An adjunctive therapy approach prevents antibiotic resistance emergence in opportunistic pathogens colonizing the gut

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Abstract: Therapeutic antibiotic use drives the spread of antibiotic resistance, a major threat to public health. Ideally, clinicians could treat infections with antibiotics without fueling transmission of resistant pathogens. Here, we show proof of concept for an adjunctive therapy approach that allows treatment of target pathogens without the emergence and onward transmission of resistance. Like many of the bacterial species responsible for the antimicrobial resistance crisis, vancomycin-resistant *Enterococcus* (VRE) is a colonizing opportunistic pathogen and an important cause of drug-resistant healthcare-associated infections. VRE causes life-threatening infections in the bloodstream, but spreads via fecal-oral transmission because it asymptomatically colonizes the gastrointestinal (GI) tract. Thus, there is a physical separation between the VRE targeted by treatment (those in the blood) and the VRE contributing to onward transmission (those in the GI tract). An oral adjuvant that can bind or inactivate antibiotic in the GI tract would make possible intravenous patient treatment without promoting transmissible resistance. We tested this idea in a mouse model of VRE GI tract colonization using cholestyramine, which we show binds daptomycin, one of the few remaining front-line antibiotics against VRE. Adjunctive cholestyramine therapy reduced the fecal shedding of daptomycin-resistant VRE by up to 80-fold in mice treated with daptomycin. These results provide proof of concept for an approach that could reduce the spread of antibiotic resistance for many important hospital pathogens.

Introduction

Vancomycin-resistant *Enterococcus faecium* (VR *E. faecium*) is an important cause of antibiotic-resistant infections in healthcare settings (Arias and Murray, 2012; García-Solache and Rice, 2019; O'Driscoll and Crank, 2015). The antibiotic daptomycin is one of the few remaining first-line therapies for VRE infection (O'Driscoll and Crank, 2015), but daptomycin-resistance is spreading in VRE populations (Judge et al., 2012; Kamboj et al., 2011; Kinnear et al., 2019; Woods et al., 2018). Therapeutic daptomycin use is thought to be a key driver of resistance (Kinnear et al., 2020; Woods et al., 2018). Managing the evolution of daptomycin-resistance in healthcare settings is crucial to future control of VRE infections.

E. faecium is an opportunistic pathogen that colonizes the human GI tract asymptomatically, spreads via fecal-oral transmission, and causes symptomatic infections when

introduced to sites like the bloodstream or the urinary tract (Arias and Murray, 2012). *E. faecium* colonizing the gut may be exposed to daptomycin during therapeutic use, potentially contributing to the transmission of daptomycin-resistant *E. faecium*. Daptomycin is administered intravenously to treat infections caused by pathogens including VRE and *Staphylococcus aureus*. Daptomycin is primarily eliminated by the kidneys, but 5-10% of the dose enters the intestines through biliary excretion (Woodworth et al., 1992). We hypothesize that this therapeutically unnecessary intestinal daptomycin exposure could drive resistance evolution in *E. faecium* colonizing the gut. Increased resistance in colonizing populations is important, because gut *E. faecium* populations are sources for nosocomial infections and transmission between patients (Olivier et al., 2015).

If unintended intestinal daptomycin exposure drives resistance evolution in *E. faecium*, this offers an opportunity to intervene. The opportunity emerges from a key feature of this system—the bacteria causing infection are physically separated from the population contributing to transmission. If daptomycin could be inactivated in the intestine without altering plasma concentrations, daptomycin could be used to kill bacteria at the target infection site without driving resistance in off-target populations. Preventing resistance evolution in these reservoir populations could protect patients from acquiring resistant infections, and it could limit the shedding of resistant strains and so onward transmission to other patients. We hypothesized that co-administering an oral adjuvant that reduces daptomycin activity would prevent selection for daptomycin resistance in the gut during systemic daptomycin treatment. We tested this strategy using the adjuvant cholestyramine in a mouse VR *E. faecium* gut colonization model.

Results

Generation of daptomycin-resistant VR E. faecium in the mouse GI tract

To directly test the proposition that systemic daptomycin treatment could select for resistance in the GI tract, and to generate daptomycin-resistant VR *E. faecium* mutants for subsequent experiments, we inoculated mice orally with daptomycin-susceptible VR *E. faecium* strains. Beginning one day after *E. faecium* inoculation, mice received daily doses of either subcutaneous daptomycin (50, 100, or 400 mg/kg), oral daptomycin (5, 50, 100, or 400 mg/kg), or a control mock injection for five days. We used a range of doses and routes of administration to maximize the likelihood of observing resistance emergence in one of the mice. The 50 and

100 mg/kg subcutaneous doses were selected to generate pharmacokinetics similar to clinical human doses (Mortin et al., 2007; Samonis et al., 2008), and the 5mg/kg oral approximates the 5-10% of a daptomycin dose that is secreted into the intestines during standard intravenous treatment (Woodworth et al., 1992). We used two susceptible VR *E. faecium* strains, BL00239-1 (MIC_c = 2.0 (Minimum Inhibitory Concentration computed, see Methods)) and PR00708-14 (MIC_c = 2.7), which were originally isolated at the University of Michigan Hospital from a clinical bloodstream infection and a different patient's clinical perirectal swab, respectively. Mouse fecal samples were collected to quantify VR *E. faecium* shedding and determine daptomycin susceptibility of isolated *E. faecium* clones.

