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Combination with Rifampin or Gentamicin
Against Biofilm-Forming *Enterococcus faecalis* or *E.*
faecium in an *In Vitro* Pharmacodynamic Model
Using Simulated Endocardial Vegetations and an *In*
Vivo Survival Assay Using *Galleria mellonella* Larvae

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Running Title: Enterococcal Infective Endocarditis

Activity of Daptomycin or Linezolid in Combination with Rifampin or Gentamicin against Biofilm-forming *Enterococcus faecalis* or *E. faecium* in an In Vitro Pharmacodynamic Model using Simulated Endocardial Vegetations and In Vivo Survival Assay using *Galleria mellonella* Larvae

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1 **Abstract** Enterococci are the third most frequent cause of infective endocarditis. A high-
2 inoculum stationary phase in vitro pharmacodynamic model with simulated endocardial
3 vegetations was used to simulate human pharmacokinetics of daptomycin 6 or 10mg/kg/day,
4 or linezolid 600mg q12h alone and in combination with gentamicin 1.3mg/kg q12h, rifampin
5 300mg q8h or 900mg q24h. Biofilm-forming vancomycin-susceptible *Enterococcus faecalis*
6 and vancomycin-resistant *E. faecium* (VRE) were tested. At 24, 48 and 72h, all daptomycin-
7 containing regimens demonstrated significantly more activity (decline in CFU/g) than any
8 linezolid-containing regimen against biofilm-forming *E. faecalis*. The addition of gentamicin
9 to daptomycin (6 and 10mg/kg) in the first 24 hours significantly improved the bactericidal
10 activity. In contrast, addition of rifampin delayed the bactericidal activity of daptomycin
11 against *E. faecalis*; and against VRE, antagonized all regimens at 24h. Also, against VRE,
12 addition of gentamicin to linezolid at 72h improved activity and was bactericidal. Rifampin
13 significantly antagonized the activity of linezolid against VRE at 72h. In in vivo *Galleria*
14 *mellonella* survival assays, linezolid and daptomycin improved survival. Daptomycin 10mg/kg
15 improved survival significantly over linezolid against *E. faecalis*. Addition of gentamicin
16 improved efficacy of daptomycin against *E. faecalis* and linezolid and daptomycin against
17 VRE. We conclude that in enterococcal infection models, daptomycin has more activity than
18 linezolid alone. Against biofilm-forming *E. faecalis*, the addition of gentamicin in the first 24h
19 causes the most rapid decline in CFU/g. Of interest, addition of rifampin delayed or
20 antagonized activity of daptomycin against biofilm-forming *E. faecalis* and VRE respectively
21 in the first 24h.

22

23 **Introduction.**

24 Despite major advances in medicine and surgery, infective endocarditis (IE) remains a
25 concerning disease associated with considerable morbidity and mortality.(1) Bacterial causes
26 of IE and bacteremia have changed over the past few decades and now streptococci,
27 staphylococci, and enterococci have emerged as the major pathogens.(2) Among these,
28 Enterococcus has become the most challenging to treat. Barriers in treating these infections
29 include the need for multiple agents to demonstrate bactericidal activity and microbiological
30 cure (1); biofilm production among these bacteria (3, 4); and resistance to the mainstays of
31 therapy (i.e., ampicillin, penicillin, and vancomycin) (5). Biofilm production in enterococci is
32 common in *E. faecalis*, with worldwide rates reported between 26-100%, and 93% reported
33 in the US.(3) The 2005 American Heart Association recommendations for drug-resistant
34 enterococcal IE include linezolid and quinupristin-dalfopristin, which are both bacteriostatic
35 against enterococci.(1).

36 Daptomycin, at high doses, demonstrates bactericidal activity against enterococci in
37 other types of infection, and against *S. aureus* in endocarditis.(6, 7) This is due to
38 daptomycin's mechanism of action as it disrupts the cell-membrane potential and is growth
39 phase independent.(8) There is promising data demonstrating in vitro synergy with
40 gentamicin and daptomycin combination therapy against VRE (9-13), and case reports also
41 support these findings.(11, 14, 15) Therefore, the addition of gentamicin, a ribosomal active
42 agent may provide a synergistic approach in VRE IE infections. Additionally, since *E.*
43 *faecalis* often produce biofilm, (3) it is of interest to evaluate daptomycin's activity in
44 combination with rifampin. (16-18) Finally, since daptomycin demonstrates concentration-
45 dependent killing, evaluation of approved doses (6mg/kg) and higher doses (10mg/kg) may
46 result in increased activity and resistance prevention, (19) as there is established efficacy in
47 other infection types (20) with appropriate safety data. (21)

48 We therefore evaluated the in vitro activity of daptomycin and linezolid alone and in
49 combination with gentamicin or rifampin against enterococci in an in vitro model with
50 sequestered high inoculum stationary phase infection using simulated endocardial
51 vegetations (SEV).(20, 22, 23) We also tested these regimens in an in vivo survival assay
52 using *Galleria mellonella* larvae. We used a vancomycin-susceptible biofilm-producing *E.*
53 *faecalis* and a vancomycin-resistant *E. faecium*. We also evaluated biofilm production of
54 these isolates.

55 MATERIALS AND METHODS

56 **Bacterial strains.** We evaluated a vancomycin-susceptible, ampicillin-susceptible *E.*
57 *faecalis*, ATCC 29212 (also gentamicin-susceptible and rifampin-susceptible) and a
58 vancomycin-resistant (VRE) *E. faecium* clinical isolate from the Providence Veterans Affairs
59 Medical Center (also penicillin-resistant, gentamicin-susceptible, and rifampin resistant). Both
60 isolates were linezolid and daptomycin susceptible.

61 **Antimicrobial agents.** Linezolid (lot# 11C03U04, 10H10Z16; Pfizer, Inc.; NY) was
62 obtained commercially, and daptomycin was obtained from Cubist Pharmaceuticals, Inc.,
63 (Lexington, MA). Rifampin (lot 085K1929) and gentamicin (lot 050K03421, 097K06887V)
64 were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of each
65 antibiotic were freshly prepared at the beginning of each week and kept frozen at -4°C.

