J Antimicrob Chemother 2022; **77**: 3265–3269
https://doi.org/10.1093/jac/dkac311 Advance Access publication 19 September 2022

Journal of Antimicrobial Chemotherapy

Strain-to-strain variability among *Staphylococcus aureus* causing prosthetic joint infection drives heterogeneity in response to levofloxacin and rifampicin

María Ángeles Meléndez-Carmona [1], Mikel Mancheño-Losa [2]*, Albert Ruiz-Sorribas[3], Irene Muñoz-Gallego [1], Esther Viedma[1], Fernando Chaves[1], Françoise Van Bambeke [3] and Jaime Lora-Tamayo [2,4]

[1]Department of Clinical Microbiology, Hospital Universitario 12 de Octubre, Instituto de Investigación Biomédica 'i+12' Hospital 12 de Octubre, Madrid, Spain; [2]Department of Internal Medicine, Hospital Universitario 12 de Octubre, Instituto de Investigación Biomédica 'i+12' Hospital 12 de Octubre, Madrid, Spain; [3]Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium; [4]CIBER Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author. E-mail: mikel.mancheno@gmail.com

Received 16 February 2022; accepted 19 August 2022

Introduction: Levofloxacin and rifampicin are the preferred treatment for prosthetic joint infection (PJI) caused by *Staphylococcus aureus*, especially when managed with implant retention (DAIR). However, a significant variability of success has been reported, which could be related to intrinsic characteristics of the microorganism. Our aim was to evaluate the variability in the anti-biofilm response to levofloxacin and rifampicin in a clinical collection of *S. aureus*.

Material and methods: Eleven levofloxacin- and rifampicin-susceptible *S. aureus* isolates causing PJI managed with DAIR were included. Levofloxacin, rifampicin and levofloxacin + rifampicin were tested in an *in vitro* static biofilm model in microtitre plates, where 48 h biofilms were challenged with antimicrobials during 24 h. Additionally, two genetically similar strains were tested in the CDC Biofilm Reactor, where 48 h biofilms were treated during 56 h. Antimicrobial activity was assessed by viable biofilm-embedded cells recount, and by crystal violet staining.

Results: All antimicrobial regimens showed significant anti-biofilm activity, but a notable scattering in the response was observed across all strains (inter-strain coefficient of variation for levofloxacin, rifampicin and levofloxacin + rifampicin of 22.8%, 35.8% and 34.5%, respectively). This variability was tempered with the combination regimen when tested in the biofilm reactor. No correlation was observed between the minimal biofilm eradicative concentration and the antimicrobial activity. Recurrent *S. aureus* isolates exhibited higher biofilm-forming ability compared with strains from resolved infections (7.6 $\log_{10}$ cfu/cm^2±0.50 versus 9.0 $\log_{10}$ cfu±0.07).

Conclusions: Significant variability may be expected in response to levofloxacin and rifampicin among biofilm-embedded *S. aureus*. A response in the lower range, together with other factors of bad prognosis, could be responsible of treatment failure.

Introduction

Staphylococcus aureus remains a major cause of prosthetic joint infection (PJI). In this context, common antibiotic susceptibility tests do not reliably predict the antimicrobial response that is generally expected of infections caused by planktonic bacteria.[1]

Despite these difficulties, the combination of rifampicin plus a fluoroquinolone (e.g. levofloxacin) is currently the treatment of choice for acute staphylococcal PJI managed with debridement, antibiotics and implant retention (DAIR).[2] Unfortunately, the heterogeneity observed among studies addressing the efficacy of DAIR yields quite a wide range of failure rate.[3,4] While this probably reflects the diversity among studies, it may also be caused by the confluence of multiple host parameters,[5] and also other important variables related to the causative staphylococcal strain, its genotypic background and virulence intrinsic characteristics.[6,7]

Our group recently tested the activity of both rifampicin and levofloxacin against intracellular strains of *S. aureus* responsible for PJI.[8] While all antimicrobials were active, a striking heterogeneity was observed between specific strains.

To complement these findings, we conducted a study to assess variability in response to levofloxacin and rifampicin among

