruginosa* were grown on cell imaging cover slips (Eppendorf) under static conditions for 48 h in BM broth. Next one-half of the medium was replaced by the fresh one containing antimicrobials at final concentrations as indicated and cultivation was continued for the next 24 h. The samples were then stained for 5 min with the SYTO® 9 (ThermoFisher Scientific) at final concentration of 0.02 μg/ml (green fluorescence) and propidium iodide (Sigma) at final concentration of 3 μg/ml (red fluorescence) to differentiate between viable and non-viable bacteria. To differentiate between gram-positive and gram-negative bacterial species ViaGram^{TM} Red^{+} (ThermoFisher Scientific) was used. The microscopic images were obtained with a 1-μm Z-stacks.

Evaluation of antibacterial activity

The minimum inhibitory concentration (MIC) of antimicrobials was determined by the broth microdilution method in 96-well microtiter plates (Eppendorf) according to the recommendation of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) rules for antimicrobial susceptibility testing [72]. Briefly, the 10^8 cells/mL bacterial suspension was subsequently diluted 1:300 with BM broth supplemented with various concentrations of antimicrobials in microwell plates to obtain a 3×10^5 cells/mL suspension. The

586 concentrations of antimicrobials ranged from 0.25 to 512 mg/L. Besides the usual double
587 dilutions, additional concentrations were included in between. The cultures were next incubated
588 at 35°C for 24 h. The MIC was determined as the lowest concentration of antimicrobials for
589 which no visible bacterial growth could be observed after 24 h incubation.

590 To determine the MBC of antimicrobials the CFU/mL were further evaluated in the
591 culture liquid from those wells without visible growth. 10 µl of the culture liquid from the wells
592 with no visible growth were inoculated into 3ml of LB broth followed by cultivation for 24h.
593 The MBC was determined as the lowest concentration of compound for which no visible
594 bacterial growth could be observed according to the EUCAST of the European Society of
595 Clinical Microbiology and Infectious Diseases (ESCMID) [73].

596

597 **Drop plate assay**

598 To evaluate the viability of both detached and planktonic cells, a series of 10-fold
599 dilutions of liquid culture from each well were prepared in 3 technical repeats and dropped by 5
600 µl onto LB agar plates. CFUs were counted from the two last drops typically containing 5-15
601 colonies and further averaged. To evaluate the viability of the biofilm-embedded cells, the wells
602 were washed twice with 0.9% NaCl in order to remove the non-adherent cells. The biofilms were
603 also suspended in 0.9% NaCl by scratching the well bottoms with subsequent treatment in an
604 ultrasonic bath for 2 min to facilitate the disintegration of bacterial clumps [63]. Viable cells
605 were counted by the drop plate method as described above.

606

607 **Statistical analysis**

608 Experiments were carried out in six biological repeats with newly prepated cultures and
609 medium in each of them. The fraction of non-viable cells in microscopic images was estimated
610 as the relative fraction of the red cells among all cells in the combined images obtained by

611 overlaying of the green and the red fluorescence microphotographs (10 images per each sample)
612 by using BioFilmAnalyzer software[74]. The statistical significance of the discrepancy between
613 monoculture and mixed biofilms treatment efficacy was determined using the Kruskal-Wallis
614 statistical test with significance threshold at $p < 0.05$.
615

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