Only very high daptomycin doses (400 mg/kg subcutaneous, ≥50 mg/kg oral) consistently reduced fecal VR *E. faecium* below the level of detection during treatment; with lower doses, VR *E. faecium* shedding was often detectable throughout treatment (Figure 1A). For Strain BL00239-1, *E. faecium* clones with increased daptomycin resistance were isolated from two of three mice following treatment with 100 mg/kg subcutaneous daptomycin (Figure 1B-C). We chose one of these daptomycin-resistant clones to use in subsequent experiments (strain BL00239-1-R, MIC_c = 8.6). Sequencing of the core genome showed that the resistant strain acquired a mutation in the major cardiolipin synthase *clsA* gene (R211L, CGA→CTA), which has been previously described in association with daptomycin resistance (Adams et al., 2015), and a transposon insertion into the methionine sulfoxide reductase *msrA* gene (Zhao et al., 2010). For the second strain, mice were treated with subcutaneous daptomycin (50, 100, or 200 mg/kg) or a mock injection. We screened for the emergence of increased resistance by plating mouse fecal suspensions on daptomycin-supplemented agar. Samples from two mice treated with 200 mg/kg daptomycin produced colonies on daptomycin-supplemented plates, and we isolated a resistant clone from one of these samples. We confirmed that the isolated clone (PR00708-14-R) had an increased daptomycin MIC_c relative to PR00708-14 by broth microdilution (MIC_c = 12.0). Genome sequencing revealed that PR00708-14 and PR00708-14-R differed by several mutations in hypothetical proteins and noncoding regions, which to our knowledge have not previously been associated with daptomycin resistance. These experiments show that daptomycin resistance can emerge *de novo* in *E. faecium* colonizing the GI tract following systemic daptomycin treatment.