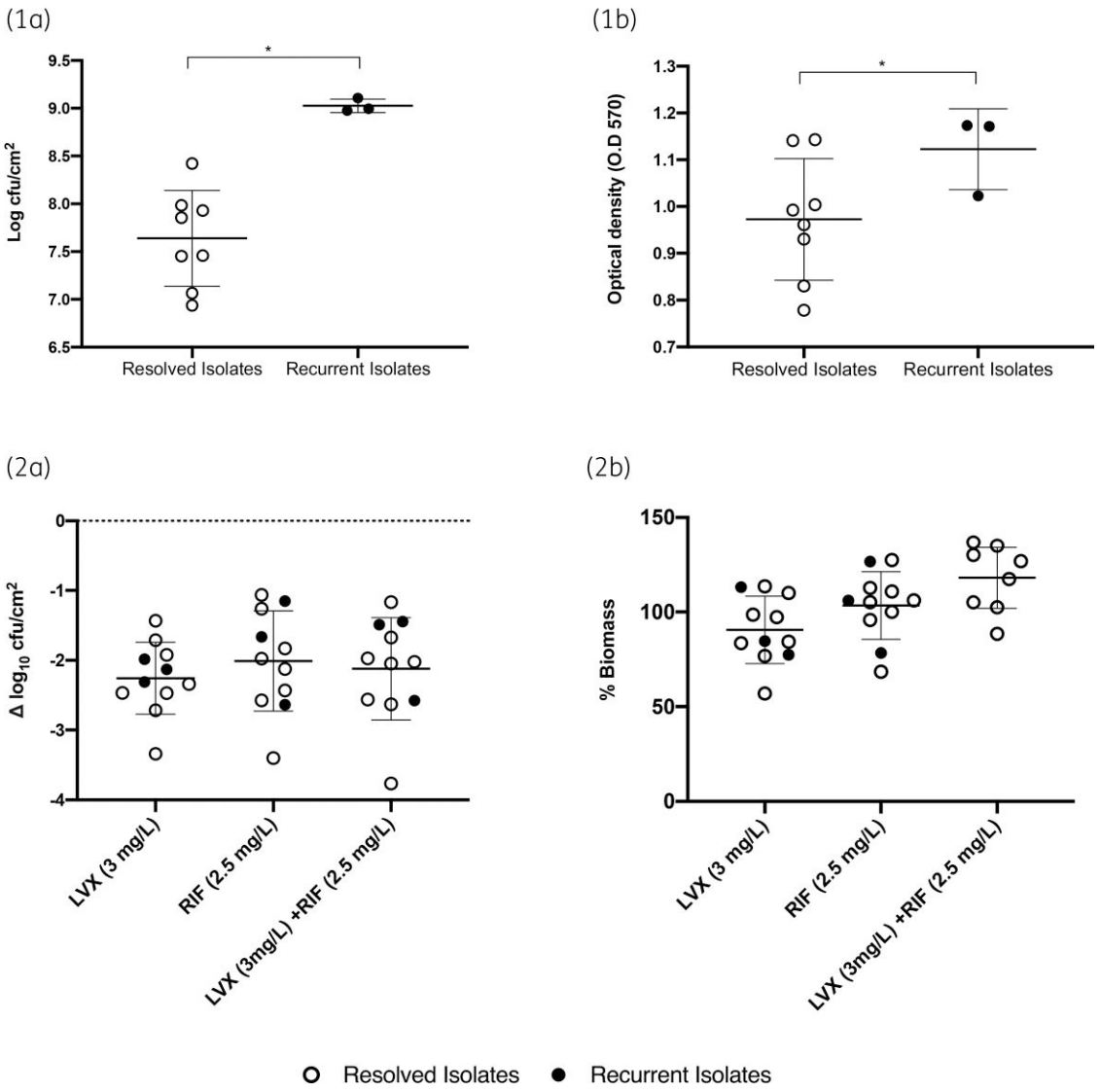


Figure 1. Biofilm-forming ability of *S. aureus* and efficacy of antimicrobials against biofilms (static model). Panel 1: biofilms were incubated during 48 h in the absence of drugs. The ordinate shows (1a) cfu counting (expressed in $\log_{10}$ cfu/cm^2) and (1b) crystal violet absorbance (measured at 570 nm). Panel 2: 48 h biofilms were incubated with levofloxacin (LVX, 3 mg/L) and rifampicin (RIF, 2.5 mg/L), alone and in combination, during 24 h. Results are expressed as (2a) the reduction of $\log_{10}$ cfu/cm^2 compared with control (Δ $\log_{10}$ cfu/cm^2). (2b) Biomass [assessed by crystal violet; expressed as the percentage of the value measured in control conditions (no antibiotics added)]. White circles indicate resolved cases, whereas black circles denote recurrent cases. Each dot represents the result of three replicates of each isolate. Bar denotes mean ± SD of the isolates grouped. Statistical analysis was performed by using unpaired Student's *t*-test and by ANOVA with Tukey's *post hoc* test per treatment. *$P < 0.05$.

a wide spread of staphylococcal strains causing PJI in two experimental models of non-intracellular staphylococcal biofilm.

Material and methods

Further details are included in the Supplementary data, available at *JAC* Online.

Bacterial isolates

The *S. aureus* isolates were obtained from a prospective multicentre study.[6] We selected 11 strains responsible for acute PJI cases managed with DAIR and rifampicin+levofloxacin (Table S1). Three cases (27%) eventually failed due to the same *S. aureus* (confirmed by PFGE) that caused the original infection (recurrent isolates), and eight were considered cured (resolved isolates) after a median follow-up of 36.5 months (IQR 13.8–17.7).

Antimicrobials and susceptibility testing

Levofloxacin and rifampicin drug powders were reconstituted following CLSI guidelines.[9] Susceptibility was measured by the Etest method following EUCAST guidelines.[10] Biofilm susceptibility was also performed by determining the minimum biofilm eradication concentrations (MBEC) and minimum biofilm inhibitory concentration (MBIC) as previously described.[1]

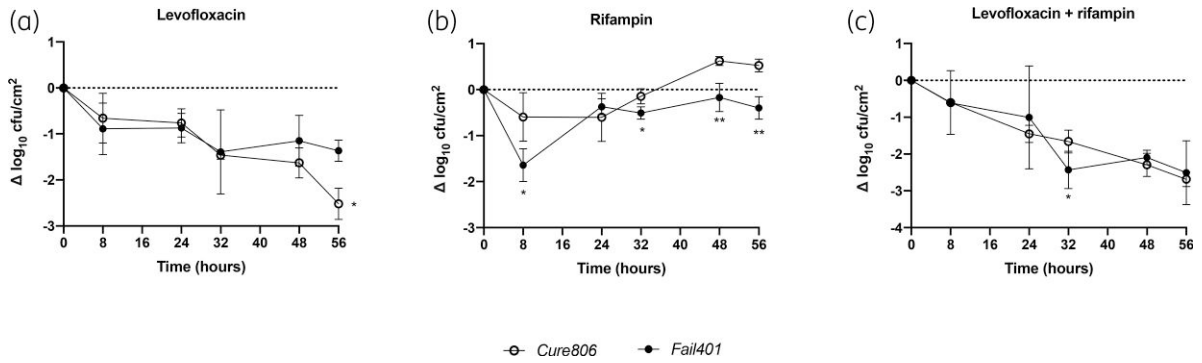


Figure 2. Bacterial killing of biofilm-embedded cells from coupons treated with monotherapies of (a) levofloxacin (LVX), (b) rifampicin (RIF), and (c) LVX-RIF combination in the CDC biofilm reactor. Experimental concentration for LVX and RIF was 3 and 2.5 mg/L, respectively. Results are expressed using the $\log_{10}$ change method from time 0 to each timepoint (mean ± SD). Biofilm bacterial densities at the beginning of the experiment were 5.4 ± 0.4 $\log_{10}$ cfu/cm^2 and 6.5 ± 0.08 $\log_{10}$ cfu/cm^2 ($P = 0.001$) for *Cure*806 and *Fail*401, respectively, and they remained stable throughout the following 56 h when no antibiotics were administered. Shapiro–Wilks test was tested for normality and the unpaired Student's *t*-test or Mann–Whitney *U*-test were used to compare differences between two groups. *$P < 0.05$; **$P < 0.01$.

Static in vitro biofilm model

Biofilms were obtained by growing a suspension of *S. aureus* for 48 h in a 12-well plate containing titanium-alloy (Ti6Al4V) disc coupons in RPMI-1640 medium supplemented with KH_2PO_4 50 mM + Na_2HPO_4 74.1 mM+1% glucose (pH 7, 37°C) as previously described (Figure S1).[11] After 48 h, mature biofilms were exposed to antibiotics for 24 h, namely levofloxacin, rifampicin, alone and in combination at concentrations that would be expected in human cortical bone at standard doses (levofloxacin, 750 mg/day; rifampicin, 600 mg/day): 3 mg/L and 2.5 mg/L, respectively (Table S2).[12] Biofilm-embedded bacteria were recovered by two alternating cycles of vortexing and sonicating (40 Hz), then serially diluted and plated to allow cfu counting after overnight incubation at 37°C. In addition, biofilm biomass was quantified using modified crystal violet staining.[11] The biomass was expressed as a percentage of the absorbance value measured under control conditions (no antibiotics added) at 570 nm.