66 **Medium.** As previously described, Mueller-Hinton broth (Becton Dickinson, Sparks,
67 MD) supplemented with calcium and adjusted to physiologic conditions of 50 mg/L calcium
68 chloride (ionized Ca; 1.03-1.23 mmol/L) and 12.5 mg/L magnesium was used for all
69 susceptibility analyses and in vitro pharmacodynamic analyses.(24) Bacto Tryptic Soy Broth
70 (TSB; Becton Dickinson) supplemented with 1% glucose and 50mg/L calcium chloride was
71 used to optimize biofilm production in the biofilm assay.(25, 26) Colony counts were
72 determined using Tryptic Soy Agar (TSA, Difco, Becton Dickinson). For the in vivo study,
73 strains were grown overnight at 30°C in brain heart infusion (BHI) with agitation. Inoculum
74 was confirmed by plating serial dilutions on BHI agar.

75 **Susceptibility.** Minimum inhibitory concentrations (MICs) and minimum bactericidal
76 concentration (MBC) testing was determined at both standard ($\sim 10^6$ CFU/mL) and high
77 inoculum ($\sim 10^9$ CFU/mL) in triplicate using microbroth dilution according to CLSI
78 methods.(27). All samples were incubated at 35° C for 24 hours prior to interpretation of
79 results.

80 **Biofilm Formation.** In growth conditions (media; see above) that optimize biofilm
81 production in *Enterococcus*, quantification of biofilm formation was conducted using the
82 microtiter plate assay first described by Christensen *et al.* (28) and modified as follows.
83 Briefly, stationary cultures of an overnight growth of the *Enterococcal* strains (1% vol/vol)
84 were diluted into fresh cation- and glucose-supplemented TSB. The inoculated medium was
85 dispensed into wells of sterile flat-bottom 96-well polystyrene tissue culture plates (Costar no.
86 3596; Corning Inc., Corning, NY, USA). Biofilm production in *Enterococcus* has been linked
87 to several genes including, *fsr*, *gelE*, and *sprE*.(29) Previous findings support that expression
88 of these genes were found at 24h of growth.(29) We examined two sets of plates, incubated
89 at 35°C a minimum of 24h and 48h, respectively. The attached bacteria was then fixed and
90 stained with crystal violet. After drying, the optical density (OD) of stained adherent bacterial
91 films was read using a μ Quant™ Microplate Spectrophotometer microtiter dish reader (Bio-
92 Tek Instruments, Inc. Winooski, Vermont, USA.). The optical density (OD) of bacterial films
93 were classified into the following categories: no biofilm production, weakly (+), moderately
94 (++) , or strongly (+++) adherent, based upon the ODs of bacterial films (30). The test was
95 carried out in triplicate. The results were averaged.

96 **In vitro pharmacodynamic infection model with Simulated Endocardial**
97 **Vegetations (SEVs).** As previously described, organism stocks containing approximately
98 10^{10} CFU/mL were prepared by inoculating 5mL test tubes of normal saline with colonies
99 harvested from fresh overnight growth on TSA.(20, 22, 24, 31, 32) SEVs containing 10^9
100 CFU/g were prepared by combining 0.05mL of the organism suspension with 0.4mL of
101 human cryoprecipitate antihemolytic factor (AHF) from volunteer donors (Rhode Island Blood
102 Bank, Providence, RI), 0.05mL of aprotinin suspension, and 0.025 mL of platelet suspension
103 (platelets mixed with normal saline, 250,000 to 500,000 platelets per clot) in 1.5 mL
104 eppendorf tubes. Bovine thrombin (5,000 units/mL, 50 μ L), was added to each tube after

105 insertion of a sterile monofilament line into the mixture. The resultant SEVs were removed
106 from eppendorf tubes with a sterile 21-gauge needle and introduced into the model. This
107 methodology results in SEVs containing approximately 3-3.5 g/dL of albumin and 6.8-7.4
108 g/dL of total protein (22).

109 **In vitro pharmacodynamic infection model.** An in vitro infection model consisting
110 of a 250 mL one-compartment glass apparatus with ports where the SEVs are suspended,
111 was utilized for all simulations. The apparatus was pre-filled with media and antibiotics were
112 administered as boluses over a 72-hour period into the central compartment via an injection
113 port. The models were placed in a 35°C water bath throughout the procedure with a
114 magnetic stir bar for thorough mixing of the drug in the model. Fresh media was
115 continuously supplied and removed from the model via a peristaltic pump (Masterflex, Cole-
116 Parmer Instrument Company, Chicago, IL USA) set to simulate the half-lives of the
117 antibiotics. Two SEVs were removed from each model at 0, 4, 8, 24, 32, 48, 56 and 72
118 hours. Once removed, SEVs were then immediately homogenized in trypsin, plated onto
119 TSA, and incubated at 35°C for 24 hours before colony count enumeration. This method
120 results in a lower limit of detection of 2.0 log₁₀ CFU/g (23). Antimicrobial carryover was
121 minimized by serial dilution (10-10,000) of plated samples in conjunction with vacuum
122 filtration, when necessary, where samples were washed through a 0.22 µm filter with sterile
123 water. These filters were then plated onto TSA and incubated at 35° C for 24 hours.
124 Colonies were counted on filter paper; the limit of detection is 1.0 log₁₀ CFU/g.

125 Daptomycin was administered to simulate a 6mg/kg dose (peak, 98.6µg/mL) and 10mg/kg
126 (141 µg/mL) every 24 hours (q24h) with pump rate set to achieve a half-life of 8 hours (21,
127 33). Linezolid was administered to simulate 600mg q12h with a half-life of 6 hours and a
128 peak concentration 21 µg/mL.(27) Gentamicin was administered to simulate 1.3 mg/kg q12h
129 (approximate: peak 6 µg/mL, trough 0.4µg/mL) a half-life of 2 hours.(24) Rifampin was

130 administered to simulate a dose of 300mg q8h (approximate peak, 14.5 $\mu\text{g}/\text{mL}$) and a half-life
131 of 4 hours.(24) Additionally, a regimen simulating rifampin 900mg once daily in combination
132 with linezolid or daptomycin 6mg/kg was performed in duplicate to assess the effects of
133 rifampin dosage schedule and concentration.

