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## 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 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  $\mu$ 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  $\mu$ 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 log<sub>10</sub> 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 log<sub>10</sub> 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  $\text{CFU}/\text{g}$  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 turbidimetric 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<sub>max</sub>), and minimum concentration (C<sub>min</sub>) 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  $\sim 1$  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 $\mu$ 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  $\leq 0.05$  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<sub>max</sub> 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 (10<sup>9</sup> 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 $\mu$ g/mL), daptomycin 10mg/kg was 715-

222 1430 (MIC range 1-2 $\mu$ g/mL), and linezolid was 348 (MIC 1 $\mu$ 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 3log<sub>10</sub> 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 10<sup>6</sup>).(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

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Antimicrobial	MIC in mg/L <sup>a</sup>	
	<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.

<sup>a</sup>The standard inoculum was  $5 \times 10^5$  CFU/mL, and the high inoculum was  $5 \times 10^9$  CFU/mL. Data for the high inoculum are presented parenthetically.

NA = not applicable

Regimen <sup>a</sup>	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

<sup>a</sup>based on a 75 kg patient

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

Note that positive values indicate growth.

<sup>a</sup> Indicates statistically significant difference from growth control.

Antimicrobial	Mean change in bacterial density (log <sub>10</sub> 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 <sup>a</sup>	-4.28 <sup>a</sup>	-5.07 <sup>a</sup>	-2.11 <sup>a</sup>	-4.56 <sup>a</sup>	-5.86 <sup>a</sup>
Daptomycin 6mg/kg+ rifampin	-1.88 <sup>a</sup>	-2.99 <sup>a</sup>	-5.13 <sup>a</sup>	-1.84 <sup>a</sup>	-3.33 <sup>a</sup>	-5.30 <sup>a</sup>
Daptomycin 6mg/kg + gentamicin	-4.36 <sup>a</sup>	-6.02 <sup>a</sup>	-6.15 <sup>a</sup>	-2.38 <sup>a</sup>	-4.96 <sup>a</sup>	-5.05 <sup>a</sup>
Daptomycin 10mg/kg	-2.23 <sup>a</sup>	-4.17 <sup>a</sup>	-6.07 <sup>a</sup>	-3.57 <sup>a</sup>	-4.90 <sup>a</sup>	-5.63 <sup>a</sup>
Daptomycin 10mg/kg + rifampin	-1.65 <sup>a</sup>	-3.48 <sup>a</sup>	-5.46 <sup>a</sup>	-2.09 <sup>a</sup>	-3.71 <sup>a</sup>	-5.41 <sup>a</sup>
Daptomycin 10mg/kg + gentamicin	-2.32 <sup>a</sup>	-6.07 <sup>a</sup>	-5.67 <sup>a</sup>	-2.99 <sup>a</sup>	-4.08 <sup>a</sup>	-5.04 <sup>a</sup>
Linezolid	+0.02	-0.19	-0.95	+0.07	-1.08 <sup>a</sup>	-2.90 <sup>a</sup>
Linezolid + rifampin	-0.07	-0.40	-1.96 <sup>a</sup>	+0.45	+0.48	-0.79
Linezolid + gentamicin	+0.13	-0.15	-0.88 <sup>a</sup>	-0.14	-0.67 <sup>a</sup>	-4.08 <sup>a</sup>