Dynamic in vitro pharmacokinetics/pharmacodynamics (PK/PD) biofilm model

Biofilms were grown on 24 titanium-alloy disc coupons using the CDC biofilm reactor system (Biosurface Technologies Corp., Bozeman, MT, USA), as described elsewhere.[13] Briefly, biofilms were formed after a 48 h conditioning phase, which included 24 h of batch culture [TSB (Tryptic Soy Broth) with 1% glucose] followed by 24 h of continuous flow of medium (20% TSB), mixed by a stir bar at 130 rpm, and at 37°C. The therapeutic phase was then started (time 0) and antibiotics were administered as bolus every 24 h, with the same C_{max} as in the previous model. During this phase, 20% TSB was pumped at a rate that simulated the half-life of each antibiotic (Table S2). Three coupons were collected aseptically at 0, 8, 24, 32, 48 and 56 h. Biofilm-embedded bacteria were recovered as previously described.[13]

Appropriate levofloxacin and rifampicin concentrations in the reactor throughout the experiment were confirmed by the bioassay method in Difco Antibiotic Medium No. 1 (Becton Dickinson), using *Escherichia coli* ATCC 21922 and *Staphylococcus epidermidis* ATCC 27626, respectively.

The emergence of resistant strains at all timepoints was examined using agar plates with levofloxacin and rifampicin concentrations of 1 mg/L.

Statistical analysis

Statistical analysis and graphs were done using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Unpaired Student's *t*-test or Mann–Whitney *U*-test and the analysis of variance (ANOVA) with Tukey's *post hoc* test per treatment were used to compare differences between groups. *P* values of <0.05 were considered to be significant.

Results

Static biofilm model

The strains studied exhibited heterogeneous biofilm formation with interstrain variability of 9.6% (Figure 1). Recurrent *S. aureus* isolates showed biofilms with higher cell densities (9.0 ± 0.07 $\log_{10}$ cfu/cm^2 versus 7.6 ± 0.50 $\log_{10}$ cfu/cm^2; $P = 0.001$) and larger biomass (OD 1.1 ± 0.09 versus 0.97 ± 0.13; $P = 0.05$) than those originating from resolved infections.

All antimicrobial regimens led to a significant reduction in cfu, with no significant differences between them (Figure 1). By contrast, no significant reduction in biomass was observed with either regimen. Of note, a remarkable dispersion in the response to antibiotics on cfu counting was observed across all strains evaluated, with interstrain coefficients of variation for levofloxacin, rifampicin and levofloxacin + rifampicin of 22.8%, 35.8% and 34.5%, respectively (see range values, Table S3). Lower MBEC values did not correlate with a greater decrease in cfu/cm^2 (Figure S2).

Dynamic biofilm model (CDC biofilm reactor)

For this model, we chose two staphylococcal strains with a common genotypic background, (CC45, same *agr* genotype) and different outcome (failure in *Fail*401 and success in *Cure*806) (Table S1). PK validation of the model was sound (Table S2). Levofloxacin [Figure 2(a)] was more effective than control against both strains, but higher activity was observed against *Cure*806 at 56 h as compared with *Fail*401: −2.5 ± 0.3 $\log_{10}$ cfu/cm^2 versus −1.4 ± 0.2 $\log_{10}$ cfu/cm^2 ($P = 0.08$). In the case of rifampicin monotherapy [Figure 2(b)], there was initial bacterial killing in both cases, which was significantly more pronounced in the *Fail*401 isolate than the *Cure*806 isolate (at 8 h, −1.6 ± 0.4 $\log_{10}$ cfu/cm^2 versus −0.6 ± 0.5 $\log_{10}$ cfu/cm^2; $P = 0.002$) Both staphylococci showed subsequent regrowth from 24 h onwards, in parallel with the emergence

of resistance, which included 100% of the bacterial population at 48 h (Figure S3). The levofloxacin+rifampicin combination was the most effective therapy at 56 h for the *Fail*401 strain and showed an indifferent effect against *Cure*806 strain, with no major differences between the two isolates [Figure 2(c)]. No resistant strains were detected when using levofloxacin either alone or in combination with rifampicin.

Discussion

In the present study, we observed marked variability in response to antibiotics in a clinical collection of biofilm-embedded *S. aureus*. As expected, levofloxacin and rifampicin, alone and in combination, were active against these staphylococcal biofilms, but not to the same extent. The differences occurred despite the fact that the strains were susceptible to and had similar exposures to both antimicrobials. Our experiments focused on the anti-biofilm activity of antimicrobials by excluding other aspects of infections (e.g. intracellular bacteria, host immunity or host metabolomics), therefore the observed variability relied on the genotypic and phenotypic differences of the strains studied. Additionally, the inclusion of a number of strains with different genetic backgrounds reflects the variability found in the clinical setting.[14–16]

In the static model, we were able to test a number of strains by assessing the density of viable biofilm-embedded bacteria as well as overall biomass. While the differences in antimicrobial activity observed were probably reflections of different realities (viable cells versus biomass), heterogeneity of antimicrobial efficacy was still observed with both methods. In contrast, the CDC biofilm reactor is a more demanding model, in which the number of strains that can be tested is limited. However, again, significant variability in the response to rifampicin and levofloxacin monotherapies was observed. Importantly, these differences were attenuated when the strains were challenged with the two drugs in combination. This might suggest that the microbiological variability intrinsic to a given monotherapy could be neutralized by the addition of a second active antibiotic, thus ensuring reliable and reproducible bacterial killing. Determining antimicrobial susceptibility of biofilms in clinical practice remains not feasible. While MBEC and MBIC values have been proposed as alternative indices,[1] a clear correlation with the outcome of these infections has not been proved.[6] Indeed, our results do not support their utility, stressing the important differences of biofilm formation and maturity in the Calgary Device as compared with the clinical setting.[17]

The variability shown in our experiments underlines the importance of the specific infection-causing strain, together with other important aspects, such as the quality of surgical management, the adequacy of antimicrobial treatment and the host's baseline conditions. In this regard, it was interesting to note that strains from PJI cases with a poor prognosis had greater biofilm-forming ability than those from cases with a good prognosis, supporting the relevance of each bacterial strain's background for the patient's prognosis.