134 For combination regimen experiments the elimination rate was set for the drug with
135 the shortest half-life, the drug with the longer half-life was supplemented. All model
136 experiments were performed in triplicate unless otherwise noted, to ensure reproducibility. In
137 addition, simulations in the absence of antibiotics were performed at the shortest half-life to
138 assure adequate growth of the organisms in the model.

139 **Pharmacodynamic Analysis.** Reductions in $\log_{10}\text{CFU}/\text{g}$ over 72 hours were
140 determined by plotting time-kill curves and compared between regimens. Bactericidal activity
141 (99.9% kill) was defined as a $\geq 3\text{-}\log_{10}\text{CFU}/\text{g}$ reduction in colony count from the initial
142 inoculum. Bacteriostatic activity was defined as a $< 3\text{-}\log_{10}\text{CFU}/\text{g}$ reduction in colony count
143 from the initial inoculum while inactive was defined as no observed reductions from initial
144 inoculum. The time to achieve 99.9% kill was determined by non-linear regression (using a
145 minimum of 4 data points) if $r^2 \geq 0.95$, or by visual inspection. Enhancement of activity was
146 defined as an increase in kill of $\geq 2\text{-}\log_{10}\text{CFU}/\text{g}$ by combination of antimicrobials versus the
147 most active single agent of that combination. Improvement was defined as a 1 to $2\text{-}\log_{10}$
148 CFU/g increase in kill in comparison to the most active single agent, while combinations that
149 result in $\geq 1\text{-}\log_{10}$ bacterial growth in comparison to the least-active single agent was
150 considered to represent antagonism. The terms “improvement” and “enhancement” were
151 used because our simulations involve therapeutically obtained serum concentration and this
152 does not permit the mathematical modeling necessary to consider the standard terms
153 “additivity” and “synergy” (34). Indifference was defined as $<1\text{-}\log_{10}\text{CFU}/\text{g}$ change in activity.

154 **Resistance.** Development of resistance was evaluated for each monotherapy and
155 combination model at 24, 48, and 72 hours. MIC testing (using Etests) of daptomycin,
156 linezolid, gentamicin and rifampin were conducted with isolates obtained from the 24, 48 and
157 72 hour time points to identify any MIC shifts. Plates were examined for growth after 24
158 hours of incubation at 35°C.

159 **Pharmacokinetic Analysis.** Samples for pharmacokinetic analyses were obtained
160 through the injection port at 0.5, 1, 2, 4, 6, 8, and 24 hours for verification of target antibiotic
161 concentrations. All samples were stored at -80°C until analysis. Daptomycin concentrations
162 were determined by a previously described and validated HPLC method (Center for Anti-
163 Infective Research and Development, Hartford, CT) (20). Gentamicin concentrations were
164 determined by a homogeneous particle-enhanced turbidmetric immunoassay (PETIA;
165 Architect, Multigent®; Abbott Diagnostics Abbott Park, IL, USA) at the Providence Veteran
166 Affairs Medical Center. The gentamicin assay was known to have a range of detection of 0.3
167 to 10.0 µg/mL and a between day sample precision and percent coefficient of variation
168 (CV%) of 1.35% and < 2.75%, respectively. Linezolid and rifampin concentrations were
169 evaluated using HPLC (University of Florida, Gainesville, FL) as previously described (23,
170 24). Only single drug concentrations were evaluated, all in duplicate. The half-lives,
171 maximum concentration (C_{max}), and minimum concentration (C_{min}) of the antibiotics were
172 determined by the trapezoidal method utilizing PK Analyst software (Version 1.10, MicroMath
173 Scientific Software, Salt Lake City, UT).

174 **In vivo *Galleria mellonella* survival assay.** Efficacy of daptomycin or linezolid in
175 enterococcal infection was tested using *Galleria mellonella* survival assay. *Galleria*
176 *mellonella* caterpillars at the final-instar stage of development were acquired from the vendor
177 (Vanderhorst Wholesale Inc., St. Mary's, OH) and used within 7 days of shipment. All
178 experiments were performed according to previously described protocols with minor

179 modifications (35, 36). Sixteen larvae of appropriate weight (0.25-0.35g) were randomly
180 selected to comprise each group. Larvae were inoculated with either $\sim 4 \times 10^6$ CFU of *E.*
181 *faecalis* or $7-9 \times 10^6$ CFU of *E. faecium* followed by tested drug, or PBS as control ~ 1 hour
182 after inoculation. These inocula were chosen after an initial virulence pilot study of these
183 strains, as they were able to kill at least 90% of the larvae within 72h. One group, injected
184 twice with PBS, and one untouched group were used as controls in each experiment. All
185 injections were performed with a volume of 10 μ L using a Hamilton syringe. After injection, *G.*
186 *mellonella* were incubated at 37°C and survival was measured daily. Each experiment was
187 repeated at least twice and representative experiments are presented. Any experiment with
188 more than two dead larvae in any control group was discarded. Doses simulated free peak
189 concentrations seen in humans of daptomycin 6mg/kg, daptomycin 10mg/kg, or linezolid
190 600mg (Table 4). Gentamicin 1.3mg/kg and rifampin 300mg were also tested in combination
191 with either linezolid or daptomycin 6mg/kg.

192 **Statistical Analysis.** For the in vitro model, changes in CFU/g at 8, 24, 48, and 72 hours
193 and time to 99.9% kill were compared by two-way analysis of variance with Tukey's Post-Hoc
194 test. Statistical analyses were performed using SPSS Statistical Software (Release 20
195 SPSS, Inc., Chicago, IL). Survival in the *G. mellonella* model was plotted using Kaplan-Meier
196 curves, and groups were compared using log-rank test (GraphPad Prism 5 software). For all
197 experiments, a p value of ≤ 0.05 was considered significant.

