Antibiotics at Clinical Concentrations Show Limited Effectivity Against Acute and Chronic Intracellular *S. aureus* Infections in Osteocytes

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23 Running head: ABX Treatment of Intra-osteocytic *S. aureus* Infections

24 **Keywords:** Intracellular *S. aureus* infection, chronic osteomyelitis, intracellular antibiotic
25 treatment

Abstract

Case numbers of osteomyelitis are rising and chronic infections remain difficult to cure. While it is known that the major pathogen *Staphylococcus aureus* can persist intracellularly in osteocytes, the effectivity of antibiotics against intracellular *S. aureus* in human osteocytes remains largely unknown. Rifampicin, vancomycin, levofloxacin, ofloxacin, amoxicillin, oxacillin, doxycycline, linezolid, gentamicin and tigecycline were assessed for their MIC and minimum bacterial concentrations (MBC) against 11 *S. aureus* clinical isolates and the reference strain ATCC 25923, at pH 5.0 and 7.2 to mimic lysosomal and cytoplasmic environments, respectively. Five antibiotics reached clinically relevant concentrations above their respective MIC and were tested in osteocyte infection models. Osteocytes were treated at 1, 4 and 10x the MIC for 1 and 7 days immediately following infection (acute model), or after 14 days of infection (chronic model). The effectivity of each antibiotic on intracellular bacteria was measured in terms of colony forming unit (CFU) reduction, small colony variant (SCV) formation and specific bacterial mRNA expression change. Only rifampicin, levofloxacin and linezolid reduced intracellular CFU numbers significantly in the acute model. The effect was larger after 7 days compared to 1 day of treatment. However, no treatment reduced the quantity of bacterial mRNA, nor prevented non-culturable bacteria from returning to a culturable state. This indicates that *S. aureus* adapts phenotypically during intracellular infections of osteocyte-like cells, adopting a reversible quiescent state and is protected against antibiotics even at 10x MIC. Thus, new therapeutic approaches are necessary to cure *S. aureus* intracellular infections in osteocytes.

48 # Introduction

49 Osteomyelitis caused by pathogenic bacteria is a rare and often difficult to treat infection of
50 bone tissue. These infections can present in different contexts, such as periprosthetic joint
51 infection, diabetic foot infection (DFI) and fracture related infection, and are therefore strongly
52 associated with co-morbidities, such as diabetes, rheumatoid arthritis and trauma (1). Since
53 the prevalence of these co-morbidities is increasing, so is the incidence of osteomyelitis cases
54 (2, 3). Especially challenging are recurrent and chronic infections, which can reappear years
55 after the initial infection. They make up approximately 40% of osteomyelitis cases (4) and have
56 a high treatment failure rate, up to 37% (5, 6).

57 The main causative pathogens of osteomyelitis are Staphylococci, of which *Staphylococcus*
58 *aureus* is both the most common and problematic. In periprosthetic joint infection, up to 50%
59 of osteomyelitis is caused by methicillin-resistant *S. aureus* (MRSA) and *S. aureus* has the
60 highest treatment failure rate in periprosthetic joint infection, up to 4 times higher than the next
61 most common pathogen, *Staphylococcus epidermidis* (7, 8). A major reason for the high
62 treatment failure rate of osteomyelitis caused by *S. aureus* is the adaptability of the pathogen.
63 On the one hand, *S. aureus* can survive in reservoirs, such as in abscesses, biofilms and the
64 osteocyte lacunocanalicular network, while on the other hand, the pathogen can establish
65 intracellular infections in a number of immune and bone cell types, including osteocytes (9).
66 In intracellular infections, two thirds of available antibiotics have been reported to be ineffective
67 (10). This could be caused by a sub-therapeutic antibiotic concentration reaching the bacteria
68 due to low drug penetration into the host cell or the failure to accumulate in the same
69 compartment in which bacteria reside. *S. aureus* can persist intracellularly in phagosomes,
70 with or without lysosomal fusion, or in the cytoplasm (11, 12). The effectivity of antibiotics can
71 change intracellularly due to enzymes affecting the drug and a low intracellular pH, which is
72 on average 0.3 pH units lower in the cytoplasm than extracellularly (pH 7.0-7.4) and as low as
73 pH 4.5 in lysosomes (13, 14), making intracellular effectivity difficult to predict (15).

74 *S. aureus* can adapt phenotypically by forming alternative growth phenotypes, such as small
75 colony variants (SCV) and persister cells, especially in biofilms and intracellularly (16-19).
76 Chronic osteomyelitis is associated with increased SCV formation (8, 20), which has been
77 observed intracellularly in bone cells *ex vivo* and *in vitro* (16, 21). Furthermore, it has been
78 shown that this phenotype displays adaptations resulting in higher antibiotic tolerance and
79 immune protection that are thought to promote chronic infections (15, 17, 22, 23). Antibiotic
80 tolerance is defined as the ability of a bacterial population to survive but not replicate during
81 antibiotic treatment, in contrast to resistance, where a bacterial population can replicate in the
82 presence of an antibiotic (24). Another phenotypic adaptation in chronic infections is that of
83 persister cells, which are a subpopulation of dormant bacteria with reduced metabolism,
84 rendering them impervious to most current antibiotics (18, 25). Thus, to treat chronic
85 osteomyelitis effectively, both the intracellular location and phenotypic adaptation of the
86 causative pathogen are potential factors to consider.

87 Only a few studies have reported intracellular antibiotic effectivity in bone cells, the majority of
88 these being in osteoblasts (26). The treatment of intracellular *S. aureus* infection in osteocytes
89 is yet to be thoroughly investigated. Osteocytes form a syncytial network throughout the bone
90 and are the most abundant and long-lived resident population (27), theoretically providing an
91 ideal niche for long-term infections. Intracellular infections of osteocytes have been reported
92 *in vivo* (28), *ex vivo* and *in vitro* (16, 29). Furthermore, intracellular colonies of *S. aureus* were
93 isolated up to at least 15 days post-infection in osteocyte-like cells and showed evidence of
94 phenotypic adaption into SCV (16, 29), indicating chronic intracellular persistence of *S. aureus*
95 in osteocytes.

96 In this study, we investigated the effectivity of clinically relevant antibiotics at concentrations
97 predicted to be achievable in the bone in an intracellular osteocyte model. Additionally, both
98 acute and chronic infections of *S. aureus* were modelled, as well as short and long-term
99 antibiotic treatments. The antibiotics rifampicin, vancomycin, levofloxacin, ofloxacin,
100 amoxicillin, oxacillin, doxycycline, linezolid, gentamicin and tigecycline were chosen,

representing different mechanisms of action, levels of evidence and reported effectivity in previous studies, as recently reviewed (26). Furthermore, we tested multiple osteomyelitis-related clinical isolates of *S. aureus* to take into consideration possible phenotypic adaptations, such as SCV, which might influence the bacterial susceptibility towards antibiotics and clinical outcome.

Results

MIC and Minimal Bactericidal Concentration (MBC)

To determine which of the chosen antibiotics could satisfy the dosage criteria of being both clinically relevant and achievable in the bone, we determined the MIC and MBC for rifampicin, vancomycin, levofloxacin, ofloxacin, amoxicillin, oxacillin, doxycycline, linezolid, gentamicin and tigecycline against 11 clinical isolates of *S. aureus* from DFI patients and the ATCC 25923 reference strain. As *S. aureus* can persist intracellularly in phagolysosomes, as well as the cytosol, we also tested the MIC and MBC for each antibiotic at both pH 5.0 and 7.2. The results are summarised in **Table 1**; clinical isolates 5 and 10 did not grow at pH 5.0, hence no MIC or MBC could be determined under these conditions.

Compared to the ATCC 25923 strain, all clinical isolates were similarly susceptible to vancomycin, linezolid, gentamicin and tigecycline. However, five strains (clinical isolates 2-5,10) were resistant to levofloxacin, ofloxacin and oxacillin (30). The MBC for some strains was at least 4-fold higher than the MIC for rifampicin, oxacillin, doxycycline, linezolid and tigecycline, indicating a large bacteriostatic range under the culture conditions used. Some antibiotics showed pH dependency, with levofloxacin, ofloxacin, doxycycline and tigecycline being at least 4-times more effective at pH 7.2 than at pH 5.0 and rifampicin, oxacillin and gentamicin were more effective at pH 5.0 than pH 7.2, which is consistent with previous studies (26). There were strong variations in the antibiotic susceptibility of the bacterial strains: clinical isolates 1, 6-9, and the ATCC reference strain were susceptible to most antibiotics, while isolates 2-5, 10 and 11 were resistant to at least 3 antibiotics.

The MIC and MBC values for each strain were compared to the known concentrations of the antibiotics that can be achieved in the bone (26, 31-35). Only antibiotics that could reach a potentially therapeutic concentration in at least 6 strains (i.e. >4x MIC (36)), namely doxycycline, oxacillin, levofloxacin, rifampicin and linezolid, were further tested in an

131 intracellular infection model. Our capacity to test amoxicillin further was limited, as while this
132 can reach 27.8-37.4 mg/l in bone (31, 34), we only had access to the trihydrate and not the
133 more soluble amoxicillin-sodium form, limiting testing up to 2 mg/l, which was lower than the
134 MIC for some strains at pH 7.2 (**Table 1**).