Our study has some limitations. First, the concentrations of antimicrobials were lower than those used elsewhere,[13] which may have affected the overall response. Nevertheless, these concentrations would be expected in bone tissue and are also the same ones we used in a previous intracellular *in vitro* model.[8] Second, the number of isolates included in the resolved group ($n=8$) is unbalanced with respect to the recurrent group ($n=3$), which may potentially lead to unstable statistical analyses. Finally, we did not carry out an analysis of virulence factors, which could account for the observed variability, nor a transcriptomic analysis of the genome of each strain when embedded in biofilm and in response to the antimicrobial stress. This could shed some light on the variability observed and should be addressed in future studies.

To conclude, our results emphasize the significant variability that may be observed in response to antimicrobials in the biofilm setting. This heterogeneity may be the consequence of specific genotypic and phenotypic features of the strain responsible for the infection beyond the standard antimicrobial susceptibility profile, and could potentially be a significant parameter of prognosis of the infection.

Acknowledgements

We are thankful to the staff of the Department of Clinical Microbiology from the Hospital Universitario 12 de Octubre for their invaluable support and dedication. We are also grateful to Janet Dowson for reviewing the English manuscript.

Funding

This work was supported by the Plan Nacional I+D+I (PI18/01623), Instituto de Salud Carlos III, Ministerio de Economía y Competitividad. M.A.M and M.M.-L. were both supported by a 'Río Hortega' research contract (CM20/00146 and CM19/00226, respectively, Instituto de Salud Carlos III). M.A.M received a Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC) mobility fellowship to support part of her work carried out in the 'Pharmacologie cellulaire et moléculaire' laboratory in Brussels. A.R.S. was funded by the Orthenzy project, Win2Wal program from 'Region Wallonne', Belgium. F.V.B. is a research director from the Belgian Fonds de la Recherche Scientifique (FRS-FNRS, grant J.0162.19).

Transparency declarations

We declare that we have no conflicts of interest.

Supplementary data

Supplementary Methods, Figures S1–S3 and Tables S1–S3 are available as Supplementary data at *JAC* Online.

References

1 Ceri H, Olson ME, Stremick C *et al.* The Calgary Biofilm Device. *J Clin Microbiol* 1999; **37**: 1771–6. https://doi.org/10.1128/JCM.37.6.1771-1776.1999

2 Osmon DR, Berbari EF, Berendt AR *et al.* Diagnosis and management of prosthetic joint infection: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis* 2013; **56**: e1–25. https://doi.org/10.1093/cid/cis966

3 Lora-Tamayo J, Euba G, Cobo J *et al.* Short- versus long-duration levofloxacin plus rifampicin for acute staphylococcal prosthetic joint infection managed with implant retention: a randomised clinical trial. *Int J Antimicrob Agents* 2016; **48**: 310–6. https://doi.org/10.1016/j.ijantimicag.2016.05.021

4 Byren I, Bejon P, Atkins BL *et al*. One hundred and twelve infected arthroplasties treated with 'DAIR' (debridement, antibiotics and implant retention): antibiotic duration and outcome. *J Antimicrob Chemother* 2009; **63**: 1264–71. https://doi.org/10.1093/jac/dkp107

5 Shohat N, Goswami K, Tan TL *et al*. 2020 Frank Stinchfield Award: identifying who will fail following irrigation and debridement for prosthetic joint infection: a machine learning-based validated tool. *Bone Joint J* 2020; **102**: 11–9. https://doi.org/10.1302/0301-620X.102B7.BJJ-2019-1628.R1

6 Muñoz-Gallego I, Viedma E, Esteban J *et al*. Genotypic and phenotypic characteristics of *Staphylococcus aureus* prosthetic joint infections: insight on pathogenesis and prognosis of a multicentre prospective cohort. *Open Forum Infect Dis* 2020; **7**: ofaa344. https://doi.org/10.1093/ofid/ofaa344

7 Tuchscherr L, Kreis CA, Hoerr V *et al*. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J Antimicrob Chemother* 2016; **71**: 438–48. https://doi.org/10.1093/jac/dkv371

8 Meléndez-Carmona MÁ, Muñoz-Gallego I, Viedma E *et al*. Intraosteoblastic activity of levofloxacin and rifampin alone and in combination against clinical isolates of meticillin-susceptible *Staphylococcus aureus* causing prosthetic joint infection. *Int J Antimicrob Agents* 2019; **54**: 356–60. https://doi.org/10.1016/j.ijantimicag.2019.06.018

9 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing—Twenty-Seventh Edition*: *M100*. 2017.

10 EUCAST. Breakpoint tables for interpretation of MICs and zone diameters-Version 12.0; 2022.

11 Ruiz-Sorribas A, Poilvache H, Kamarudin NHN *et al*. *In vitro* polymicrobial inter-kingdom three-species biofilm model: influence of hyphae on biofilm formation and bacterial physiology. *Biofouling* 2021; **37**: 481–93. https://doi.org/10.1080/08927014.2021.1919301

12 Landersdorfer CB, Bulitta JB, Kinzig M *et al*. Penetration of antibacterials into bone: pharmacokinetic, pharmacodynamic and bioanalytical considerations. *Clin Pharmacokinet* 2009; **48**: 89–124. https://doi.org/10.2165/00003088-200948020-00002

13 El Haj C, Murillo O, Ribera A *et al*. Evaluation of linezolid or trimethoprim/sulfamethoxazole in combination with rifampicin as alternative oral treatments based on an *in vitro* pharmacodynamic model of staphylococcal biofilm. *Int J Antimicrob Agents* 2018; **51**: 854–61. https://doi.org/10.1016/j.ijantimicag.2018.01.014

14 Post V, Wahl P, Uçkay I *et al*. Phenotypic and genotypic characterisation of *Staphylococcus aureus* causing musculoskeletal infections. *Int J Med Microbiol* 2014; **304**: 565–76. https://doi.org/10.1016/j.ijmm.2014.03.003

15 Luedicke C, Slickers P, Ehricht R *et al*. Molecular fingerprinting of *Staphylococcus aureus* from bone and joint infections. *Eur J Clin Microbiol Infect Dis* 2010; **29**: 457–63. https://doi.org/10.1007/s10096-010-0884-4

16 Muñoz-Gallego I, Lora-Tamayo J, Pérez-Montarelo D *et al*. Influence of molecular characteristics in the prognosis of methicillin-resistant *Staphylococcus aureus* prosthetic joint infections: beyond the species and the antibiogram. *Infection* 2017; **45**: 533–7. https://doi.org/10.1007/s15010-017-1011-6

17 Coenye T, Goeres D, Van Bambeke F *et al*. Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clin Microbiol Infect* 2018; **24**: 570–2. https://doi.org/10.1016/j.cmi.2018.01.003

Supplementary Material and Method.