198 RESULTS

199 **Susceptibility testing.** Daptomycin, linezolid, gentamicin, and rifampin MICs for the
200 two strains of enterococci are shown in Table 1. Against *E. faecalis*, there was minimal
201 increase (1 and 2 dilutions respectively) in MICs with daptomycin and linezolid in the
202 presence of high inocula. Against VRE *faecium*, there was an increase in the high inocula
203 MICs of daptomycin and linezolid by 3 dilutions and 2 dilutions, respectively. There was
204 minimal increase (0-2 dilution) in the gentamicin and rifampin MICs when the isolates were
205 evaluated at high inocula. This is consistent with published studies. (10, 23).

206 **In vitro pharmacokinetics and pharmacodynamics.** The pharmacokinetic
207 parameters of the antimicrobial agents were within the targeted range and can be found in
208 Table 2. All obtained C_{max} values were within 5% of targeted. The average and standard
209 deviation of area under the concentration-time curve (AUC) for daptomycin 6mg/kg was
210 1028+/-36, daptomycin 10mg/kg was 1430+/-47, and linezolid was 348 +/- 16.

211 **Biofilm production.** The *E. faecalis* isolate is a biofilm-positive control and produced
212 consistent biofilm (++) at 24 and 48h. The *E. faecium* isolate did not produce biofilm (0) at
213 24hours and was weakly adherent (+) at 48 hours.

214 **In vitro pharmacodynamic infection model with Simulated Endocardial**
215 **Vegetations (SEVs).** The antimicrobial activity of daptomycin and linezolid were evaluated
216 alone and in combination with gentamicin or rifampin against a high inoculum (10⁹ CFU/g) of
217 enterococci in a simulated IE vegetation model (Figure 1). Bactericidal activity ($\geq 3 \log_{10}$
218 decrease in CFU/g) was achieved by daptomycin 6 and 10mg/kg against *E. faecalis* at 24h
219 and by daptomycin 10mg/kg against *E. faecium* at 8h. Linezolid monotherapy did not achieve
220 bactericidal activity against either isolate tested at any time point. The AUC/MIC ratio for
221 daptomycin 6mg/kg was 514-1028 (MIC range 1-2 μ g/mL), daptomycin 10mg/kg was 715-

222 1430 (MIC range 1-2 μ g/mL), and linezolid was 348 (MIC 1 μ g/mL). Percent time above the
223 MIC (%T>MIC) was 100% for daptomycin and linezolid regimens.

224 Against biofilm-forming *E. faecalis*, daptomycin-containing regimens demonstrated
225 significantly more activity (as measured by a decline in the mean CFU/g) than linezolid-
226 containing regimens from 8 hours through the end of the experiment ($p\leq 0.005$). (Figure 1a.)
227 Addition of gentamicin significantly increased activity for daptomycin 10mg/kg at 24h (95% CI
228 0.954-3.4029; $p=0.033$). Addition of gentamicin to daptomycin 6mg/kg was significantly more
229 active than any other regimen tested at 8h ($p\leq 0.001$). At 24h, there was a 3log₁₀ CFU/g
230 difference in activity between added gentamicin or rifampin to daptomycin 6mg/kg ($p=0.010$),
231 though the difference was no longer significant at 48h. There was no significant difference
232 between linezolid monotherapy and linezolid plus rifampin or gentamicin regimens at any
233 time point during the 72h experiment, though adding rifampin to linezolid met the definition
234 for improvement at 72h. Changing the schedule of rifampin dosing from 300mg three times
235 daily to 900mg once daily had no effect on either regimen.

236 Against VRE *faecium*, at 24 and 48h, daptomycin-containing regimens had
237 significantly ($p\leq 0.005$) more activity than any of the linezolid-containing regimens (Figure 1b).
238 Addition of gentamicin improved linezolid activity, such that at 72h, linezolid plus gentamicin
239 is only significantly different than daptomycin 6mg/kg (the most active regimen) (95%CI
240 0.0144-3.4556, $p=0.047$) out of the daptomycin-containing regimens. It was not, however,
241 significantly more active than linezolid monotherapy. The addition of gentamicin was
242 significantly more active than the addition of rifampin with daptomycin 6mg/kg at 24h (95%CI
243 0.2349-2.9984, $p=0.013$). Rifampin antagonized all regimens at 24h. Addition of rifampin also
244 significantly antagonized linezolid activity at 48 and 72 hours (95%CI 0.0546-3.9921,
245 $p=0.040$ and 95%CI 0.0595-4.1772, $p=0.040$). At 72h, activity of linezolid plus rifampin was
246 not significantly different from the growth control. Changing rifampin dosing from three times

247 daily to once daily did not significantly increase activity, however linezolid plus rifampin once
248 daily was significantly more active than the growth control at 72h (95%CI 0.1546-4.6654,
249 $p=0.028$).

250 Gentamicin and rifampin monotherapy did not demonstrate any significant activity
251 against either isolate during the study. Resistance occurred in the rifampin and gentamicin
252 monotherapy models by 24h. The linezolid and daptomycin MICs varied at each time point
253 but never exceeded 4 $\mu\text{g/mL}$. In combination with both daptomycin and linezolid, rifampin
254 MICs increased throughout the 72h experiments against VRE, from 4 to $>32 \mu\text{g/mL}$.
255 Gentamicin MICs remained constant throughout the combination regimen experiments.

256 **In vivo *Galleria mellonella* survival assay.** Results demonstrated that all
257 antimicrobial regimens tested improved survival in all assays ($p<0.0001$) (Figures 2 and 3).
258 Against *E. faecalis*, monotherapy only with daptomycin 10mg/kg improved survival
259 significantly over linezolid alone ($p=0.0032$) (Figure 2a). Gentamicin added efficacy to
260 daptomycin 6mg/kg ($p=0.0361$), but not to linezolid (Figure 2 c and e), as observed in the in
261 vitro model. Against *E. faecium*, gentamicin added efficacy to both daptomycin 6mg/kg and
262 linezolid regimens ($p=0.0009$ and 0.0015) (Figure 3c and e). Addition of rifampin was not
263 significant for daptomycin or linezolid against either strain (Figure 2b, d, and 3b, d). Though
264 there was no antagonism observed for rifampin, other results concur with our IVPD findings.