135

136 Infected Host Cell Survival

137 For infection experiments, clinical isolate 8 was chosen, since it was relatively susceptible to
138 most antibiotics in an extracellular environment. To ensure that neither the bacterial infection
139 itself, nor the antibiotic treatments affected host cell viability, we conducted live/dead staining
140 at each time point and measured the *18S* rRNA expression. There was no significant change
141 in host cell *18S* rRNA expression due to the infection or any treatment, as shown for
142 levofloxacin as a representative example of all treatments at any time point (**Supp. Fig. 1A**).
143 No difference in viability of the host cells between the infected and uninfected controls or any
144 time point within the assay were observed (**Supp. Fig. 1B**).

145

146 Intracellular Antibacterial Activity

147 Next, we tested the five candidate antibiotics (doxycycline, oxacillin, levofloxacin, rifampicin
148 and linezolid) for intracellular antibacterial activity at 1, 4 and 10x their respective MIC. The
149 treatment period was either 1 day or 7 days and started either immediately (acute model) or
150 14 days (chronic model) after the infection. As an outcome, we measured the numbers of
151 colony forming units (CFU), the proportion of SCV, and mRNA expression of bacterial genes.
152 A schematic for the infection models is depicted in **Figure 1**.

153 *Acute Infection Model*

154 To evaluate the effectivity of treatments given immediately during an acute infection, we first
155 determined the effect on CFU reduction, caused by the different antibiotics and antibiotic
156 concentration levels.

157 For all experiments there was a natural decline in CFU recovery between 1- and 7-days post-
158 infection (**Fig. 2**), consistent with phenotypic loss of culturability. Beyond this, doxycycline had
159 no effect on the number of intracellular CFU at any dosage after either 1 or 7 days of treatment
160 (**Fig. 2A, Table 2**). Even though oxacillin caused a significant CFU reduction after 1 day of
161 treatment at all concentrations, the reduction was less than 1-log (**Fig. 2B, Table 2**), which
162 would not be clinically useful. After 7 days of treatment with oxacillin, no significant effect was
163 observed, indicating that oxacillin most likely does not reduce intracellular *S. aureus* number
164 to a clinically relevant extent. Similarly, levofloxacin was ineffective at all concentrations after
165 1 day of treatment. However, 7 days of levofloxacin treatment at all concentrations resulted in
166 a significant and clinically relevant intracellular CFU-reduction of up to 2.98-log (**Fig. 2C, Table**
167 **2**), indicating that levofloxacin might be an effective treatment, when given for a prolonged
168 period. Rifampicin caused the highest significant dose-dependent CFU reduction after 1 day
169 of treatment of up to 2.28-log (**Fig. 2D, Table 2**), which increased up to 3.32-log after 7 days
170 of treatment. The highest significant CFU reduction of 3.74-log was achieved after 7 days of
171 treatment with linezolid, however, linezolid had a significant but smaller impact after 1 day of
172 treatment of 1.54 log (**Fig. 2E, Table 2**). Rifampicin and linezolid therefore seemed to be able
173 to reduce intracellular *S. aureus* CFU immediately after 1 day, but had an increased impact
174 after 7 days, making them the most potent candidate treatments to reduce CFU numbers
175 during an acute infection.

176 When evaluating the impact of antibiotic treatments on bacterial mRNA levels, we did not find
177 concordance with CFU recovery. Even at the highest antibacterial concentration used for 7
178 days of treatment no antibiotic treatment reduced intracellular levels of *aroE* or *gyrB* mRNA
179 compared to the untreated control (**Fig. 2F**).

180 Next, we analysed the phenotypes of the recovered colonies. Overall, the proportion of SCV
181 (%SCV) increased from 3.01% ± 2.06% at the day of the infection to 7.22% ± 3.29% after 7
182 days in the untreated controls (*p = 0.0012*) (**Supp. Fig 2A**). Doxycycline, oxacillin and linezolid
183 did not significantly impact the %SCV at any concentration after 1 or 7 days of treatment (**Fig.**
184 **3A, B and E**). However, levofloxacin caused a dose-dependent increase in the %SCV with up
185 to 28.21% ± 15.67% at day 7, which was significantly higher than the untreated control (*p =*
186 *0.0007*) (**Fig. 3C**). Rifampicin, in contrast, significantly reduced the proportion of SCV to 1.73%
187 ± 2.44% after 7 days of treatment at 1x MIC (*p = 0.0197*) (**Fig. 3D**). For rifampicin at 4x MIC
188 and 10x MIC no effect could be quantified since most wells did not grow any colonies. Example
189 images of WT and SCV are shown in **Figure 3F**. The formation of SCV has been shown to be
190 associated with an increased expression of *sigB* or *asp23* (37, 38). After 7 days of treatment,
191 there was a non-significant and less than 2-fold change in the expression of *sigB* or *asp23*
192 and no change caused by any treatment (**Supp. Fig 2B-C**), showing no evident relationship
193 between colony morphology and gene expression in this experiment.

194 *Chronic Infection Model*

195 Following 14 days of infection, free of antimicrobial treatment, cells were treated for a further
196 1 or 7 days at 1, 4 and 10x MIC with doxycycline (**Fig. 4A**), oxacillin (**Fig. 4B**), levofloxacin
197 (**Fig. 4C**), rifampicin (**Fig. 4D**) or linezolid (**Fig. 4E**) as in the acute model. CFU numbers and
198 mRNA expression were evaluated as in the acute model.

199 Only very low CFU numbers, if any, were recovered, so that the change in CFU number and
200 %SCV could not statistically analysed. The initial bacterial intracellular bacterial number in the
201 doxycycline and levofloxacin experiments (see **Fig. 2A, C**) were higher than in the oxacillin,
202 levofloxacin and linezolid experiments, which might contribute to the overall higher CFU
203 numbers evident in the chronic infection model (**Fig. 4A, C**). For oxacillin (**Fig. 4B**), rifampicin
204 (**Fig. 4D**) and linezolid (**Fig. 4E**), less than 100 colonies were recovered after 1 day and no
205 colonies (with the exception of one well for oxacillin) were cultured after 7 days.

206 Interestingly, bacterial mRNA expression was similar to levels in the acute model and did not
207 significantly change with any treatment throughout the time course, as shown in **Figure 4F**.
208 Since the mRNA levels were similar to the acute model, the reduction in CFU might not be
209 due to a bacterial decline but a switch to a non-culturable phenotype.

210

211 ## Bacterial Resuscitation

212 To further test if antibiotic-treated, chronically infected cells harboured viable bacteria, a
213 resuscitation assay was used. Differentiated SaOS-2 osteocyte-like cells harbouring quiescent
214 *S. aureus* were treated with each antibiotic at 10x MIC in a multi-well assay plate. Following
215 removal of the antibiotic and any lysostaphin in the media, plates were monitored for bacterial
216 outgrowth. As shown in **Figure 5,** bacterial resuscitation occurred in all cases. The infected
217 control (**Fig. 5A**), and cells treated with oxacillin (**Fig. 5C**) and linezolid (**Fig. 5F**) had a similar
218 outgrowth of ~50% after 7 days and >80% after 11 days. Levofloxacin treated cells (**Fig. 5D**)
219 showed a similar behaviour after 7 days but had a lower proportion of outgrowth after 11 days.
220 Cells that were exposed to doxycycline (**Fig. 5B**), showed an overall lower outgrowth at day 5
221 and 11, while rifampicin (**Fig. 5E**) only produced outgrowth at day 11. It is noteworthy that
222 most wells that had culturable bacteria in the lysate also had culturable bacteria in the
223 supernatant, except for rifampicin-treated wells, where culturable bacteria were only
224 recovered from the host cell lysate.

225 In addition to the observation of outgrowth, bacterial DNA levels were measured. Compared
226 to the DNA levels at a culturable state immediately after infection, the DNA levels decreased
227 significantly for all conditions between 2-3-logs when the bacteria were non-culturable at day
228 1. However, this change was smaller than the change in CFU numbers and larger than the
229 mRNA changes in the chronic model (see **Fig. 4**). DNA levels increased significantly with the
230 increase in culturability for all conditions (**Fig. 5A-D**) with the exception of rifampicin (**Fig. 5E**).
231 The ability to regain a culturable phenotype after treatment and persistence of high levels of

232 mRNA expression confirms that the antibiotics failed to kill intracellular bacteria in this model
233 and instead may have promoted a switch to a non-culturable but metabolically active
234 phenotype.

