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

Introduction.

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

In vitro pharmacodynamic infection model with Simulated Endocardial Vegetations (SEVs). As previously described, organism stocks containing approximately 10^{10} CFU/mL were prepared by inoculating 5mL test tubes of normal saline with colonies harvested from fresh overnight growth on TSA.(20, 22, 24, 31, 32) SEVs containing 10^{9} CFU/g were prepared by combining 0.05mL of the organism suspension with 0.4mL of human cryoprecipitate antihemolytic factor (AHF) from volunteer donors (Rhode Island Blood Bank, Providence, RI), 0.05mL of aprotinin suspension, and 0.025 mL of platelet suspension (platelets mixed with normal saline, 250,000 to 500,000 platelets per clot) in 1.5 mL eppendorf tubes. Bovine thrombin (5,000 units/mL, 50 µL), 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^{o}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^{o}C for 24 hours before colony count enumeration. This method
120 results in a lower limit of detection of 2.0 $\log_{10}$ 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^{o} C for 24 hours.
124 Colonies were counted on filter paper; the limit of detection is 1.0 $\log_{10}$ 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

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

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

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

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

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

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

modifications (35, 36). Sixteen larvae of appropriate weight (0.25-0.35g) were randomly selected to comprise each group. Larvae were inoculated with either ~4x10^6 CFU of *E. faecalis* or 7-9x10^6 CFU of *E. faecium* followed by tested drug, or PBS as control ~1 hour after inoculation. These inocula were chosen after an initial virulence pilot study of these strains, as they were able to kill at least 90% of the larvae within 72h. One group, injected twice with PBS, and one untouched group were used as controls in each experiment. All injections were performed with a volume of 10µL using a Hamilton syringe. After injection, *G. mellonella* were incubated at 37°C and survival was measured daily. Each experiment was repeated at least twice and representative experiments are presented. Any experiment with more than two dead larvae in any control group was discarded. Doses simulated free peak concentrations seen in humans of daptomycin 6mg/kg, daptomycin 10mg/kg, or linezolid 600mg (Table 4). Gentamicin 1.3mg/kg and rifampin 300mg were also tested in combination with either linezolid or daptomycin 6mg/kg.

Statistical Analysis. For the in vitro model, changes in CFU/g at 8, 24, 48, and 72 hours and time to 99.9% kill were compared by two-way analysis of variance with Tukey's Post-Hoc test. Statistical analyses were performed using SPSS Statistical Software (Release 20 SPSS, Inc., Chicago, IL). Survival in the *G. mellonella* model was plotted using Kaplan-Meier curves, and groups were compared using log-rank test (GraphPad Prism 5 software). For all 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 Cmax 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 (10^9 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 1-2µg/mL), daptomycin 10mg/kg was 715-

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

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

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

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

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

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

DISCUSSION

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

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

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

Studies have shown that daptomycin demonstrates activity in enterococcal infections, and may provide an option in patients with allergies or contraindications to other therapies. In a retrospective cohort study of VRE bloodstream infections, treatment with daptomycin or linezolid demonstrated no difference in mortality; however, infection with *E. faecium* and concurrent treatment with rifampin or gentamicin were independent risk factors for mortality.(54) Antagonistic activity is often observed when rifampin is added to bactericidal agents in high inoculum infections, due to high rates of mutations conferring resistance (~1 in 10^6).(31, 55, 56) The in vitro model demonstrated antagonism with rifampin. The in vivo model used a lower bacterial burden, so antagonism from rifampin resistance may not be as evident. In contrast, previous in vitro studies have shown synergy with daptomycin and rifampin, and non-antagonism with daptomycin and gentamicin.(6)

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

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

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

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

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

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

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

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Conflicts of Interest and Disclosures

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Antimicrobial	MIC in mg/L [a]	
	E. faecalis ATCC 29212	*E. faecium* 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.
[a] The standard inoculum was $5x10^5$ CFU/mL, and the high inoculum was $5x10^9$ 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
[a]based on a 75 kg patient

TABLE 3. Inoculum change from starting inoculum of $5x10^9$ 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.