Bacterial isolates

The *S. aureus* isolates causing PJI were obtained from a prospective multicentre study (Muñoz-Gallego I, *et al*. *Open Forum Infect Dis* 2020; **7**:ofaa344). We selected 11 *S. aureus* isolates exposed to similar antimicrobial and surgical management (**table 1)**. All were acute PJI cases (either early post-surgical or hematogenous) managed with DAIR (debridement <21 days after onset of symptoms) and their treatment included RIF (600 mg once daily) plus LVX (750 mg once daily). Three cases (27%) eventually failed due to the same *S. aureus* that caused the original infection (recurrent isolates), and eight were considered cured (resolved isolates) after a median follow-up of 36.5 months (IQR 13.8-17.7). The genotypic background of the strains (*agr* functionality and clonal complex) had been determined in a previous study (Muñoz-Gallego I, *et al*. *Open Forum Infect Dis* 2020; **7**:ofaa344). The *agr* operon activity was explored phenotypically by assessing the delta-haemolysin production (Traber KE, *et al*. *Microbiology* 2008; **154**: 2265-2274). DNA microarrays allowed the assignment of isolates to clonal groups (*S. aureus* Genotyping Kit 2.0, Alere, Jena, Germany). All isolates were stored in cryovials at -80ºC. Prior to the experiments, each isolate was subcultured onto tryptic soy agar (TSA, BioMérieux, Spain) plates and incubated at 37ºC for 24h. Three colonies were then selected and grown overnight in 10 mL of tryptic soy broth (TSB, Sigma Aldrich, Spain), from which early log-phase growth was obtained for two hours reaching a bacterial growth of 10^8 colony forming units (cfu)/mL.

Susceptibility testing

Susceptibility to oxacillin, LVX and RIF was measured by the Etest method following EUCAST guidelines (EUCAST. Breakpoint tables for interpretation of MICs and zone diameters- Version 12.0.2022). Biofilm susceptibility to LVX and RIF was also performed by determining the minimum biofilm eradication concentrations (MBEC) and minimum biofilm inhibitory concentration (MBIC) with the MBEC assay (Calgary Biofilm Device; Innovotech, Edmonton, AB, Canada), as previously described (Ceri H, *et al*. *J Clin Microbiol* 1999; **37**:

1771-6) (**table 1**).

Antimicrobial agents

LVX and RIF were obtained from Sigma-Aldrich (Madrid, Spain). Drug powders were reconstituted following CLSI guidelines, using sterile distilled water for LVX and methanol for RIF (CLSI. M100 2017). All stock solutions of each antibiotic were prepared prior to experiments and stored at -20ºC.

Static *in vitro* biofilm model

Biofilms were obtained by growing a suspension of *S. aureus* for 48h in a 12-well plate containing titanium disc coupons (Titanium Alloy (Ti-6AL-4V ELI) Disc Coupon) in RPMI 1640 medium supplemented with KH_2PO_4 50 mM + Na_2HPO_4 74.1 mM + 1% Glucose (Sigma, St Louis, Mo) at pH 7 and 37ºC (Ruiz-Sorribas A, *et al*. *Biofouling* 2021; **37**: 481-93) **(Supplementary Figure 1).** The medium was refreshed every 24 hours. No spontaneous bacterial killing was observed at 72h (data not shown). After 48h, the medium was removed, and mature biofilms were exposed to antibiotics for 24 hours. Antimicrobial regimens included LVX, RIF, alone and in combination at concentrations that would be expected in human cortical bone ($C_{cortical}$) at standard doses of LVX (750 mg once daily) and RIF (600 mg once daily): 3 mg/L and 2.5 mg/L, respectively (**Supplementary Table 1**) (Landersdorfer CB, *et al*. *Clin Pharmacokinet* 2009;**48**: 89-124). Bacterial viability in the biofilm was measured by cfu count at 48h (as control) and 24h after exposure to antibiotics. For this, the titanium coupons were rinsed with phosphate buffered saline (PBS: NaCl 137mM, KCl 2.7 mM, Na_2HPO_4 8mM, KH_2PO_4 1.5 mM) at pH 7.4, then subjected to two alternating cycles of 5 min sonication (40 KHz; Branson 3510 Ultrasonic bath) each separated by 1min of vortexing in 2 mL PBS. Serial dilutions were then plated on tryptic soy agar (Ph.Eur., USP, JP, VWR Chemicals BDH) to allow cfu counting after overnight incubation at 37ºC. Bacterial counts were expressed as $\log_{10}$ cfu/cm^2 (the biofilm-growing surface area on one side and edge of each coupon totalled 1.57 cm^2). The $\log_{10}$ change method was used to evaluate the efficacy of antimicrobials ($\Delta \log_{10}$ cfu/cm^2 = $\log_{10}$ cfu/cm^2 antibiotic - $\log_{10}$ cfu/cm^2 control).

In addition, biofilm biomass was quantified using modified crystal violet staining (Ruiz-Sorribas A, *et al*. *Biofouling* 2021; **37**: 481-93). Once the coupons were removed from the wells and rinsed, they were fixed at 60º C for 24h and stained with crystal violet solution at 0.5% (V/V, final concentration 115 mg/L) in water. The crystal violet fixed to the biofilm was then solubilized by adding 66% glacial acetic acid and incubated at room temperature for 1 h. Coupons were removed from the wells and absorbance was read at 570 nm. The biomass assessed by crystal violet staining was expressed as a percentage of the absorbance value measured under control conditions (no antibiotics added). Both cfu counts and CV staining were evaluated against each staphylococcal isolate in three independent experiments.