235

236 # Discussion

237 The treatment of chronic and recurrent bone infection is becoming increasingly challenging
238 with a rise in case numbers and a high proportion of treatment failure. One pathway for this
239 failure is intracellularly persisting bacteria, especially within osteocytes, which are the most
240 abundant, longest lived and arguably the most immune privileged bone cell type. Therefore,
241 the effectivity of antibiotics used at clinically relevant concentrations against intracellular *S.*
242 *aureus,* the most common pathogen responsible of causing osteomyelitis was investigated in
243 the context of osteocyte infections.

244 The 10 antibiotics tested represent a variety of classes and mechanisms of action. These,
245 include protein biosynthesis inhibitors, such as the oxazolidinone linezolid, the tetracycline
246 doxycycline, the glycylcycline tigecycline and the aminoglycoside gentamicin, enzyme
247 inhibitors such as the fluoroquinolones ofloxacin and levofloxacin and the ansamycine
248 rifampicin, and cell wall inhibitors, such as the beta lactams amoxicillin and oxacillin and the
249 glycopeptide vancomycin. All of these antibiotics are currently used clinically for the treatment
250 of osteomyelitis (26).

251 Rifampicin is one of the most demonstrably effective antibiotics against intracellular *S. aureus*
252 (26). MICs for rifampicin have been reported between 0.0005-0.016 mg/l (32, 39-44), being
253 even lower at pH 5.0 (39-42), and represent a large potential therapeutic window as up to 5-
254 6.5 mg/l that can be achieved in the bone (31, 32, 35). We found an MIC range between 0.005-
255 0.032 mg/l at pH 7.2 and 0.00125-0.008 at pH 5, with a 2-8-fold higher sensitivity at pH 5.0
256 compared to pH 7.2. In the osteocyte infection model, rifampicin reduced the intracellular CFU
257 number after 24h, with up to a 2.28-log reduction, and up to a 3.7-log reduction after 7 days
258 of treatment. Rifampicin was also the only drug to significantly reduce SCV appearance. It is
259 remarkable that this effect was achieved with the low concentration range of 0.016-0.16 mg/l,
260 while other studies used 2-32 mg/l and often only found a 1-log reduction after 24h (40, 42-
261 45). Furthermore, it was the only drug treatment that did not yield culturable bacteria in the
262 media after antibiotic removal and yielded continuously low relative bacterial DNA quantities.

263 Linezolid was the only protein biosynthesis inhibitor tested that was effective in reducing
264 intracellular bacterial culturability at a clinically relevant concentration, in particular after a
265 longer treatment duration of 7 days. The MICs were between 1-4 mg/l, similar to other studies
266 (32, 39, 40, 43, 46, 47), with little pH dependency, however the MBCs were up to 64-fold
267 higher than the MIC. After 24h of treatment the maximal reduction of 1.54-log was similar to
268 most reported findings (40, 43, 46, 48-51), though the 7 day effectivity was considerably
269 improved, with up to a 3.74-log reduction at 40 mg/l and 2.87 at 16 mg/l. These levels of
270 effectivity are greater than that reported in a primary osteoblast model at 20 mg/l (21) and was
271 the greatest reduction achieved after 7 days of treatment in this study. Valour *et al.*, however,
272 reported an effectivity of up to 3-log after 1 day of treatment in MG-63 osteoblasts (32). Our
273 study suggests that linezolid might be effective to reduce CFU when it is given for a longer
274 duration, and appears to impact the growth phenotype, as persister cells readily reverted and
275 escaped after antibiotic removal.

276 Even though the fluoroquinolone ofloxacin showed a similar MIC of 0.5 mg/l against the
277 ATCC25923 strain to that found for the three clinical isolates tested, six clinical isolates were
278 resistant to this antibiotic according to EUCAST (30), which is consistent with other reports
279 (32, 39, 52, 53). Furthermore, susceptible strains showed an approximately 10-fold reduced
280 susceptibility at pH 5.0, resulting in a higher MIC than the 3 mg/l achievable in bone (32).
281 Therefore, ofloxacin was not investigated further in the osteocyte model. However, Valour *et*
282 *al*. found ofloxacin to be very effective (3-5-log reduction) after 24h against an *S. aureus* strain
283 with an MIC also of 0.5 mg/l (32). The newer fluoroquinolone, levofloxacin, similarly showed a
284 lower susceptibility of 10-fold at pH 5.0 compared to pH 7.2 and cross-resistance to ofloxacin
285 in our study, which is consistent with other studies (39, 40, 54). However, when susceptible,
286 the MIC was between 0.5-5 mg/l at both pH levels tested, a range that easily falls below the
287 maximum concentration of 10 mg/l achievable in bone (31). In the osteocyte model, the CFU
288 number after 24h of levofloxacin treatment was reduced up to 1-log, similar to previous reports
289 (39, 40, 54), but this was reduced further, up to 2.98-log, after 7 days of treatment. Still,

290 levofloxacin failed to reduce the bacterial mRNA levels at any stage and showed a significant
291 dose-dependent increase in SCV emergence after 7 days of treatment. Therefore,
292 fluoroquinolones might be effective in reducing intracellular CFU numbers if the bacterial strain
293 is non-resistant, however, they also might induce phenotypic switches to less antibiotic and
294 immune-susceptible phenotypes and should therefore only be used after careful
295 consideration.

296 Doxycycline showed a strongly strain-dependent MIC between <0.03125–0.5 mg/l and 3
297 strains had a MIC of 8 mg/l or higher, which were classified as resistant (30). There was only
298 a slight pH dependency. Since the maximum bone concentration is reportedly up to 3 mg/l,
299 we tested doxycycline effectivity in the osteocyte infection model. However, it did not prove to
300 be effective, with less than a 1-log reduction in CFU and no effect on mRNA expression of the
301 bacterial genes tested.

302 The three cell wall inhibitors (oxacillin, amoxicillin and vancomycin) tested were found to be
303 ineffective in reducing intracellular bacterial numbers. Oxacillin had low MICs of 0.03125-0.25
304 mg/l for most strains and was much more effective at pH 5.0 than at pH 7.2 (up to 16-fold),
305 which is consistent with other studies (39, 40, 55) and much lower than the maximum bone
306 concentration of 4-10 mg/l (31, 32). However, in this study the outcomes were similar at all
307 concentrations tested (0.625-2.5 mg/l) after 1 day and 7 days with a less than 1-log CFU
308 reduction. This is comparable to findings reported by Barcia-Macay *et al.*, who found a similar
309 reduction at 0.125 and 1.25 mg/l but only up to a 2-log reduction at 63 mg/l in a THP-1
310 macrophage model (40). In contrast, others found less than a 1-log CFU reduction at 10 and
311 64 mg/l (43, 55). Only Valour *et al.* found an intracellular CFU reduction between 1.7 and 4.9-
312 log at higher concentrations of 3.3-30 mg/l (32). Overall, we conclude that at clinically-relevant
313 concentrations, oxacillin is most likely ineffective against intracellular *S. aureus.* Gentamicin
314 was used as a standard control antibiotic and is often described as only being effective against
315 extracellular pathogens, and is therefore used to clear extracellular bacteria in intracellular
316 infection assays (56). However, the cell:plasma ratio measured in macrophages between 4.4

317 and 6.8 is comparable to other antibiotics that have intracellular activity, such as linezolid and
318 oxacillin (40, 57). Similarly to previous studies, we observed a 2- to 8-fold reduced effectivity
319 of gentamicin at pH 5.0 compared to pH 7.2 (40, 41, 57), which might contribute to the low
320 intracellular activity of 1-3-log reported in bone cells (21, 45, 58-60), when given at
321 concentrations that exceed those that can be expected in the bone due to poor bone
322 penetration (31). Therefore, gentamicin does not seem to be a suitable candidate to treat
323 chronic osteomyelitis.

324 Tigecycline showed a consistently low MIC of 0.125 mg/l, which is comparable to other
325 studies, as well as reduced effectivity at pH 5.0 of up to 8-fold (32, 39, 43). It was not tested
326 further because the MIC at pH 5 and the MBC were higher than the achievable bone
327 concentration of 0.3 mg/l (32). Two studies found only a less than 1-log reduction in CFU at 1
328 and 10 mg/l, which is higher than the maximal concentration within bone, indicating a low
329 potential for the treatment of bone infections. In contrast, tigecycline used at 0.3 mg/l led to an
330 intracellular bacterial reduction of 3.9-log in MG-63 osteoblasts (32), indicating that despite
331 low bone penetration, this antibiotic might be effective through mechanisms such as
332 intracellular accumulation. In spite of this, the relatively low therapeutic window of 0.125 mg/l
333 MIC and maximum concentration of 0.3 mg/l makes the potential effectivity of tigecycline
334 doubtful.

335 Vancomycin showed low variation in MIC due to strain and pH differences, with all values
336 falling between 0.5-2 mg/l, comparable to other studies (32, 39-41, 43). Since the maximum
337 bone concentration is estimated to be between 1.1-6 mg/l (31, 32, 35), the antibiotic was not
338 tested further in the osteocyte model. Other studies found either no change or a maximum of
339 1-log reduction in CFU at concentrations up to 50 mg/l even after 7 days of treatment (21, 32,
340 40, 43), providing strong evidence that even when supra-clinical concentrations of vancomycin
341 are used that vastly exceed the concentration that can be achieved in the bone, the antibiotic
342 remains ineffective against intracellular *S. aureus*.