265

266 **DISCUSSION**

267 Infective endocarditis vegetations often carry a high bacterial burden (10^8 - 10^{10}
268 organisms per gram of tissue).(37) This high bacterial density and limited blood supply to this
269 area allow for a diminished immune response and limited antimicrobial drug access. Location
270 of the vegetation (right-sided versus left-sided endocarditis), patient comorbidities, and
271 surgical interventions determine treatment success. (38, 39). The ability of bacteria to form
272 biofilms may contribute to treatment failure, as these bacteria are inherently less susceptible
273 to antibiotics due to decreased growth rates, nutrient restriction, and adaptive stress
274 responses.(40-43)

275 Endocarditis cause by enterococci requires treatment with synergistic antimicrobials;
276 traditionally, a cell wall active agent (beta-lactam or vancomycin) and an aminoglycoside.
277 The presence of high-level resistance to vancomycin eliminates main therapeutic options in
278 the management of serious enterococcal infections. Currently, options for resistant *E.*
279 *faecalis* IE include ampicillin in combination with either imipenem/cilastatin or ceftriaxone.(1)
280 While treatment with ampicillin in combination with ceftriaxone is becoming more common
281 against high level aminoglycoside resistant (HLAR) *E. faecalis*, further investigations into
282 PK/PD activity and dosage are needed. The 2005 American Heart Association Treatment of
283 IE guidelines recommend \geq 8 weeks of linezolid or quinupristin/dalfopristin monotherapy for
284 the treatment of *Native or Prosthetic Valve Enterococcal Endocarditis Caused by Strains*
285 *Resistant to Penicillin, Aminoglycoside, and Vancomycin*.(1) In many cases these
286 treatments are not ideal; linezolid has inherent bacteriostatic activity (6, 44),
287 myelosuppression (45, 46), and documented failure in animal studies and human case
288 reports in bacteremia and IE. (47-50) Quinupristin/dalfopristin use is also limited as it
289 demonstrates inherent bacteriostatic activity against VRE (51), lack of activity against *E.*
290 *faecalis* (6), musculoskeletal toxicities in approximately 50% of the population, and the use of

291 a central line for administration.(52) Daptomycin is commonly used for the treatment of VRE
292 infections (53), although the optimal dose and combinations are unknown.

293 Studies have shown that daptomycin demonstrates activity in enterococcal infections,
294 and may provide an option in patients with allergies or contraindications to other therapies.
295 In a retrospective cohort study of VRE bloodstream infections, treatment with daptomycin or
296 linezolid demonstrated no difference in mortality; however, infection with *E. faecium* and
297 concurrent treatment with rifampin or gentamicin were independent risk factors for
298 mortality.(54) Antagonistic activity is often observed when rifampin is added to bactericidal
299 agents in high inoculum infections, due to high rates of mutations conferring resistance (~1 in
300 10⁶). (31, 55, 56) The in vitro model demonstrated antagonism with rifampin. The in vivo
301 model used a lower bacterial burden, so antagonism from rifampin resistance may not be as
302 evident. In contrast, previous in vitro studies have shown synergy with daptomycin and
303 rifampin, and non-antagonism with daptomycin and gentamicin.(6)

304 *G. mellonella* is an invertebrate model host that shares many of the advantages of
305 mammalian models while being free of the ethical and logistical constraints that accompany
306 their use.(57) Specifically, *G. mellonella* larvae can grow in 37°C thus effectively simulating
307 human temperatures and can be directly injected with the tested inoculum and compounds
308 thus allowing for exact quantification of the experimental concentrations.(58) As a result, this
309 model host is well established in the screening of the efficacy and safety of antimicrobial
310 compounds against a variety of infections (59), and has also been effectively used to test
311 antibiotics against *Enterococcus* spp. in the past.(60) *G. mellonella* possess both cellular and
312 humoral defenses and have extensive structural and functional similarities to vertebrate
313 immune systems.(61) Finally, *G. mellonella* larvae have also been proven effective in
314 identifying immunomodulatory properties of several compounds that would have otherwise
315 gone unnoticed in in vitro experiments.(62) Our in vivo model demonstrated improvement

316 with addition of gentamicin to daptomycin 6mg/kg. It is possible that this improvement would
317 not be seen with higher daptomycin doses, as survival was 100% at 9 days with the 10mg/kg
318 dose.

319 Another in vitro model with simulated endocardial vegetations by Hall et al.
320 successfully demonstrated the concentration-dependent activity of daptomycin against VRE,
321 supporting doses >6mg/kg/day, as well as demonstrating daptomycin activity superior to that
322 of linezolid.(32) A recent meta-analysis of VRE bacteremia demonstrated a trend toward
323 increased survival with linezolid treatment over daptomycin.(63) These differences, however,
324 were not statistically significant, and the studies used suffered from problems of different
325 definitions of mortality, low doses of daptomycin (average dose ~6mg/kg), and a possible
326 treatment selection bias in the cohorts.(64) A recent cohort study of patients with gram-
327 positive infective endocarditis demonstrated no significant difference in mortality between
328 standard of care antibiotics and daptomycin, given at an average of ~8mg/kg in the *E.*
329 *faecalis* group.(65) The *E. faecalis* group treated with daptomycin had a significantly shorter
330 length of stay compared to standard antibiotics (17.5 [13.5-19.5] vs. 31 [19.0-50.0]days,
331 $p=0.02$).(65) Although small, this study also demonstrated no significant increase in adverse
332 events with higher dose daptomycin. Our work demonstrates no statistically significant
333 differences in any daptomycin regimen at 72h. High-dose daptomycin has some in vitro
334 evidence to support its use in complicated enterococcal bacteremia and IE, as 10mg/kg, but
335 not 6mg/kg, can prevent MIC increases in daptomycin non-susceptible *S. aureus*
336 isolates.(66)