Dynamic *in vitro* PK/PD biofilm model

For this model we selected one resolved PJI *S. aureus* isolate (*Cure806*) and one failed PJI *S. aureus* isolate (*Fail401*) with similar clinical, microbiological, and genotypic characteristics (**Table 1**). Biofilms were grown on 24 titanium disc coupons (Titanium Alloy [Ti-6AL-4V ELI] Disc Coupon) using the CDC biofilm reactor (CBR) system (Biosurface Technologies Corp., Bozeman, MT, USA), as described elsewhere (Goeres DM, *et al. Microbiology* (Reading). 2005; **151**: 757–762). Briefly, this is a glass vessel with an effective volume of 337.5 mL, connected to a 20-L carboy containing sterile media and to a waste discharge vessel. Biofilm was formed after a 48-h conditioning phase, which included 24h of batch culture at 37ºC in TSB supplemented with 1% glucose, followed by 24h of continuous flow of medium (20% TSB) pumped by a peristaltic pump (MasterFlex L/S Digital Dispensing Pump Drives, Cole-Parmer Instrument Co, USA). The infusion rate was set at 9.96 mL/min for the *Fail*401 isolate and 13.18 mL/min for the *Cure*806 isolate to ensure that the bacterial residence time inside the reactor was shorter than the generation time for the suspended bacteria (33.8 min and 25.6 min, respectively) and so to select for biofilm growth on the coupons. The broth was maintained in the reactor at 37ºC and continuously mixed by a magnetic stir bar spinning at 130 rpm to produce constant shear forces. After the conditioning phase, the therapeutic phase was started (time 0) and antibiotics were administered as bolus every 24 hours, aiming to achieve the same

C_{max} as in the previous model. During this phase, 20% TSB was pumped at a rate that simulated the half-life of each antibiotic ($t_{1/2}$) (**Supplementary Table 1**). Given the differences in $t_{1/2}$, when the two antibiotics were administered in combination, supplementary peristaltic pumps and compartments were added to the system, as described elsewhere (Blaser J, *et al*. *J Antimicrob Chemother* 1985; **15**: 125–30).

PK validation

Appropriate LVX and RIF concentrations in the reactor throughout the experiment were confirmed by the bioassay method in Difco Antibiotic Medium No. 1 (Becton Dickinson), using *Escherichia coli* ATCC 21922 and *Staphylococcus epidermidis* ATCC 27626, respectively. The Elimination $t_{1/2}$ and peak cortical concentrations were determined by GraphPad Prism (**Supplementary Table 1)**.

PD analysis

Three coupons were collected aseptically during the treatment phase at 0, 8, 24, 32, 48, and 56h. Coupons were rinsed twice in sterile saline to remove planktonic bacteria and each one was then placed in 10 mL of sterile saline (0.9% NaCl). Biofilm-embedded bacteria were recovered by three alternating 1 min cycles of vortexing and sonication (100W- 40 KHz; LT-100 PRO; Tierratech, Cantabria, España) and a final 1 min of vortexing. The samples were serially diluted and plated onto tryptic soy agar (TSA) with 5% of sheep blood and incubated at 37ºC for 24h. To prevent antimicrobial carryover from the sample, plating was done in a single streak down the centre of the plate and was allowed to absorb into the agar before spreading the inoculum. Bacterial counts were expressed as $\log_{10}$ cfu/cm^2 (biofilm-growing surface area on both sides of each coupon totalled 2.53 cm^2). The lower limit of detection of the bacterial colony count was 39.5 cfu/cm^2. The log change method was used to evaluate the efficacy of the antimicrobials from hour 0 to each time point ($\Delta \log_{10}$ cfu/cm^2 = $\log_{10}$ cfu/cm^2 at time t – $\log_{10}$ cfu/cm^2 at time 0).

Emergence of resistance

The emergence of resistant strains at all time points was examined using agar plates with LVX and RIF concentrations of 1 mg/L.

Statistical analysis

Statistical analysis and graphs were done using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). The results were tested for normality using the Shapiro-Wilks test. Log_{10} cfu/cm^2 changes in biofilm-embedded bacteria from coupons were evaluated by analysis of variance (ANOVA) with Tukey's post-hoc test per treatment. The unpaired Student's t-test or Mann-Whitney U-test were used to compare differences between two groups. The coefficient of variation (the ratio of the standard deviation to the mean) was used to express measurement variability. Spearman's rho coefficient measured correlations between antimicrobial efficacy and MBEC. All tests were two-tailed. *P* values of <0.05 were considered to be significant.

Supplementary Table 1. Clinical and microbiological characteristics of the cases of *Staphylococcus aureus* joint infection included in this study[a].

Strain code	Sex, age (years)	Prosthesis	Type of infection[c]	Duration of antimicrobial treatment (days)[d]	Outcome[e]	Levofloxacin susceptibility (mg/L)			Rifampin susceptibility (mg/L)			CC[f]	*agr* functionality[g]
						MIC	MBIC	MBEC	MIC	MBIC	MBEC		
Fail104	F, 63	Knee	EPI	60	Failure (recurrent)	0.25	≤0.5	>256	0.012	≤0.5	1	15	Positive
Fail401[b]	F, 65	Knee	EPI	144	Failure (recurrent)	0.125	≤0.5	>256	0.008	≤0.5	32	45	Positive
Fail402	M, 81	Knee	AHI	78	Failure (recurrent)	0.125	≤0.5	256	0.008	≤0.5	256	5	Positive
Cure111	M, 69	Knee	EPI	82	Resolved	0.25	≤0.5	≤0,5	0.008	≤0.5	4	30	Positive
Cure112	F, 83	Knee	AHI	90	Resolved	0.19	≤0.5	256	0.012	≤0.5	8	6	Negative
Cure116	F, 62	Knee	EPI	79	Resolved	0.19	≤0.5	256	0.012	≤0.5	8	5	Positive
Cure208	M, 53	Hip	EPI	93	Resolved	0.19	≤0.5	256	0.006	≤0.5	256	509	Negative
Cure804	F, 84	Knee	AHI	89	Resolved	0.125	≤0.5	>256	0.008	≤0.5	64	10	Positive
Cure806[b]	M, 37	Knee	EPI	81	Resolved	0.125	≤0.5	>256	0.012	≤0.5	256	45	Positive
Cure807	F, 63	Knee	EPI	57	Resolved	0.125	≤0.5	>256	0.016	≤0.5	32	45	Positive
Cure808	F, 72	Knee	EPI	100	Resolved	0.19	≤0.5	>256	0.012	≤0.5	16	1	Positive

M, male; F, female; MIC: minimal inhibitory concentration; MBIC: Minimum biofilm inhibitory concentration; MBEC: Minimum biofilm eradication concentration; CC: clonal complex.

[a]All isolates were susceptible to methicillin (MSSA).

[b]Information of strains used for the dynamic biofilm model.

[c]Type of infection; EPI: Early Postoperative Infection; AHI: Acute Haematogenous Infection.