343 Of five antibiotics tested here, only two had a significant impact on SCV induction, with
344 levofloxacin increasing and rifampicin decreasing the %SCV. Rifampicin has been previously
345 reported to induce the appearance of SCV (42, 44), however this has not always been the
346 case (21, 32). The impact of levofloxacin treatment across different studies was inconsistent
347 with either no effect or a decrease in prevalence of SCV (42, 61). However, the strain used
348 herein throughout the intracellular infection experiments showed overall a low induction of
349 SCV compared to other strains, which might have influenced the outcome. This implies that
350 the emergence of SCV in response to an antibiotic may be both strain and host cell context-
351 dependent rather than an intrinsic property of the antibiotic in question. The low SCV formation
352 overall during the experiment is most likely the cause for the lack of upregulation of *asp23* and
353 *sigB*, which have been regularly reported during chronic intracellular persistence of *S. aureus*
354 that involve an increase in SCV prevalence (16, 37, 38, 62).

355 Furthermore, this study highlights an important limitation of the utility of currently available
356 antibiotics, in that while culturability may be inhibited, intracellular bacterial viability and activity
357 may be unaffected, an observation that was especially evident in the delayed treatment model,
358 and may be able to regain culturability as shown in the outgrowth experiment. In the
359 resuscitation model, it is noteworthy that the first culturable bacteria were detected several
360 days after the removal of the antibiotics. Most cultures started to show bacteria in the media
361 after 7 days following antibiotic removal, with the exception of oxacillin, which displayed
362 outgrowth within 4 days of its removal, and rifampicin-treated cultures, which only acquired
363 intracellular culturable bacteria within the time frame of the experiment. The observation of
364 persisting high DNA counts with a decrease in culturability, up to a non-culturable state during
365 to antibiotic treatment with rifampicin and vancomycin has been recently been published (63).
366 This is further evidence that phenotype switching to low- or non-culturable phenotypes is an
367 important and emerging aspect of intracellular infections (16, 29, 64) and additional efforts
368 should ideally be employed to monitor this.

369 Limitations of this study, include the limited number of antibiotics tested and the mono-
370 therapeutic approach. Clinically utilised antibiotics were selected to represent the various
371 known mechanisms of action and included those that were concluded to be most likely
372 effective in a recent systematic review (26). Future investigations of additional antibiotics and
373 clinically-relevant combinations should be considered. Further, even though the MIC/MBC
374 were tested in multiple clinical isolates of *S. aureus*, for feasibility purposes, the intracellular
375 infection assay was only conducted with one clinical isolate. However, this isolate, an actual
376 clinical osteomyelitis strain, brings further clinical relevance to the findings. The purpose of the
377 model utilised here was for the high-throughput testing of antimicrobial effectivity against
378 intracellular *S. aureus* in a human osteocyte-like context. To achieve reproducibility, we used
379 an osteocyte-like cell line model rather than primary cells, which might behave differently.
380 However, osteogenically differentiated SaOS-2 is a validated human osteocyte cell line model
381 for infection experiments, shown to possess key properties of primary cells (29, 65, 66).

382 In conclusion, we show in a human osteocyte-like cell culture model, the most effective
383 antibiotics at clinically relevant dosages in reducing intracellular CFU were rifampicin,
384 levofloxacin, and linezolid. Furthermore, we show that treatment duration has a significant
385 impact on the effectivity of these antibiotics, which should be considered in further studies.
386 However, this study also demonstrated that treatments that seem to be effective in reducing
387 bacterial CFU numbers, might not necessarily eradicate the bacteria, as bacteria may simply
388 switch to a non-culturable state and regrow once antibiotic pressure is removed.

389

390 Material and Methods

391 Osteocyte-like Culture

392 All chemicals were purchased from Sigma-Aldrich (St Louis, USA), unless stated otherwise.
393 SaOS-2 ATCC ® HTB-85™ (obtained from the American Type Culture Collection, Manassas,

394 USA) were passaged in alpha-modification of minimum essential medium (αMEM)
395 supplemented with 10% v/v foetal calf serum (FCS; Thermo-Fisher Scientific, Scoresby,
396 Australia), 50 mg/l ascorbate 2-phosphate and standard tissue culture additives (10 mM
397 HEPES, 2 mM L-Glutamine, 1 unit/ml penicillin/streptomycin each, Thermo-Fisher Scientific)
398 at 37°C/ 5% CO_2/ 21% O_2.Cells were differentiated to an osteocyte-like stage as previously
399 described (66). Briefly, cells were cultured in 48 well plates until confluence. Then the FCS
400 concentration was reduced to 5% v/v, 1.8 mM potassium di-hydrogen phosphate (AJAY
401 Chemicals, Auburn, Australia) was added, and the cells were incubated at 37°C/ 5% CO_2/ 1%
402 O_2 (Galaxy 170 R/ New Brunswick/ Eppendorf). Cells were cultured for up to 14 days with bi-
403 weekly media change, with the last media change being into antibiotic-free media. To ensure
404 that an osteocyte-like state was achieved, the expression of the osteocyte-marker genes
405 *Col1a1*, *RunX2*, *MEPE*, *BGLAP, DMP1* and *SOST* were measured after 7 and 14 d of
406 differentiation. All osteocyte marker gene expression increased significantly over time. The
407 mineralisation was confirmed via Alizarin Red and von Kossa staining (**Supp. Fig. 3)** (16, 66-
408 69).

409 Bacterial Culture and Growth Conditions

410 Eleven *S. aureus* clinical isolates were utilised that have been taken from patient's wound or
411 bone undergoing treatment for DFI and the methicillin susceptible *S. aureus* (MSSA) ATCC
412 25923 (obtained from the American Type Culture Collection). Bacteria were grown in terrific
413 broth (TB; Invitrogen, Waltham, USA) on a 37°C/ 200 rpm rocking platform, pelleted at 20,000
414 ×g for 5 min and resuspended in phosphate buffered saline (PBS). The viable bacterial
415 concentration for each experiment was estimated from a CFU/ml *vs.* OD_{630} nm standard curve,
416 then validated by plating dilutions on nutrient broth (NB; 10 g/l Peptone, 5 g/l sodium chloride,
417 3 g/l beef extract; Chem-supply, Gillman, Australia)-agar.

418 ## MIC/MBC Assay

419 The MIC for tigecycline, linezolid, oxacillin sodium salt monohydrate, levofloxacin, ofloxacin
420 (all Glentham Life Science, Corsham, UK), doxycycline hydrate, vancomycin, amoxicillin and
421 rifampicin were determined with the microdilution method in TB at pH 5.0 (adjusted with HCl)
422 and pH 7.2 (70). For MBC evaluation, all clear wells as well as the first well showing turbidity
423 (as a positive control) were spot plated in triplicate and colony growth was observed after 24
424 and 48h. Each experiment was at least repeated once to ensure biological replication.
425 European committee on antimicrobial susceptibility testing (EUCAST) tables were used to
426 define resistance, however, TB was used instead of Mueller Hinton broth (30).

427 ## Intracellular Antibacterial Activity

428 Clinical isolate 8 was grown, as described above. The required number of bacteria for a
429 multiplicity of infection (MOI) of 50 was diluted into PBS and validated by plating dilutions onto
430 NB agar. Intracellular infection was conducted, essentially as previously described (16, 29,
431 63). Briefly, culture medium was removed, and the differentiated SaOS-2 cells were exposed
432 to the bacterial suspension or sterile PBS (uninfected control) for 2 h. Following washing with
433 PBS, all cells were re-incubated with 10 mg/l lysostaphin for 2 h in antibiotic-free media, to
434 lyse extracellular bacteria. Then the cells were treated with antibiotics at 1x, 4x, 10x MIC (i.e.,
435 0.25, 1, 2.5 mg/l doxycycline-hydrate, 0.016, 0.064, 0.16 mg/l rifampicin, 0.0625, 0.25, 0.625
436 mg/l oxacillin, 1, 4, 10 mg/l levofloxacin and 2, 8, 20 mg/l linezolid) immediately or after 14
437 days. The infected-untreated, infected-treated cells, as well as uninfected controls were
438 harvested after 1, 7, 15 and 21 days. Infected-untreated and uninfected controls were
439 additionally harvested immediately after the extracellular clearance and after 14 days. The
440 antibiotic-free media of the cells was changed weekly with the addition of 10 mg/l lysostaphin
441 throughout the experiment. Each condition was conducted in at least triplicate. To test for
442 outgrowth after antibiotic treatment in the chronic model, the 10x MIC treatment was removed
443 and the cells washed with PBS, before changing to an antibiotic- and lysostaphin-free media.

444 Media were spot plated regularly and observed for changes in colour and cloudiness daily.
445 After 11 days, cells were washed and lysed to identify any intracellular CFU that did not grow
446 into the media.