337 In conclusion, daptomycin-containing regimens generally were more active against
338 enterococcal isolates than linezolid throughout the experiments. The addition of rifampin to
339 either linezolid or daptomycin did not significantly increase antibacterial activity in an in vitro
340 sequestered high inoculum model of enterococcal endocarditis at 72h, and rifampin delayed

341 the bactericidal activity of daptomycin during the first 24 hours. The inhibition of bacterial
342 RNA synthesis may be responsible for delaying the killing activities of cell wall active
343 agents.(67) The addition of gentamicin improved the bactericidal activity of daptomycin most
344 in the first 24h against *E. faecalis*, and increased linezolid activity at 72h against VRE
345 *faecium*. It is currently unclear how linezolid, a protein synthesis inhibitor, demonstrates
346 improved activity in the presence of gentamicin. This improved activity has also been
347 observed in *S. aureus* and a vancomycin-resistant *E. faecalis*.(67-69) We feel that our work
348 supports the use of daptomycin 6 or 10mg/kg with 24 hours of gentamicin added for *E.*
349 *faecalis*, as the most active therapy for enterococcal endocarditis. Other clinical studies
350 demonstrate worse clinical outcomes when using rifampin in combination, while gentamicin
351 adds activity in the first 24 hours only, and should be limited due to concerns for
352 nephrotoxicity.

353 A limitation of this study is the use of limited isolates. In addition, we cannot conclude
354 that our in vitro results will hold true with treatment durations longer than 72 hours. Our
355 findings with daptomycin and linezolid monotherapy are consistent with published clinical, in
356 vitro and animal models. (7, 32, 70) The linezolid concentration in *G. mellonella*, while active,
357 was lower than desired due to limits on available pharmaceutical concentrations. It is
358 possible that the differences seen would not be significant if a higher concentration were
359 used. While *G. mellonella* received doses targeting the free peak concentration achieved in
360 humans, each drug was dosed only once, with survival being measured over 9 days, and
361 pharmacokinetic information including metabolism and excretion are unknown.

362 The results support daptomycin 6 or 10mg/kg, with gentamicin added for 24 hours,
363 against enterococci in simulated endocardial vegetations. Nonetheless, our results should be
364 applied to clinical practice with caution. Confirmation of these results in clinical studies is
365 needed before these regimens can be adopted for use in the care of patients.

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376

377 **Conflicts of Interest and Disclosures**

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Antimicrobial	MIC in mg/L ^a	
	<i>E. faecalis</i> ATCC 29212	<i>E. faecium</i> L2001
Daptomycin	2 (4)	1 (8)
Linezolid	1 (4)	1 (4)
Gentamicin	16 (32)	16 (32)
Rifampin	0.5 (0.5)	4 (16)
Vancomycin	2	>256

TABLE 1. MIC results using standard and high inocula for enterococcal isolates.

^aThe standard inoculum was 5×10^5 CFU/mL, and the high inoculum was 5×10^9 CFU/mL. Data for the high inoculum are presented parenthetically.

NA = not applicable

Regimen ^a	Peak concentration (mg/L)		Half-life (h)	
	Targeted	Obtained	Targeted	Obtained
Daptomycin 6mg/kg q24h	98.6	102.5 ± 1.96	8	7.92 ± 0.18
Daptomycin 10mg/kg q24h	140.0	143.2 ± 1.94	8	7.87 ± 0.21
Linezolid 600mg q12h	21.0	21.9 ± 0.86	6	6.52 ± 0.87
Gentamicin 1.3mg/kg q12h	6.0	5.7 ± 0.51	2	2.08 ± 0.17
Rifampin 300mg q8h	10.5	11.0 ± 1.23	4	3.60 ± 0.50

TABLE 2. Values of mean targeted and obtained pharmacokinetic parameters obtained with simulated endocarditis vegetations (SEV) infection models ± standard deviation

^abased on a 75 kg patient

TABLE 3. Inoculum change from starting inoculum of 5×10^9 CFU/g at 8, 24, and 72 h obtained in the SEV model.

Note that positive values indicate growth.

^a Indicates statistically significant difference from growth control.

Antimicrobial	Mean change in bacterial density (\log_{10} CFU/g)					
	<i>E. faecalis</i>			<i>E. faecium</i>		
	8h	24h	72h	8h	24h	72h
Growth Control	+1.13	+1.06	+1.29	+1.82	+1.93	+1.86
Daptomycin 6mg/kg	-2.07 ^a	-4.28 ^a	-5.07 ^a	-2.11 ^a	-4.56 ^a	-5.86 ^a
Daptomycin 6mg/kg+ rifampin	-1.88 ^a	-2.99 ^a	-5.13 ^a	-1.84 ^a	-3.33 ^a	-5.30 ^a
Daptomycin 6mg/kg + gentamicin	-4.36 ^a	-6.02 ^a	-6.15 ^a	-2.38 ^a	-4.96 ^a	-5.05 ^a
Daptomycin 10mg/kg	-2.23 ^a	-4.17 ^a	-6.07 ^a	-3.57 ^a	-4.90 ^a	-5.63 ^a
Daptomycin 10mg/kg + rifampin	-1.65 ^a	-3.48 ^a	-5.46 ^a	-2.09 ^a	-3.71 ^a	-5.41 ^a
Daptomycin 10mg/kg + gentamicin	-2.32 ^a	-6.07 ^a	-5.67 ^a	-2.99 ^a	-4.08 ^a	-5.04 ^a
Linezolid	+0.02	-0.19	-0.95	+0.07	-1.08 ^a	-2.90 ^a
Linezolid + rifampin	-0.07	-0.40	-1.96 ^a	+0.45	+0.48	-0.79
Linezolid + gentamicin	+0.13	-0.15	-0.88 ^a	-0.14	-0.67 ^a	-4.08 ^a