[d]Duration of antimicrobial therapy was considered since the performance of debridement until antimicrobial withdrawal (for patients cured) or until salvage surgical treatment (for cases with recurrent infection).

[e]Failure was considered in patients needing salvage therapy (surgical and/or medical) due to the same *S. aureus* causing the original infection.

[f] DNA microarrays allowed the assignment of isolates to clonal groups (*S. aureus* Genotyping Kit 2.0, Alere, Jena, Germany).

[g]*agr* functionality: presence of δ-haemolysin (synergistic haemolysis within the β-haemolysin zone).

Supplementary Table 2. Pharmacokinetic parameters of levofloxacin and rifampin used in the CDC biofilm reactor.

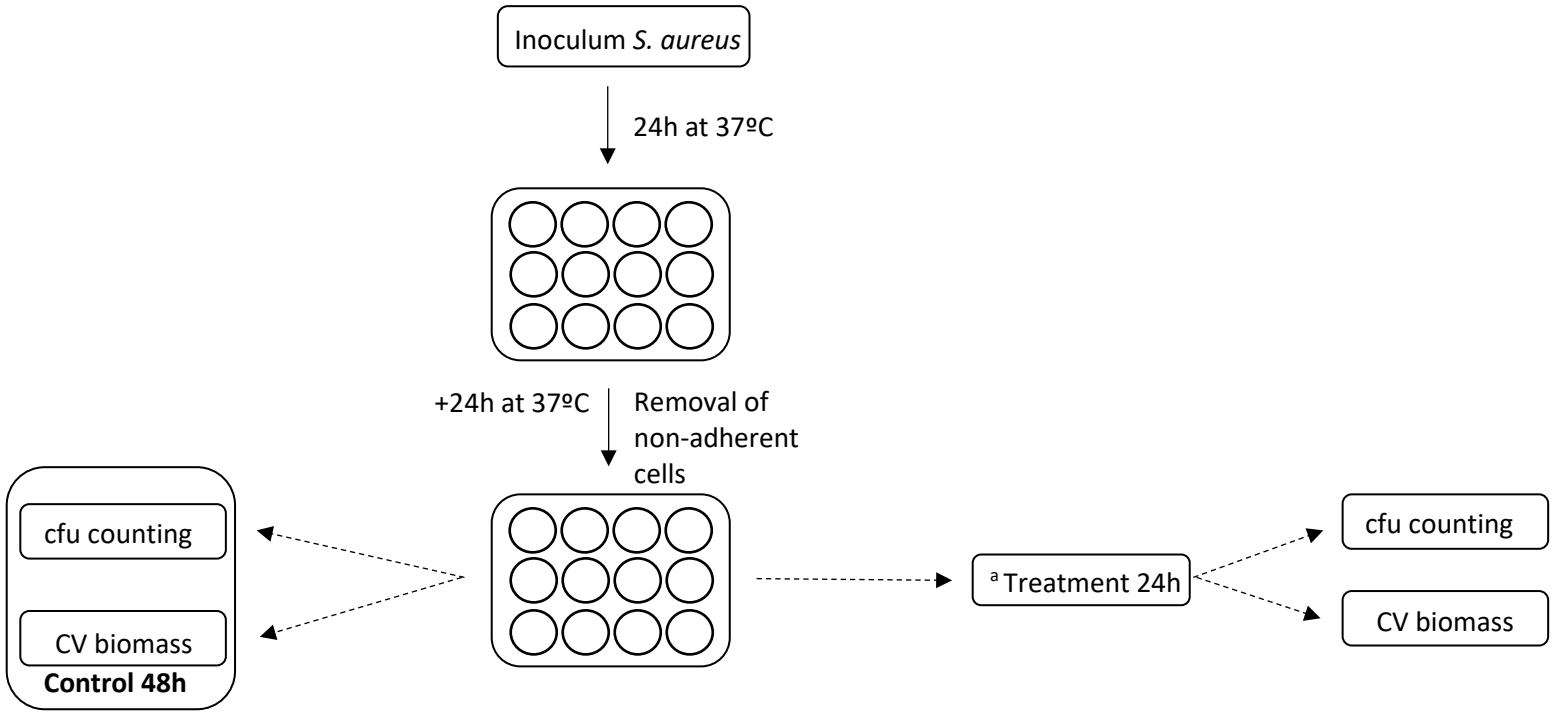
Antimicrobial agent	Daily Dosage [a] (mg)	C_{max} [b] (mg/L)	Half-life ($t_{1/2}$) (h)			Bone Penetrance (%)	Cortical bone concentrations ($C_{cortical}$)		
			Theorical[c] $t_{1/2}$	Experimental $t_{1/2}$	Error [f] (%)		Expected $fC_{cortical}$[d] (mg/L)	Experimental $fC_{cortical}$ [e] (mg/L)	Error [f] (%)
Levofloxacin	750	8	7.0	6.8	2.2	37	3	3.3	8.7
Rifampin	600	12.5	5.0	4.4	12.6	20	2.5	2.8	11.3

[a] Standard therapeutic dosage of administration to humans for staphylococcal prosthetic joint infection; [b] C_{max} in serum after administration of the antimicrobial (Rimmelé T, et al., Antimicrob Chemother. 2004; **53**:553) (Grayson ML, et al., Kucers´s the use of antibiotics. 7th ed. Boca Ratón, 2017); [c] Theorical $t_{1/2}$, half-life [44]; [d] Expected free antibiotic concentration taken into account the dosage (levofloxacin 750 mg/24h; rifampin 600 mg/24h) (Goeres DM, et al., Microbiology (Reading). 2005;**151**:757). [e] Free antibiotic concentration of antibiotics reached in CDC biofilm reactor. [f] Percentage of error respect to the PK value target (7h and 3 mg/L for LVX and 5h 2.5 mg/L for RIF). All PK values had an error less than 20%. Experimental $fC_{cortical}$ for LVX-RIF combination: 3.0 mg/L (error: 1.6%) and 2.4 mg/L (error: 3.3%) for levofloxacin and rifampin, respectively and Experimental $t_{1/2}$: 7.4h (error: 5.1%) and 4.4 (error:11.8%), respectively.