447 Quantification of Intracellular Bacteria *via* CFU Counting

448 At each time point, culture supernatants were tested for viable bacteria by spot-plating. The
449 media were then removed, and the host cells lysed in sterile water for 20 min. Cell lysates
450 were 1:10 serially diluted and plated on NB agar. Plates were evaluated after 24h and 48 h to
451 quantify colony number and phenotype. Colonies that 1) only appeared after 48h or were
452 smaller after 24h and did not increase in size compared to 48h like the wild type (WT) 2) had
453 a white colour were defined as SCV **(Fig. 3F).**

454 Measurement of Host Cell and Bacterial Gene Expression

455 Cell supernatant was removed, and RNA isolated using 300 µl TRI Reagent (Life
456 Technologies, Grand Island, USA), combining 2 wells/replicate. After DNAse digestion,
457 complementary DNA was synthesised with the the iScript gDNA clear cDNASynthesis kit
458 (BioRad, Hercules, USA), all as per manufacturer's instructions. DNA was extracted with the
459 Viagen DirectPCR® DNA Extraction System™ and 1% proteinase K (Australian Biosearch,
460 Wangara, Australia) for 10min at room temperature, 30min at 55°C and 15min at 85°C. The
461 DNA was then digested with NcoI-HF (New England BioLabs, Notting Hill, Australia). Real-
462 time PCR reactions were then performed to determine the mRNA levels of target genes or
463 DNA levels using RT2 Forget-Me-Not™ EvaGreen qPCR Mastermix (Biotium, Assay Matrix,
464 Melbourne, Australia) on a CFX Connect Real Time PCR System (BioRad). Measurement of
465 effects on bacterial housekeeping gene levels in response to a 10x MIC dose of each antibiotic
466 was made against infected but untreated control or the corresponding 1x MIC treatment.
467 Oligonucleotide primer sequences were used as previously published (16, 66, 68).

468 ## Live/Dead staining

469 Cells were cultured, as described above, on Cell Imaging Plates (Eppendorf, Germany).
470 Immediately before the infection or 1, 7, 15 and 21 days after the infection the media of infected
471 and uninfected cells were incubated in the dark at 37°C/ 5% CO_2/ 1% O_2 with eBioscience™
472 Calcein Violet 450 AM Viability Dye (Invitrogen) and Ethidium Homodimer III (Biotium) for 30
473 min. Confocal images were taken with an Olympus FV3000 confocal microscope (Olympus,
474 Tokyo, Japan) and processed with ImageJ software (Bethesda, USA) to obtain relative
475 intensities of live/dead stained cells.

476 ## Statistics

477 Each experiment was conducted with a minimum of biological triplicates. For statistical
478 analysis and graphical visualisation of the data GraphPad Prism version 9.0 (GraphPad
479 Software, La Jolla, USA) was used. Differences in CFU, gene expression and proportion of
480 SCV were analysed with either a 1- or 2-way ANOVA with a Tuckey's multiple comparison or
481 an unpaired t -Test ($\alpha = 0.05$). Graphs show mean values with SD.

482 ## Acknowledgments

483 This work was supported by funding from the National Health and Medical Research Council
484 of Australia (NHMRC; Grant No. 2011042) awarded to G.J.A. and D.Y.. A.R.Z. was supported
485 by a University of Adelaide Faculty of Health and Medical Sciences Postgraduate Research
486 Scholarship. The authors wish to thank Mr. James Lee and Professor Robert Fitridge (The
487 University of Adelaide) for providing the clinical *S. aureus* isolates for this study.

488

489 ## Disclosures

490 None of the authors has any conflicts of interest, financial or otherwise, to disclose.

491

492 ## Author Contributions

493 ARZ project conception, performed experiments, manuscript draft

494 DY, NJG, KR, SPK experimental support, manuscript development

495 LBG, RN manuscript development

496 GJA, project conception, manuscript development

497

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740 # Figure legends

741 **Figure 1:** Schematic of the experimental design of the osteocyte infection and antibiotic
742 treatment model. A) Differentiation of SaOS-2 osteoblasts into human osteocyte-like cells. B)
743 Infection with *S. aureus*, removal of extracellular bacteria with lysostaphin. C) Antibiotic
744 treatment (blue line) either immediately after infection (acute model) or after 14 days of
745 infection (yellow line) for 1 day or 7 days with 1x,4x,10x MIC, which were concentrations
746 achievable in bone.

747

748 **Figure 2:** Effects of antibiotics in a model of acute intracellular *S. aureus* infection in
749 osteocytes. Osteocyte-like SaOS-2 cells were infected with S. aureus and immediately treated
750 with antibiotics at 1x, 4x, 10x MIC for 1 day or 7 days (n ≥ 3). A-E) CFU count per well for each
751 antibiotic treatment and infected control after 1 and 7 days of treatment. F) Relative mRNA
752 expression of housekeeping genes (*aroE* and *gyrB*) after 7 days of treatment at 10x MIC for
753 each antibiotic compared to infected control cultures (CTRL). Significant differences to the
754 infected controls are indicated by *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

755

756 **Figure 3:** Effects of antibiotics on colony phenotype of *S. aureus* recovered from an
757 intracellular infection in osteocytes. The proportion of small colony variants (%SCV) from host
758 cell lysates was determined after 1 day and 7 days of antibiotic treatments at 1x, 4x and 10x
759 their respective MIC ($n \geq 3$). A-E) %SCV emergence for the infected control and all antibiotic
760 treatments. F) Example images of SCV determination (indicated with yellow arrow): Non-
761 pigmented colonies that appeared only after 48h or were smaller after 24h and did not increase
762 in size compared to 48h wild type (WT) colonies were defined as SCV. Significant differences
763 to the infected controls are indicated by *$p < 0.05$ and ***$p < 0.0001$.

764

765 **Figure 4:** Effects of antibiotics in a model of chronic intracellular S. aureus infection in
766 osteocytes. Osteocyte-like SaOS-2 cells were infected with S. aureus and cultured for 14 days.
767 Following this, cells were treated with antibiotics at 1x, 4x, 10x MIC for a further 1 or 7 days (n
768 ≥ 3). A-E) CFU count from cell lysates per well for each antibiotic treatment and infected control
769 after 1 and 7 days of treatment. An absence of colonies after a 48h incubation was recorded
770 as 'No CFU'. F) Relative mRNA expression of housekeeping genes (*aroE* and *gyrB*) after 7
771 days of treatment at 10x MIC for each antibiotic compared to infected control cultures (CTRL).
772 No significant differences between treatments and non-treated infected controls were found.

773

774 **Figure 5:** Outgrowth of intracellular *S. aureus* from a non-culturable state. SaOS-2 osteocyte-
775 like cells were infected for 14 days with *S. aureus* to a non-culturable stage and then treated
776 for 3 days with each antibiotic at their respective 10x MIC (*n* = 30 wells per condition).
777 Following washing and replenishment with antibiotic-free media, cultures were visually
778 inspected and assayed daily for up to 11 days for bacterial re-growth into media, with data
779 plotted as percentage of wells with outgrowth (right axis, blue bars). At the day of infection, 1
780 and 11 days post-antibiotic treatment, replicate culture lysates were also used to prepare DNA
781 for PCR analysis (left axis: purple dots).

782

783 **Supplementary Figure 1:** Survival of host cells during intracellular infection. A) Levels of *18s*
784 rRNA over time in infected control, uninfected control or treatment with levofloxacin (as a
785 representative example for all treatments; *n* = 4). B) Live/dead staining of infected and
786 uninfected controls using Calcein Violet 450 AM Viability Dye (live) and Ethidium Homodimer
787 III (dead) and imaging using confocal microscopy for up to 21 days post-infection. Data shown
788 as means ± standard error of the mean for 4 biological replicates, in at least 3 regions of
789 interest per well.

790

791 **Supplementary Figure 2:** Small colony variant (SCV) growth and related bacterial gene
792 expression**.** A) The proportion of SCV in the controls of an acute intracellular infection model
793 of *S. aureus* in osteocytes. After 7 days of infection, the proportion of SCV in the whole
794 population increased significantly compared to the original population at day 0 (n = 15, p =
795 *0.0012*). B-C) Relative gene expression of *sigB* (B) and *asp23* (C) to the housekeeping genes
796 (*aroE* and *gyrB*) after 7 day of treatment ($n \geq 3$). Gene expression was normalised to that of
797 the bacterial housekeeping genes *aroE* and *gyrB* genes and calculated with the $2^{-\Delta Ct}$ method.

798

799 **Supplementary Figure 3:** Osteocytic differentiation of SaOS-2 cells. A-F) Osteogenic gene
800 expression of osteocyte markers during differentiation under 1% O_2, relative to *18S* rRNA
801 levels (n = 4). *$p < 0.05$, ***$p < 0.001$ and ***$p < 0.0001$. Gene expression was normalised to
802 that of the housekeeping gene *18S* and calculated with the $2^{-\Delta Ct}$ method. G) Von Kossa stain
803 for phosphate and Alizarin Red stain for calcium to observe mineralisation of osteocytes after
804 7 and 14 days of differentiation (n = 4).