	Mean change in bacterial density (log_{10} CFU/g)					
Antimicrobial	*E. faecalis*			*E. faecium*		
	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]

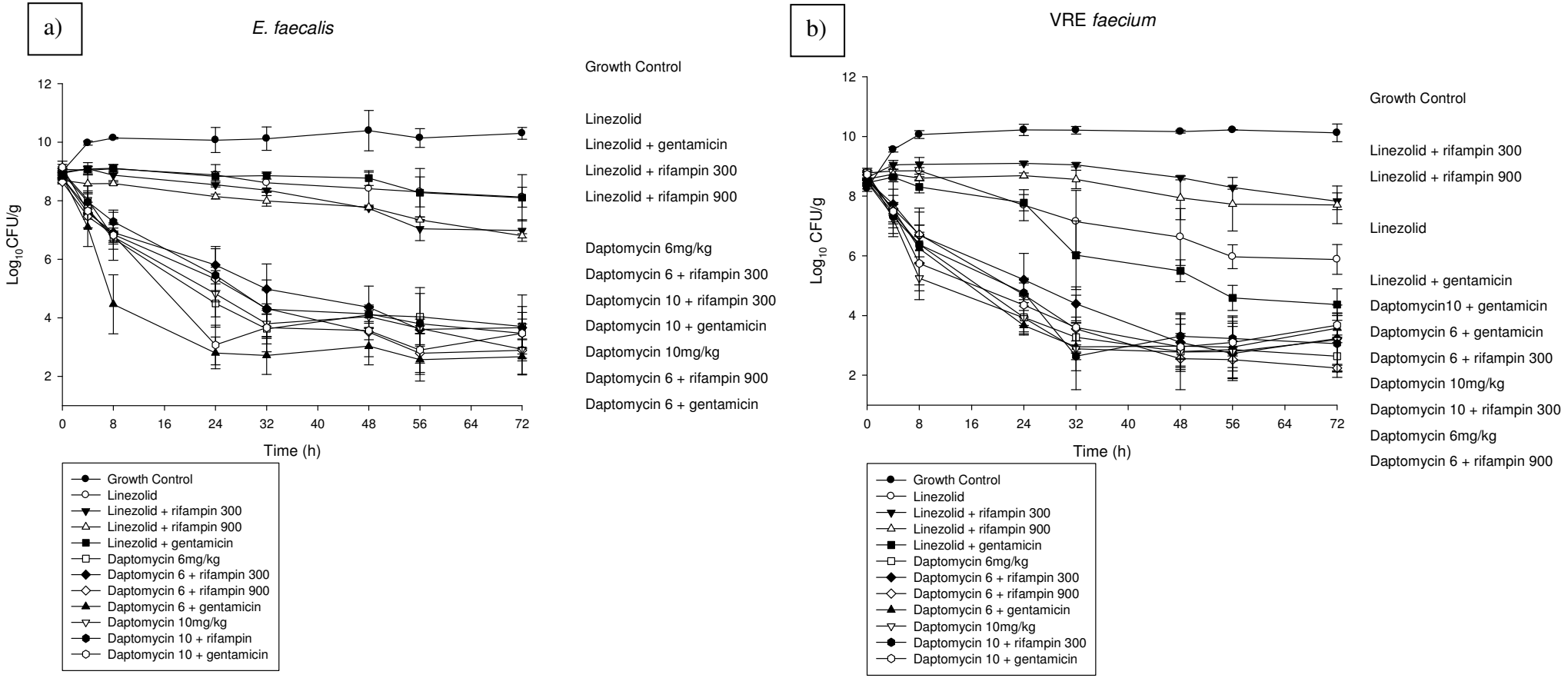

Figure 1. The activity (change in $\log_{10}$ CFU/g) of daptomycin- or linezolid- containing regimens against a) *Enterococcus faecalis*. (vancomycin- susceptible, gentamicin- susceptible, rifampin- susceptible, daptomycin- susceptible, linezolid- susceptible) or b) *Enterococcus faecium* (vancomycin- resistant, gentamicin- susceptible, rifampin- resistant, daptomycin- susceptible, linezolid- susceptible).