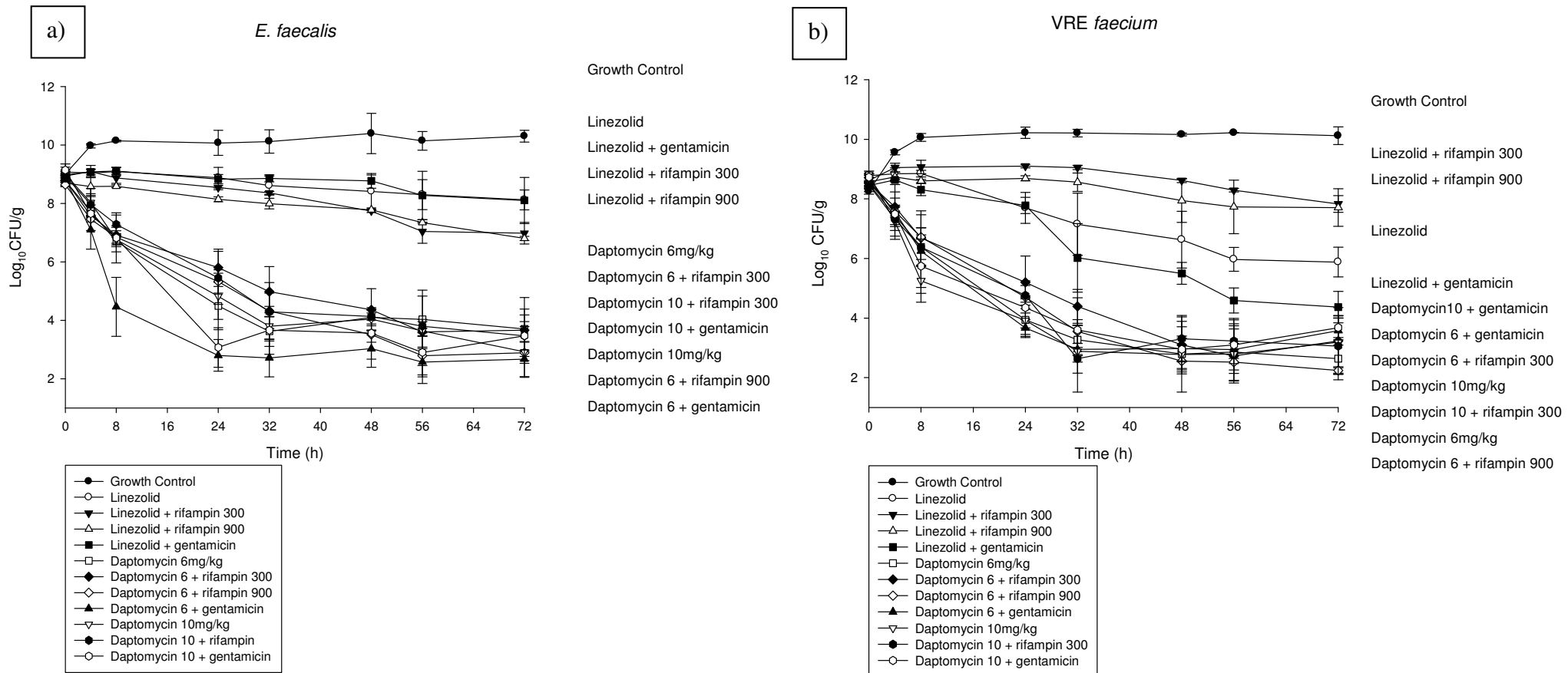


Figure 1. The activity (change in log<sub>10</sub> CFU/g) of daptomycin- or linezolid- containing regimens against a) *Enterococcus faecalis*. (vancomycin- susceptible, gentamicin- susceptible, rifampin- susceptible, daptomycin- susceptible, linezolid- susceptible) or b) *Enterococcus faecium* (vancomycin- resistant, gentamicin- susceptible, rifampin- resistant, daptomycin- susceptible, linezolid- susceptible).

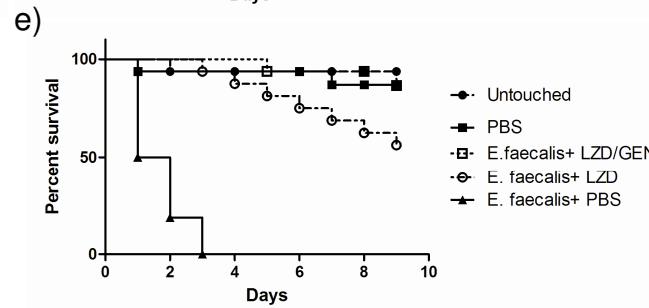
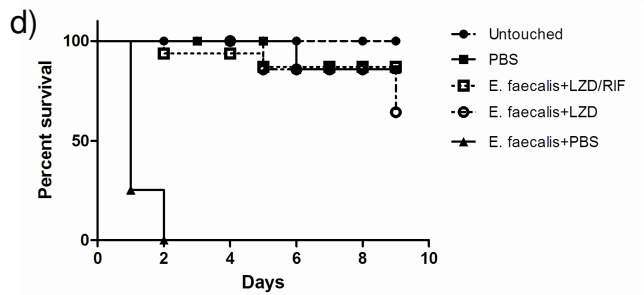
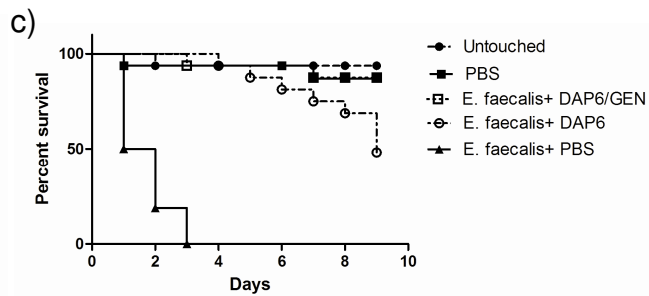
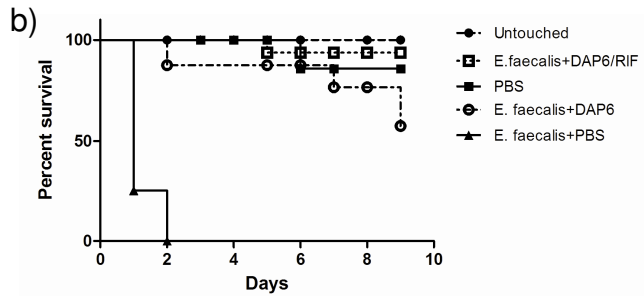
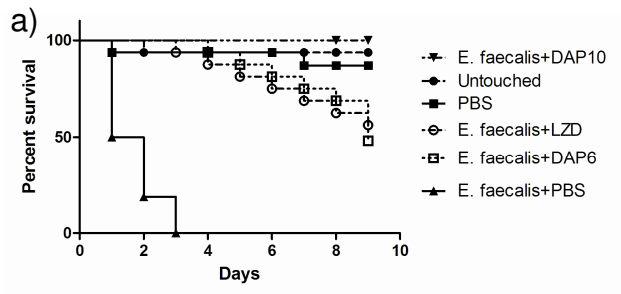