805

806 **Table 1:** Antimicrobial susceptibility of *S. aureus* and antimicrobial concentrations. Minimum inhibitory concentration (MIC) and minimum
807 bactericidal concentration (MBC) for antibiotics tested against *S. aureus* strains at pH 5.0 and pH 7.2. Values shown are in µg/ml. Cmax bone =
808 the maximal concentration achievable in bone.

809

Antibiotic	pH		Clinical Isolates											ATCC	Cmax bone
			1	2	3	4	5	6	7	8	9	10	11	25923	(31-35)
Rifampicin	5.0	MIC	0.008	0.004	0.008	0.008		0.00125	0.002	0.008	0.008		0.005	<0.004	1.3-6.5
		MBC	0.032	0.008	0.008	0.016		0.002	0.005	0.016	0.016		>0.064	0.008	
	7.2	MIC	0.032	0.016	0.016	0.016	0.008	0.005	0.016	0.016	0.016	0.005	>0.064	0.008	
		MBC	0.064	0.032	0.032	0.032	0.016	0.8	>0.064	>0.064	>0.064	0.064	>0.064	0.016	
Vancomycin	5.0	MIC	2	1	1	1		1	2	1	1		1	2	1.1-6
		MBC	2	2	4	4		8	4	2	4		8	4	
	7.2	MIC	1	1	2	1	2	1	1	1	1	0.5	1	2	
		MBC	2	1	1	1	2	2	2	1	8	0.5	8	4	
Levofloxacin	5.0	MIC	5	>500	500	500		5	5	5	5		10	5	4.6-10
		MBC	10	>500	>500	>500		10	10	50	10		50	5	
	7.2	MIC	0.5	50	50	50	100	0.5	0.5	0.5	0.5	50	1	0.5	
		MBC	1	50	50	50	>500	1	1	10	0.5	>500	50	0.5	

Ofloxacin	5.0	MIC	10	500	500	100		10	10	5	5		50	5	3
		MBC	10	>500	>500	>500		15	50	10	10		100	10	
	7.2	MIC	0.5	100	100	100	100	5	1	0.5	0.5	100	1	0.5	
		MBC	1	>500	>500	>500	>500	5	5	5	1	>500	50	1	
Amoxicillin	5.0	MIC	0.25	1	1	2		0.25	0.25	1	1		0.25	0.125	27-37
		MBC	0.5	>2	>2	>2		2	0.25	2	2		>2	0.25	
	7.2	MIC	0.0625	>2	>2	>2	1	0.125	0.0625	>2	>2	1	0.5	0.0625	
		MBC	0.0625	>2	>2	>2	2	0.25	0.25			2	>2	0.25	
Oxacillin	5.0	MIC	0.0625	0.0625	0.03125	0.125		0.03125	0.03125	0.03125	0.03125		2.5	0.0625	4-10
		MBC	0.625	0.25	0.125	1		0.0625	0.0625	0.0625	0.0625		>80	0.125	
	7.2	MIC	0.0625	0.5	1	2	>2	0.25	0.125	0.25	0.25	20	20	0.25	
		MBC	0.5	>2	>2	>2	>2	1	0.25	2	0.25	40	>80	0.5	
Doxycycline	5.0	MIC	0.125	0.25	0.125	0.25		0.25	0.25	0.25	0.25		16	0.0625	3
		MBC	2	0.25	1	8		16	16	2	0.5		>16	2	
	7.2	MIC	0.125	<0.03125	<0.03125	<0.03125	8	0.125	0.25	0.125	0.5	8	8	0.0625	
		MBC	2	0.0625	0.125	8	>16	>8	8	1	2	16	>16	0.25	
Linezolid	5.0	MIC	1	2	2	2		4	4	2	2		2	2	6.3-9.1
		MBC	64	64	16	64		64	32	8	32		64	16	
	7.2	MIC	2	1	1	1	1	4	4	2	2	<0.5	1	2	

		MBC	16	2	1	32	4	32	64	16	8	<0.5	64	16	
Gentamicin	5.0	MIC	32	>64	>64	>64		>64	>64	64	>64		>64	>64	Detectable
		MBC	>64	>64	>64	>64		>64	>64	>64	>64		>64	32	
	7.2	MIC	2	4	4	4	8	16	16	4	32	16	64	8	
		MBC	16	16	8	16	16	32	32	32	32	64	>64	8	
Tigecycline	5.0	MIC	4	8	8	8		8	8	8	8		0.125	4	0.3
		MBC	>8	8	8	8		>8	8	7	>8		4	4	
	7.2	MIC	0.125	0.125	0.125	0.125	0.25	0.125	0.125	0.125	0.125	0.125	2	0.0625	
		MBC	2	0.5	1	1	2	2	2	0.5	0.5	0.5	8	2	

811 **Table 2**: Antimicrobial effect on colony forming units (CFU) in an acute intra-osteocytic *S.*
812 *aureus* infection. Logarithmic CFU reduction in an acute infection model of SaOS-2 osteocyte
813 of each antibiotic at 1, 4 and 10x MIC after 1 and 7 days of treatment. Asterix indicates
814 significant CFU reduction compared to an untreated control with * p<0.05, ** p<0.01, ***
815 p<0.001 and *** p<0.0001.

Treatment		1-day treatment		7- days treatment	
		Mean ± SD	Log red	Mean ± SD	Log red
Doxycycline	Infected CTRL	866667±325167		36000±10000	
	1 x MIC	74667±227450	0.06	47333±15011	-0.12
	4 x MIC	753333±133167	0.06	79333±22301	-0.34
	10 x MIC	473333±46188	0.26	36000±19698	0
Oxacillin	Infected CTRL	20400±7292		1100±698	
	1 x MIC	5355±4168	0.58*	455±200	0.38
	4 x MIC	5725±1612	0.55*	390±125	0.45
	10 x MIC	3240±817	0.80*	122±63	0.96
Levofloxacin	Infected CTRL	730000±728080		13933±4291	
	1 x MIC	264667±28095	0.44	840±640	1.22***
	4 x MIC	534667±715476	0.14	103±2	2.13***
	10 x MIC	66667±14742	1.04	15±1	2.98***
Rifampicin	Infected CTRL	100000 ±59025		2080± 413	

	1 x MIC	11933± 5368	0.92***	33±30	1.80****
	4 x MIC	1620±851	1.79***	43±75	1.68****
	10 x MIC	520±320	2.28***	0	3.32****
Linezolid	Infected CTRL	19867±3521		5490±2079	
	1 x MIC	13333±4407	0.17*	498±271	1.04**
	4 x MIC	8777±7479	0.35****	7±2	2.87***
	10 x MIC	573±628	1.54****	0	3.74***

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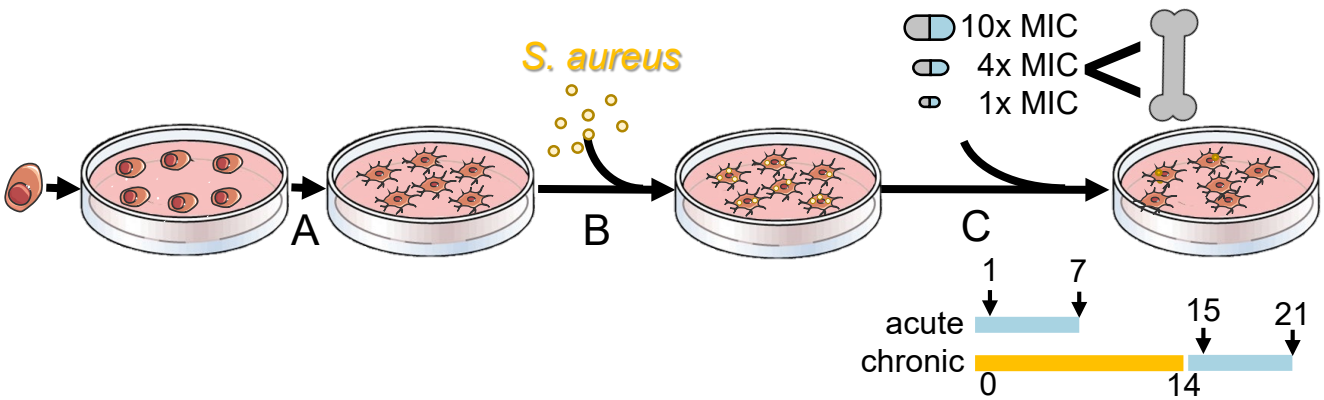

Figure 1: Schematic of the experimental design of the osteocyte infection and antibiotic treatment model. A) Differentiation of SaOS-2 osteoblasts into human osteocyte-like cells. B) Infection with *S. aureus*, removal of extracellular bacteria with lysostaphin. C) Antibiotic treatment (blue line) either immediately after infection (acute model) or after 14 days of infection (yellow line) for 1 day or 7 days with 1x,4x,10x MIC, which were concentrations achievable in bone.