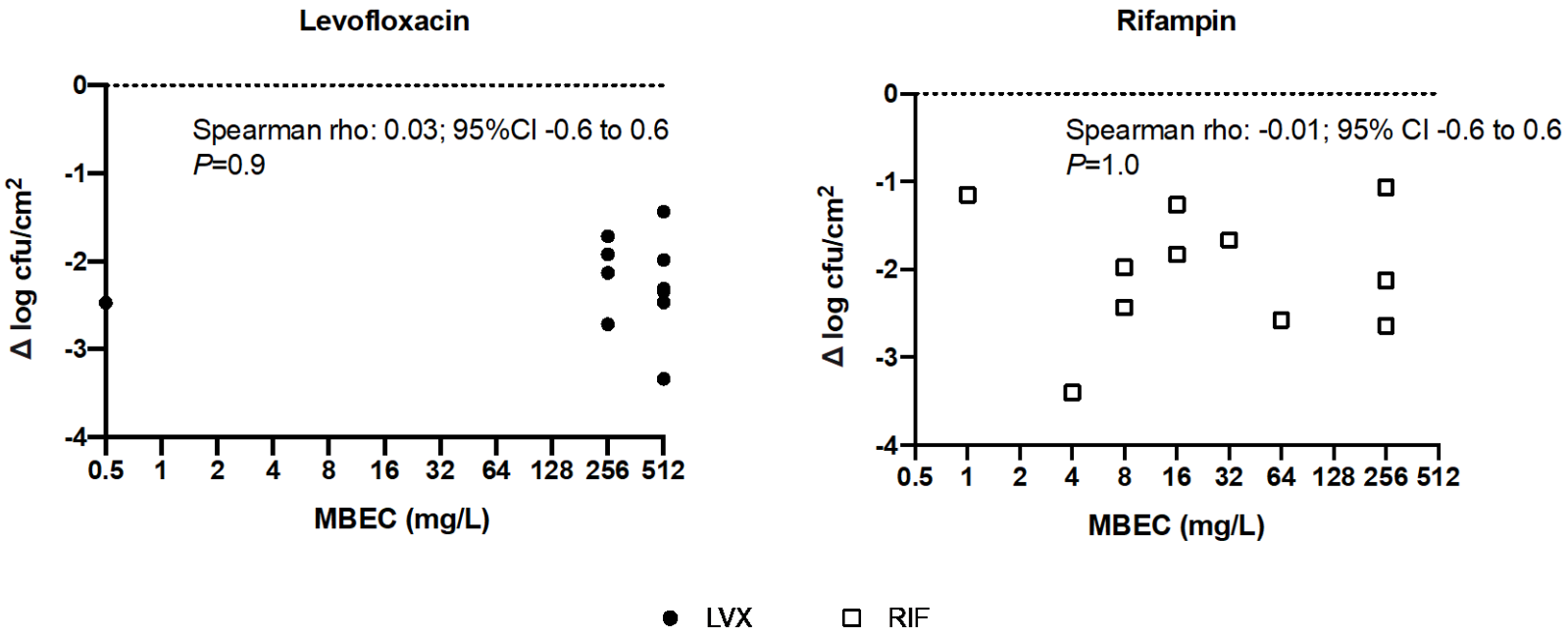
Supplementary Table 3. Biofilm bacterial density (cfu) and biomass (CV staining) values and effect of antimicrobials in the static *in vitro* biofilm model. Results are expressed as median and range. $\Delta Log_{10}cfu/cm^2$: variation of the number of colony forming units (cfu) of treated and untreated biofilms; % Residual biomass: ratio optical density (OD) of the treated biofilm / OD of the untreated biofilm.

	Total (N=11)	**Resolved (N=8)**	**Failures (N=3)**	***P***
Biofilm-forming ability				
Log_{10} cfu/cm^2	7.9 (6.9 to 9.1)	7.7 (6.9 to 8.4)	9.0 (9.0 to 9.1)	0.001*
Biomass CV (O.D)	1.0 (0.8 to 1.2)	1.0 (0.8 to 1.1)	1.2 (1.0 to 1.2)	0.05*
Levofloxacin (LVX)				
ΔLog_{10} cfu/cm^2	-2.3 (-3.3 to -1.4)	-2.4 (-3.3 to -1.4)	-2.1 (-2.3 to -2.0)	0.7
% Residual biomass	84.7 (56.9 to 113.5)	90.8 (56.9 to 113.5)	84.7 (77.4 to 113.1)	0.9
Rifampin (RIF)				
ΔLog_{10} cfu/cm^2	-2.0 (-3.4 to -1.1)	-2.1 (-3.4 to -1.1)	-1.7 (-2.6 to -1.2)	0.6
% Residual biomass	106.0 (68.5 to 127.4)	105.7 (68.5 to 127.4)	106 (78.3 to 126.6)	1.0
LVX+RIF				
ΔLog_{10} cfu/cm^2	-2.0 (-3.8 to -1.2)	-2.0 (-3.8 to -1.2)	-1.5 (-2.6 to -1.4)	0.5
% Residual biomass	117.4 (88.4 to 136.8)	122.1 (88.4 to 136.8)	117.0 (105.0 to 134.0)	0.9

Supplementary Figure 1. Scheme of the biofilm static model. [a] Treatment included: LVX at 3 mg/L, RIF at 2.5 mg/L and the combination of LVX and RIF at 3 mg/L and 2.5 mg/L, respectively.



Supplementary Figure 2. Correlation of minimal biofilm eradication concentration (MBEC) for levofloxacin (LVX) and rifampin (RIF) with the reduction of colony forming units (Δcfu/cm^2) of treated biofilms compared to control set to zero using the static *in vitro* biofilm model. Spearman´s rho= 0.03 (95% CI: -0.6 to 0.6) for MBEC versus LVX; *P*= 0.9 and Spearman´s rho= -0.01 (95%CI: -0.6 to 0.6) for MBEC versus RIF; *P*= 1.0.



Supplementary Figure 3. Emergence of resistance to rifampin (RIF) among biofilm-embedded *S. aureus* when treated with rifampin monotherapy and its combination using the *in vitro* dynamic model. Results are expressed as the absolute number of bacteria recovered from coupons (log_{10} cfu/cm^2). Data are presented as mean ± SD. Control: number of cfu/cm^2 in absence of antibiotics; RIF 2.5: number of cfu/cm^2 under treatment with RIF at 2.5 mg/L; RIF 2.5 resistance: number of resistant cfu/cm^2 under treatment with RIF 2.5; LVX 3 + RIF 2.5: number of cfu/cm^2 under treatment with RIF at 2.5 mg/L plus levofloxacin (LVX) at 3 mg/L; LVX 3 +RIF 2.5 resistance: number of resistant cfu/cm^2 under treatment with LVX 3 + RIF 2.5.