Figure 3. Efficacy of compounds against *E. faecalis* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecalis* followed by injection of the relative drug. Survival proportion with a) monotherapy of daptomycin 6mg/kg, daptomycin 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.



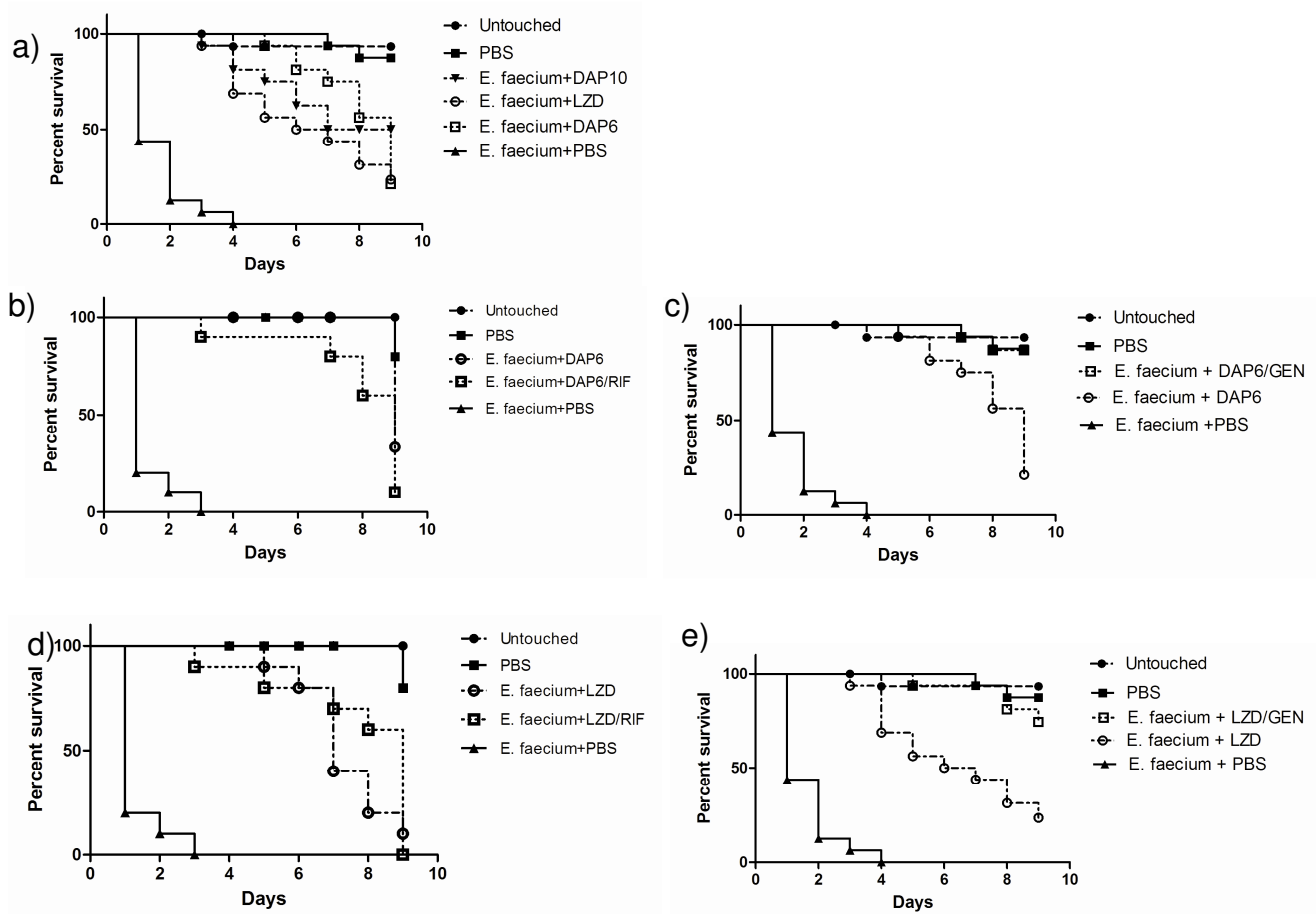


Figure 4. Efficacy of compounds against *E. faecium* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecium* followed by injection of the relative drug.

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 <sup>a</sup>
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.

<sup>a</sup> Linezolid concentrations were lower than targeted due to limits on the available pharmaceutical concentrations.