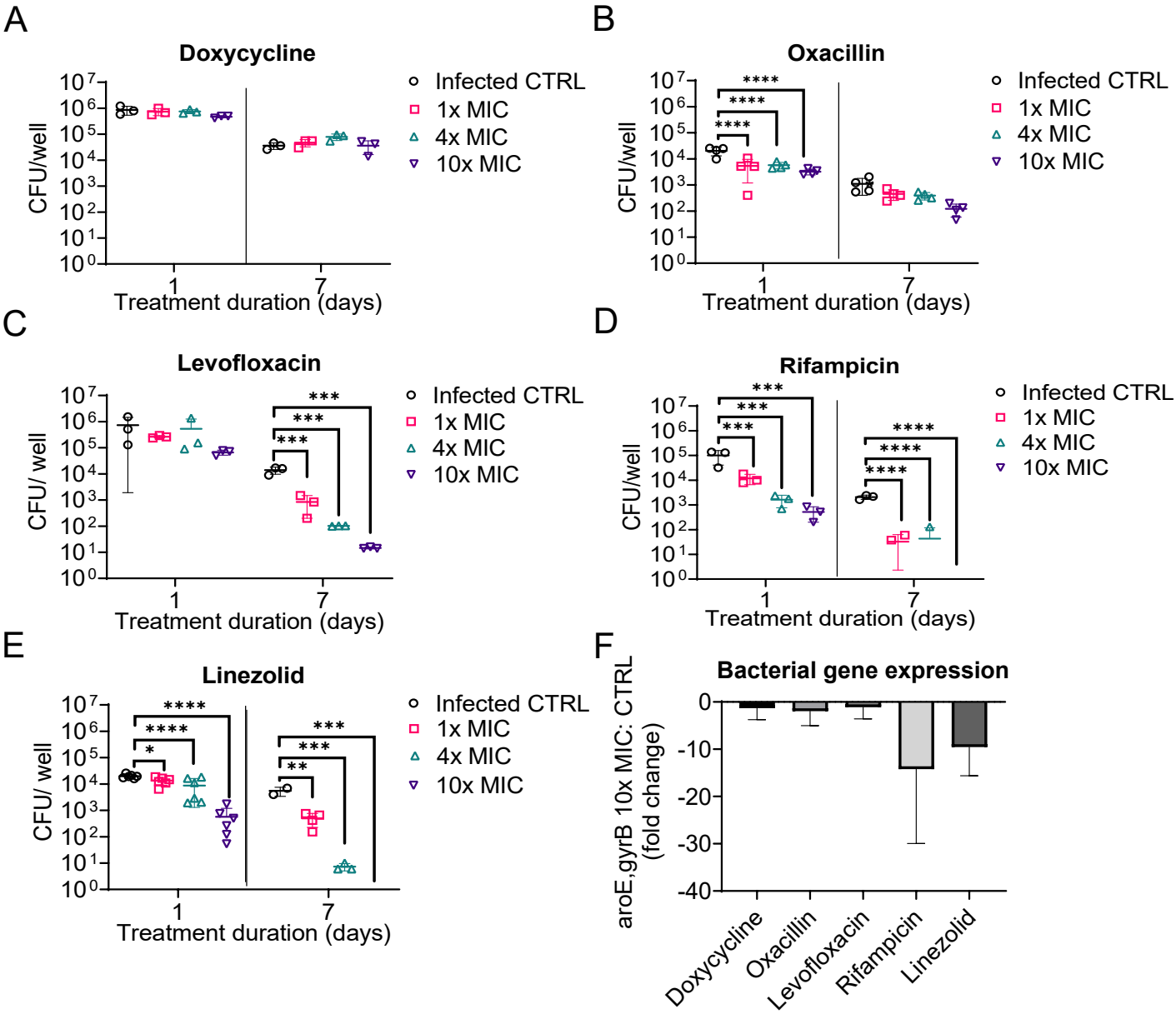

Figure 2: Effects of antibiotics in a model of acute intracellular *S. aureus* infection in osteocytes. Osteocyte-like SaOS-2 cells were infected with S. aureus and immediately treated with antibiotics at 1x, 4x, 10x MIC for 1 day or 7 days (n ≥ 3). A-E) CFU count per well for each antibiotic treatment and infected control after 1 and 7 days of treatment. F) Relative mRNA expression of housekeeping genes (*aroE* and *gyrB*) after 7 days of treatment at 10x MIC for each antibiotic compared to infected control cultures (CTRL). Significant differences to the infected controls are indicated by *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

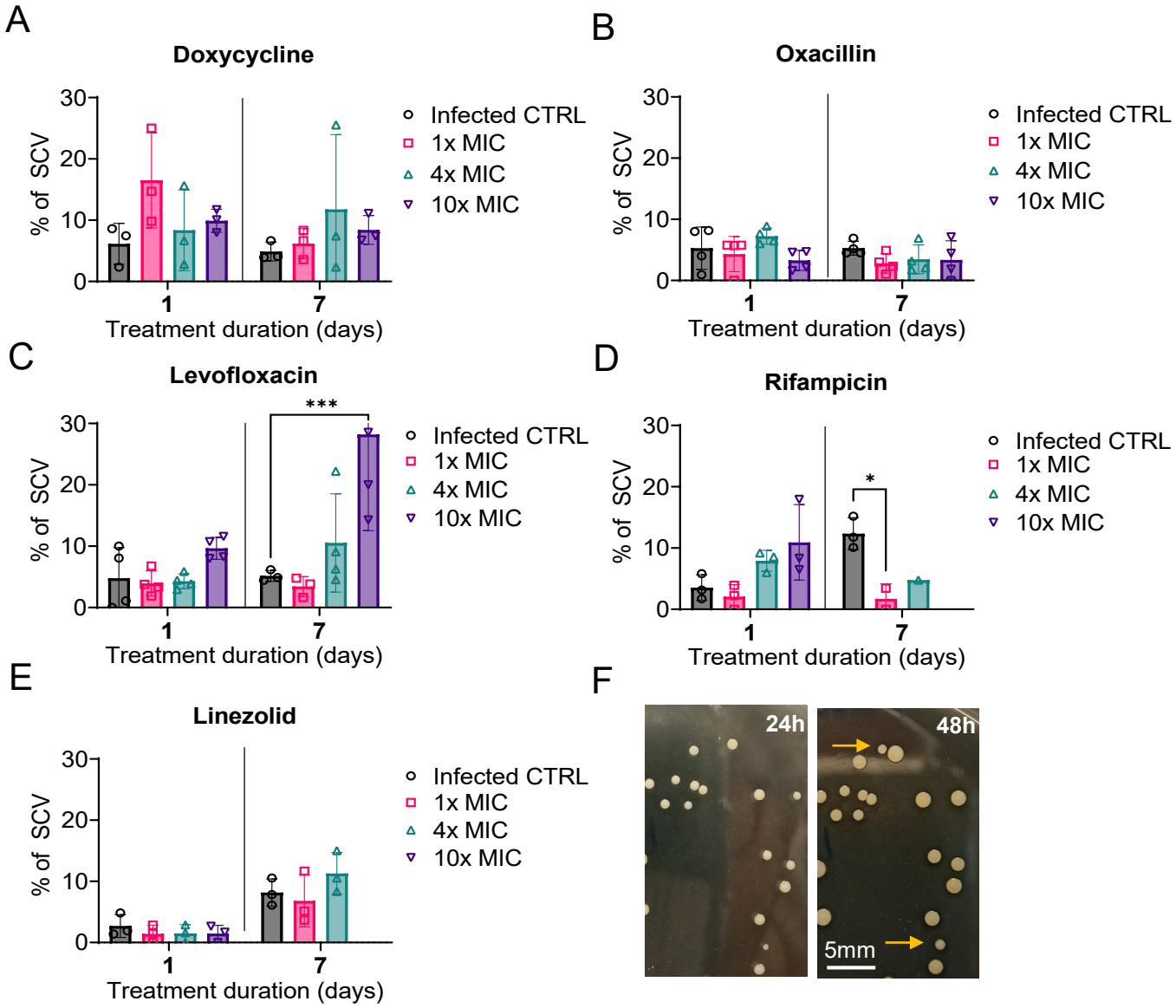

Figure 3: Effects of antibiotics on colony phenotype of *S. aureus* recovered from an intracellular infection in osteocytes. The proportion of small colony variants (%SCV) from host cell lysates was determined after 1 day and 7 days of antibiotic treatments at 1x, 4x and 10x their respective MIC ($n \geq 3$). A-E) %SCV emergence for the infected control and all antibiotic treatments. F) Example images of SCV determination (indicated with yellow arrow): Non-pigmented colonies that appeared only after 48h or were smaller after 24h and did not increase in size compared to 48h wild type (WT) colonies were defined as SCV. Significant differences to the infected controls are indicated by $^{*}p < 0.05$ and $^{***}p < 0.0001$.