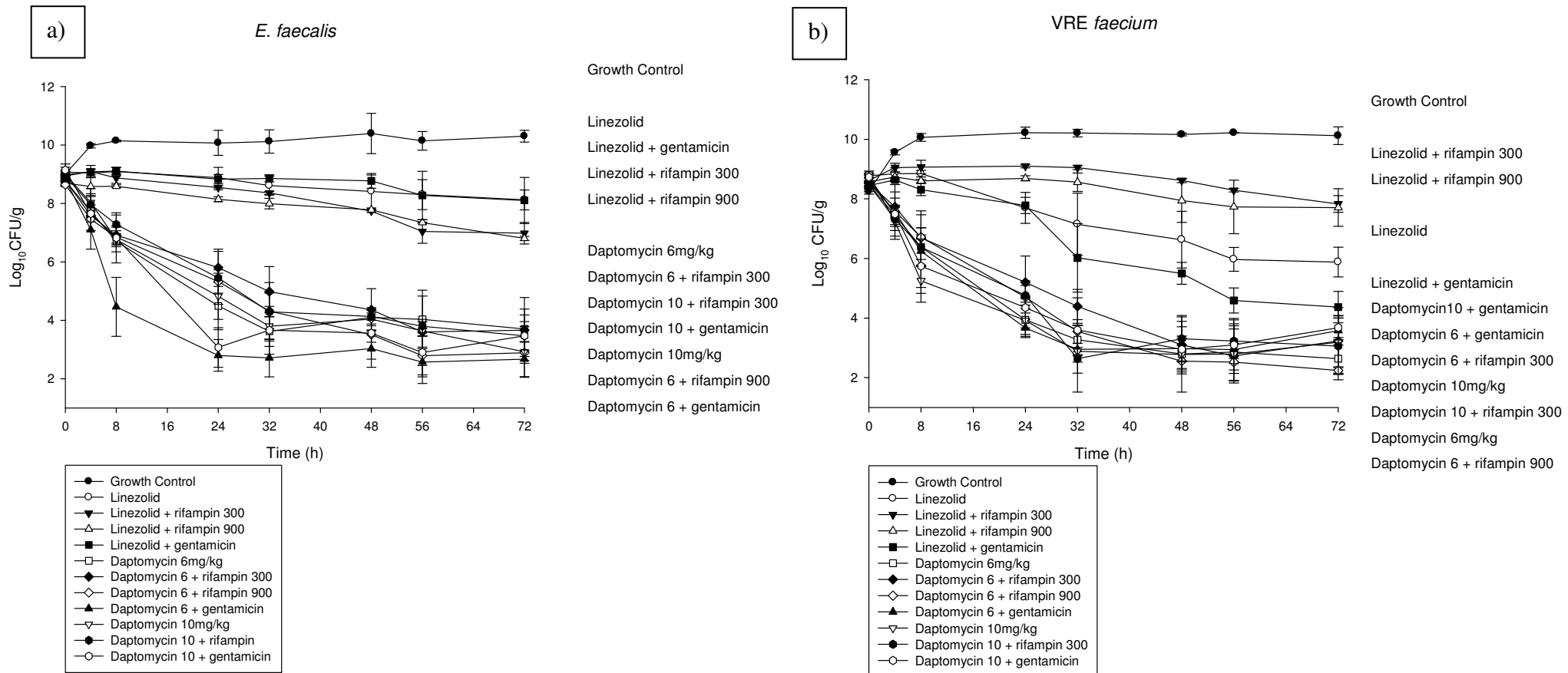


Figure 1. The activity (change in log₁₀ CFU/g) of daptomycin- or linezolid- containing regimens against a) *Enterococcus faecalis*. (vancomycin- susceptible, gentamicin- susceptible, rifampin- susceptible, daptomycin- susceptible, linezolid- susceptible) or b) *Enterococcus faecium* (vancomycin- resistant, gentamicin- susceptible, rifampin- resistant, daptomycin- susceptible, linezolid- susceptible).

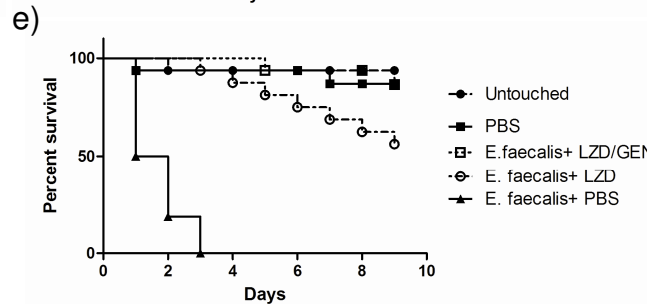
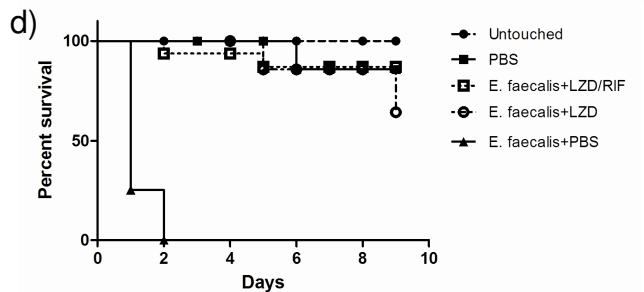
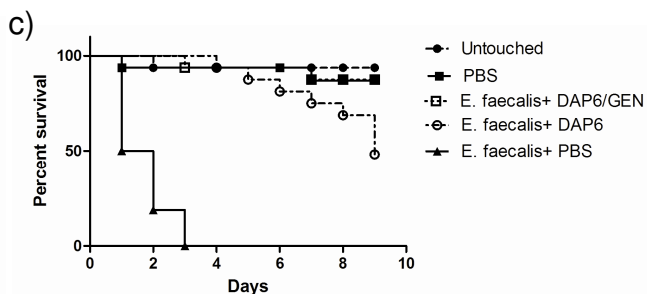
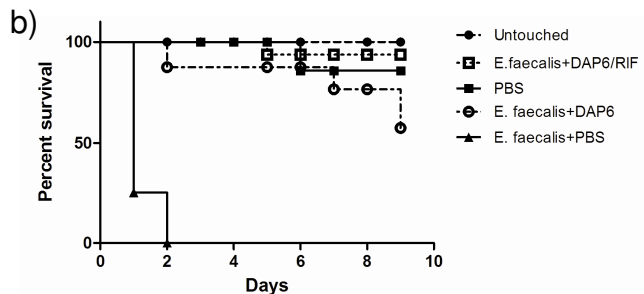
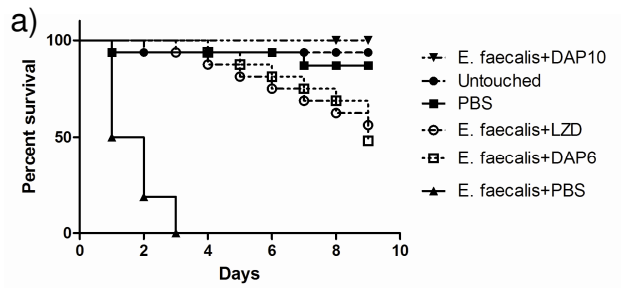