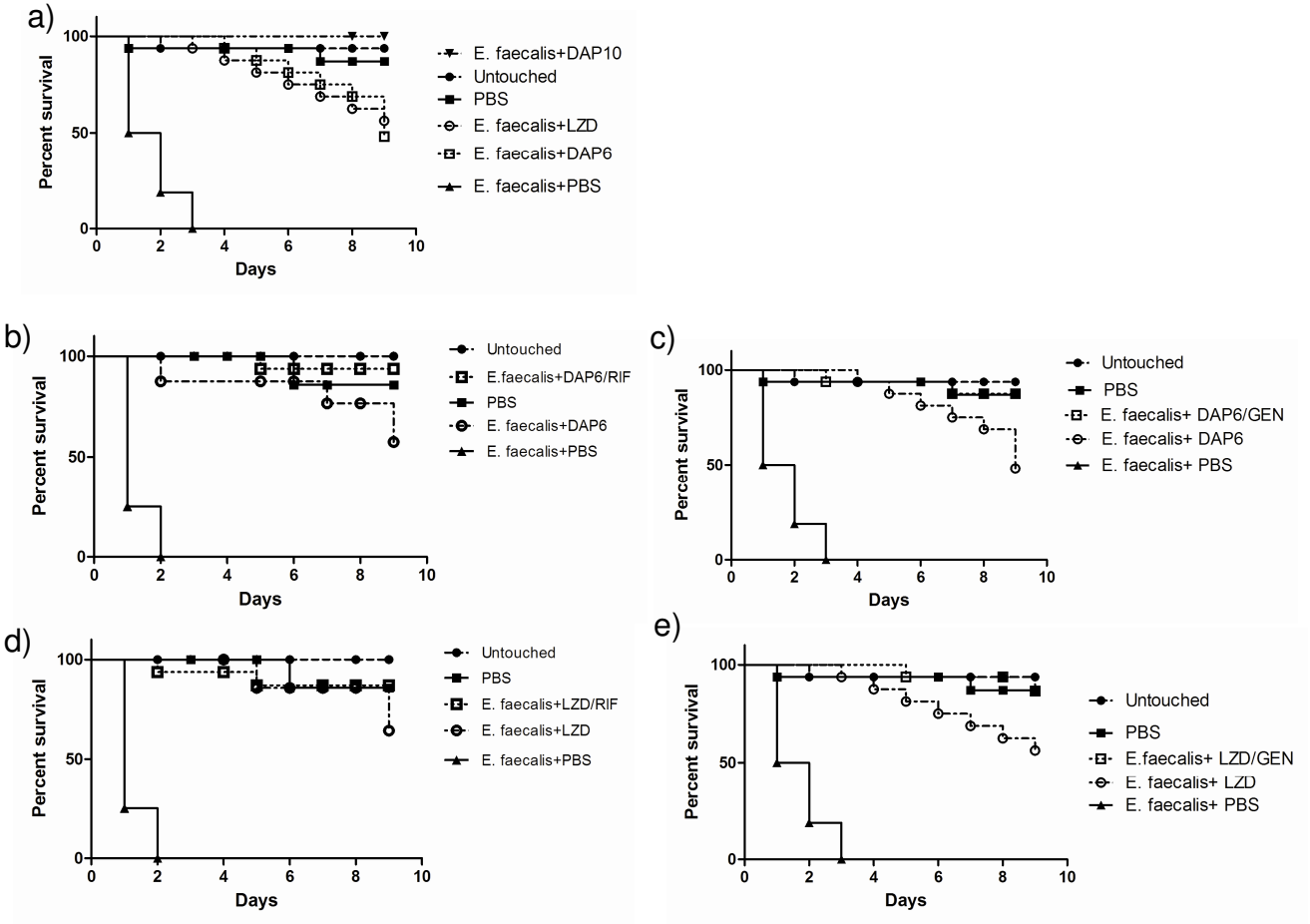

Figure 3. Efficacy of compounds against *E. faecalis* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecalis* followed by injection of the relative drug.
Survival proportion with a) monotherapy of daptomycin 6mg/kg, daptomycin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