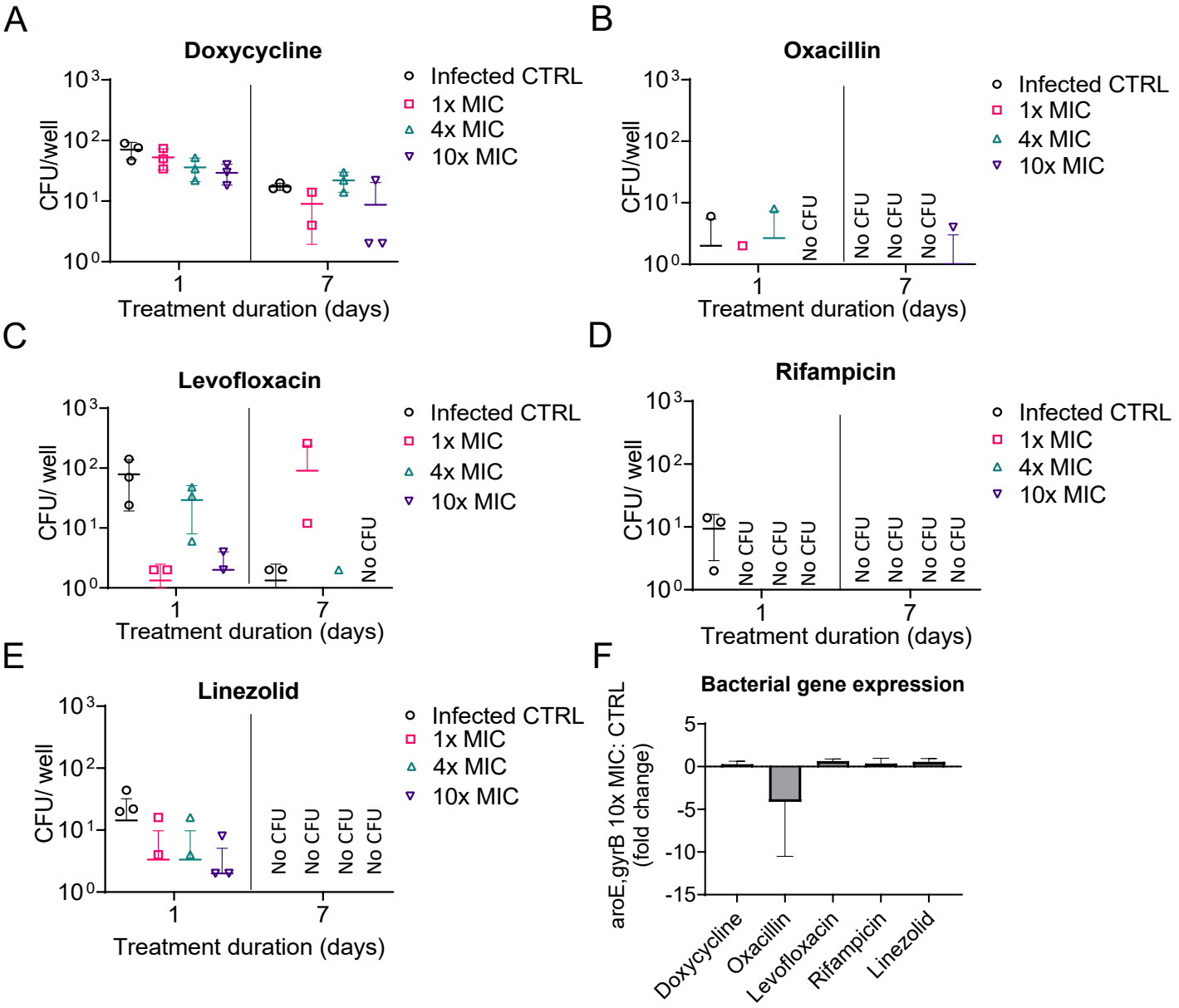

Figure 4: Effects of antibiotics in a model of chronic intracellular S. aureus infection in osteocytes. Osteocyte-like SaOS-2 cells were infected with S. aureus and cultured for 14 days. Following this, cells were treated with antibiotics at 1x, 4x, 10x MIC for a further 1 or 7 days (n ≥ 3). A-E) CFU count from cell lysates per well for each antibiotic treatment and infected control after 1 and 7 days of treatment. An absence of colonies after a 48h incubation was recorded as 'No CFU'. F) Relative mRNA expression of housekeeping genes (*aroE* and *gyrB*) after 7 days of treatment at 10x MIC for each antibiotic compared to infected control cultures (CTRL). No significant differences between treatments and non-treated infected controls were found.

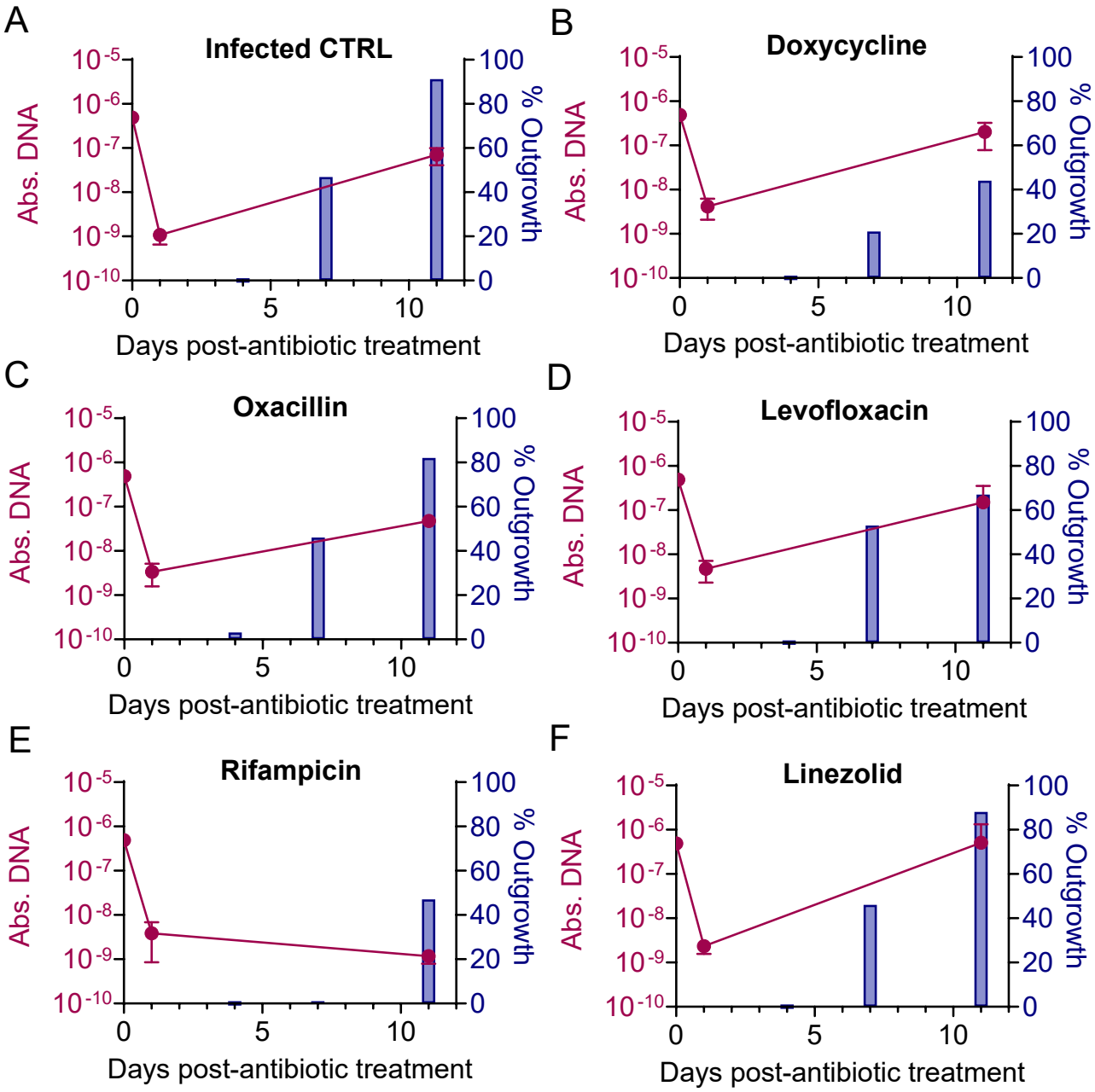

Figure 5: Outgrowth of intracellular *S. aureus* from a non-culturable state. SaOS-2 osteocyte-like cells were infected for 14 days with *S. aureus* to a non-culturable stage and then treated for 3 days with each antibiotic at their respective 10x MIC (n = 30 wells per condition). Following washing and replenishment with antibiotic-free media, cultures were visually inspected and assayed daily for up to 11 days for bacterial re-growth into media, with data plotted as percentage of wells with outgrowth (right axis, blue bars). At the day of infection, 1 and 11 days post-antibiotic treatment, replicate culture lysates were also used to prepare DNA for PCR analysis (left axis: purple dots).