Figure 3. Efficacy of compounds against *E. faecalis* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecalis* followed by injection of the relative drug. Survival proportion with a) monotherapy of daptomycin 6mg/kg, daptomycin 10mg/kg, or linezolid vs controls. b) daptomycin 6mg/kg alone and in combination with rifampin c) daptomycin 6mg/kg alone or in combination with gentamicin d) linezolid alone or in combination with rifampin and e) linezolid alone or in combination with gentamicin.

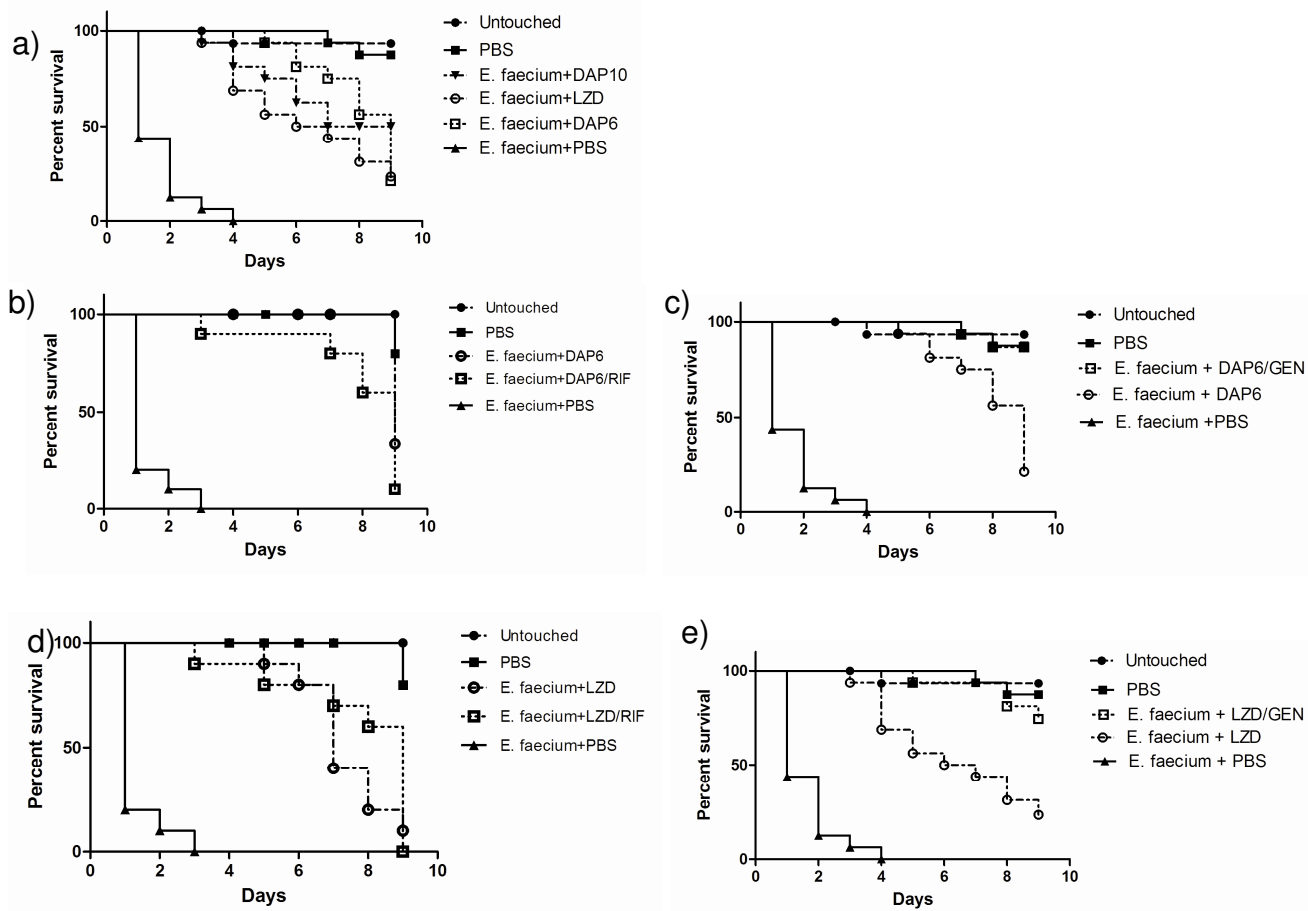


Figure 4. Efficacy of compounds against *E. faecium* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecium* followed by injection of the relative drug.

Survival proportion with a) monotherapy of daptomycin 6mg/kg, daptomycin 10mg/kg, or linezolid vs controls. b) daptomycin 6mg/kg alone and in combination with rifampin c) daptomycin 6mg/kg alone or in combination with gentamicin d) linezolid alone or in combination with rifampin and e) linezolid alone or in combination with gentamicin.

Antimicrobial and Human Dose	Targeted free peak concentration (mg/L)	Administered concentration in <i>G. mellonella</i> (mg/L)
Daptomycin 6mg/kg	9.8	9.15
Daptomycin 10mg/kg	14.0	13.07
Linezolid 600mg	14.0	8.00 ^a
Gentamicin 1.3mg/kg	6.0	5.60
Rifampin 300mg	2.6	2.50

Table 4. Targeted vs. administered peak concentrations in *G. mellonella* models.

^a Linezolid concentrations were lower than targeted due to limits on the available pharmaceutical concentrations.