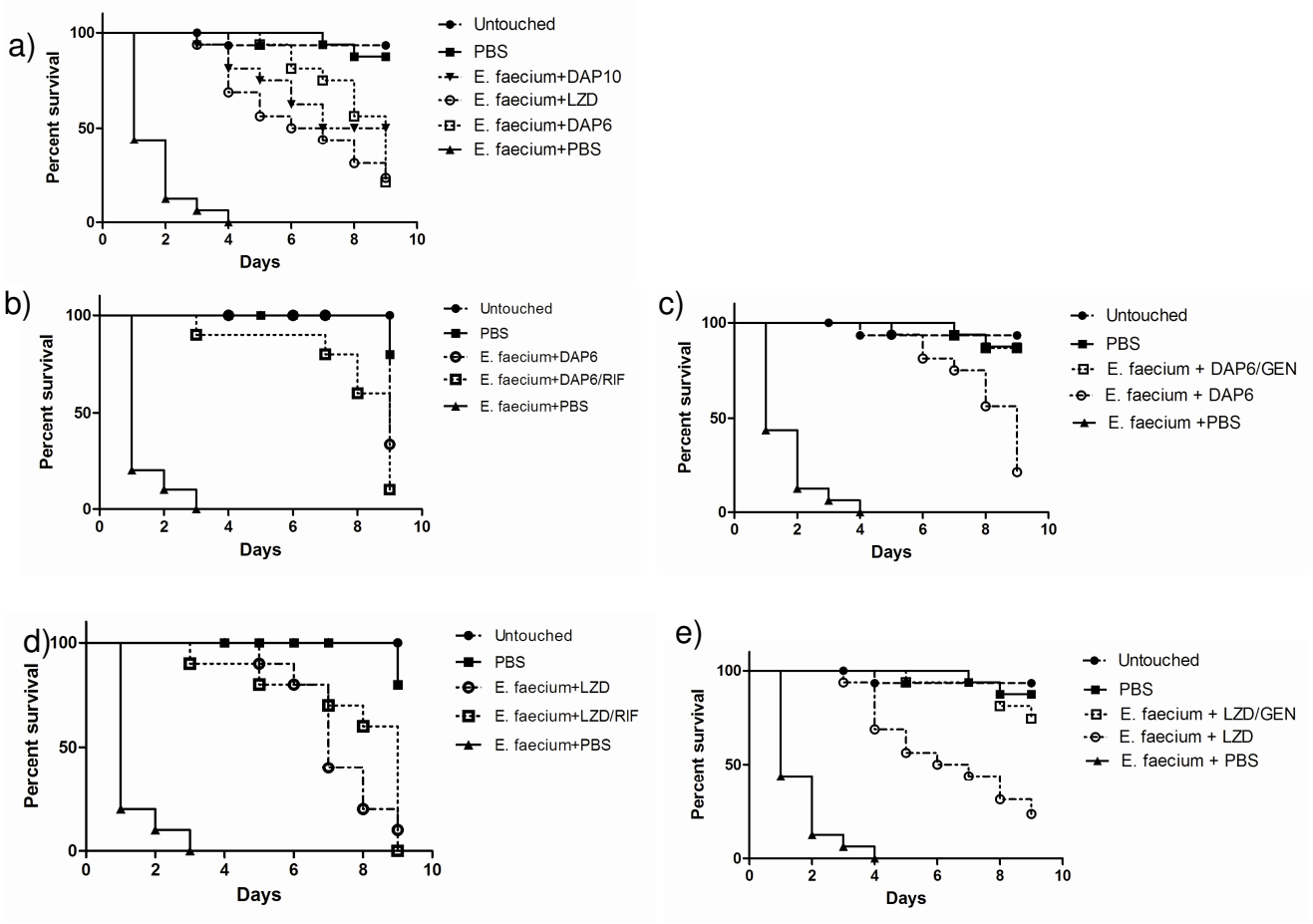

Figure 4. Efficacy of compounds against *E. faecium* on a *G. mellonella* infection model. Each line on the graph represents the survival of a group of 16 larvae injected with *E. faecium* followed by injection of the relative drug.
Survival proportion with a) monotherapy of daptomycin 6mg/kg, daptomycin 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 *G. mellonella* (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.