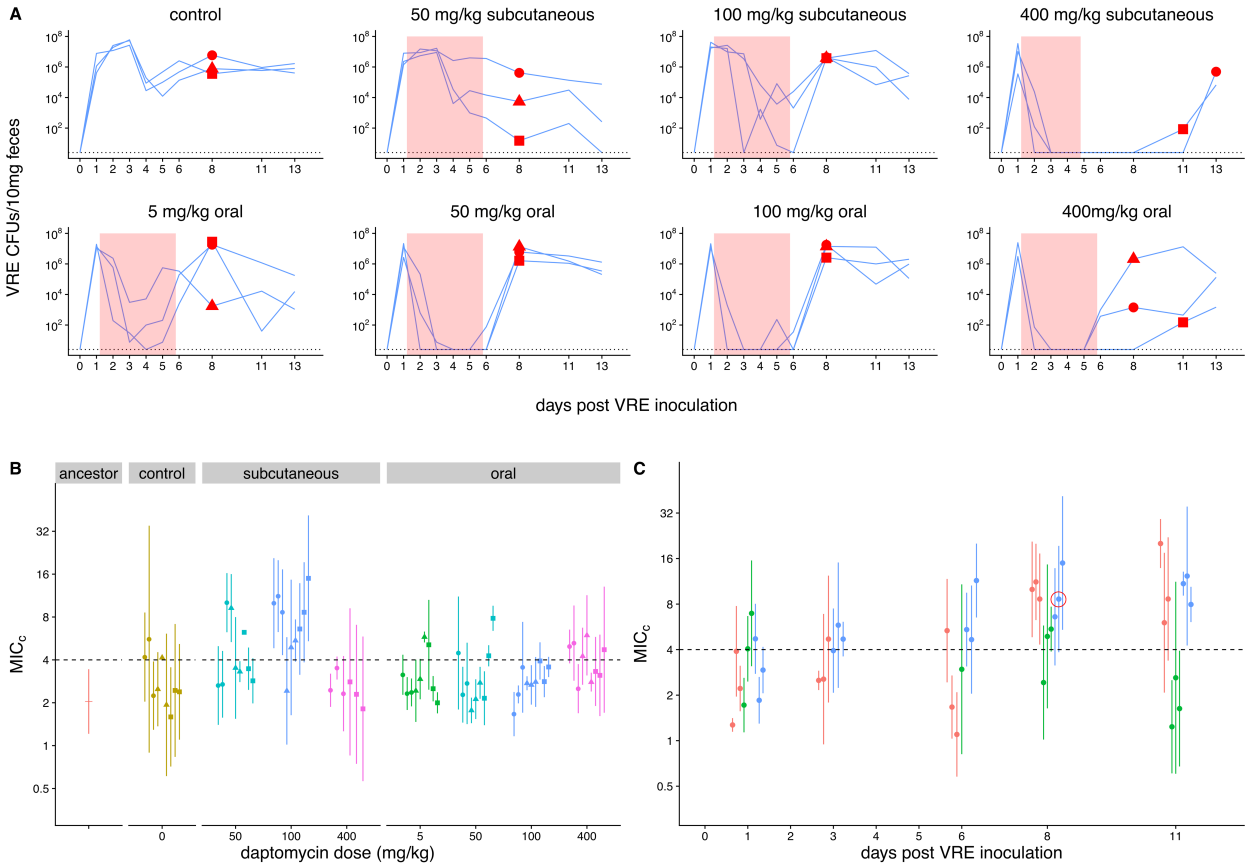

Fig. 1. Emergence of daptomycin-resistant VR *E. faecium* in mouse GI tracts following subcutaneous daptomycin treatment. **(A)** VR *E. faecium* densities in fecal samples during and after daptomycin treatment (Strain BL00239-1). Each line represents VR *E. faecium* densities from an individual mouse (n=3 per treatment). The pink shaded region indicates days of daptomycin therapy. The dotted line marks the detection limit. Red dots indicate time points where clones were isolated for analysis shown in Panel B. The 400 mg/kg subcutaneous treatment was discontinued after 4 days due to apparent toxicity, and one mouse in this treatment was euthanized at Day 4. **(B)** Following daptomycin treatment, three VR *E. faecium* clones were isolated from the feces of each mouse. Each point shows the daptomycin susceptibility of a single clone (mean of three measurements with 95% CI). Point shape indicates the mouse of origin. The dashed line marks the clinical breakpoint for daptomycin susceptibility. The ancestral clone (BL00239-1) used to inoculate mice is also shown. **(C)** For the 100 mg/kg subcutaneous treatment, VR *E. faecium* clones were isolated from each mouse at multiple time points. Each point shows the daptomycin susceptibility of a single clone (mean of three measurements with 95% CI). Color indicates mouse of origin. The dotted line marks the clinical breakpoint for daptomycin susceptibility. The resistant clone used in subsequent experiments (BL00239-1-R) is circled in red.

Daptomycin treatment enriches for daptomycin-resistant VR E. faecium in the GI tract

We used the *de novo* resistant mutants isolated above (Figure 1) to test whether daptomycin therapy selects for daptomycin-resistance in intestinal VR *E. faecium* populations when a resistant mutant is already present. We orally inoculated mice with a 1:20 mixture of the experimentally generated daptomycin-resistant and susceptible VR *E. faecium* strains (BL00239-1-R and BL00239-1). Mice were treated with subcutaneous daptomycin (50, 75, 100, or 200 mg/kg) for five or ten days after VRE inoculation. Control mice received either a mock saline injection or no injection. Fecal samples from Days 8 and 14 post-inoculation were plated in triplicate to quantify total VR *E. faecium* density, and samples were also plated on daptomycin-supplemented agar to estimate the proportion of VR *E. faecium* that were daptomycin-resistant. Control populations remained susceptible to daptomycin, but all doses and durations of daptomycin dramatically enriched for resistance in the GI tract (Figure 2A-B). At both time points, controls had significantly lower proportions of resistant bacteria than daptomycin-treated mice (Fig 2A-B; mixed effects negative binomial regression, $p < 0.001$). The absolute numbers of VRE enumerated in fecal samples at Day 8 and Day 14 did not vary significantly between treatments (negative binomial regression) (Supplemental Figure 1).

The dramatic enrichment for daptomycin-resistant VR *E. faecium* in treated mice shows that subcutaneously-administered daptomycin produced GI tract concentrations high enough to select for resistance. To quantify fecal daptomycin concentrations, we analyzed fecal samples from a subset of daptomycin-treated mice by liquid chromatography-mass spectrometry (LC-MS) (Figure 2B). Samples from all time points tested (Days 2, 6, and 8) contained detectable daptomycin, and concentrations generally peaked at the end of treatment (Day 6). Higher daptomycin doses generally corresponded to higher fecal concentrations, but concentrations were highly variable and overlapped between treatments. While fecal VR *E. faecium* densities correlated poorly with the daptomycin dose administered (Supplemental Figure 1), fecal VR *E. faecium* densities correlated with the amount of daptomycin recovered in feces (Figure 2D). These data confirmed that subcutaneously-administered daptomycin at our experimental doses generated a range of daptomycin concentrations in the GI tract that included inhibitory concentrations for the susceptible VR *E. faecium* strain.

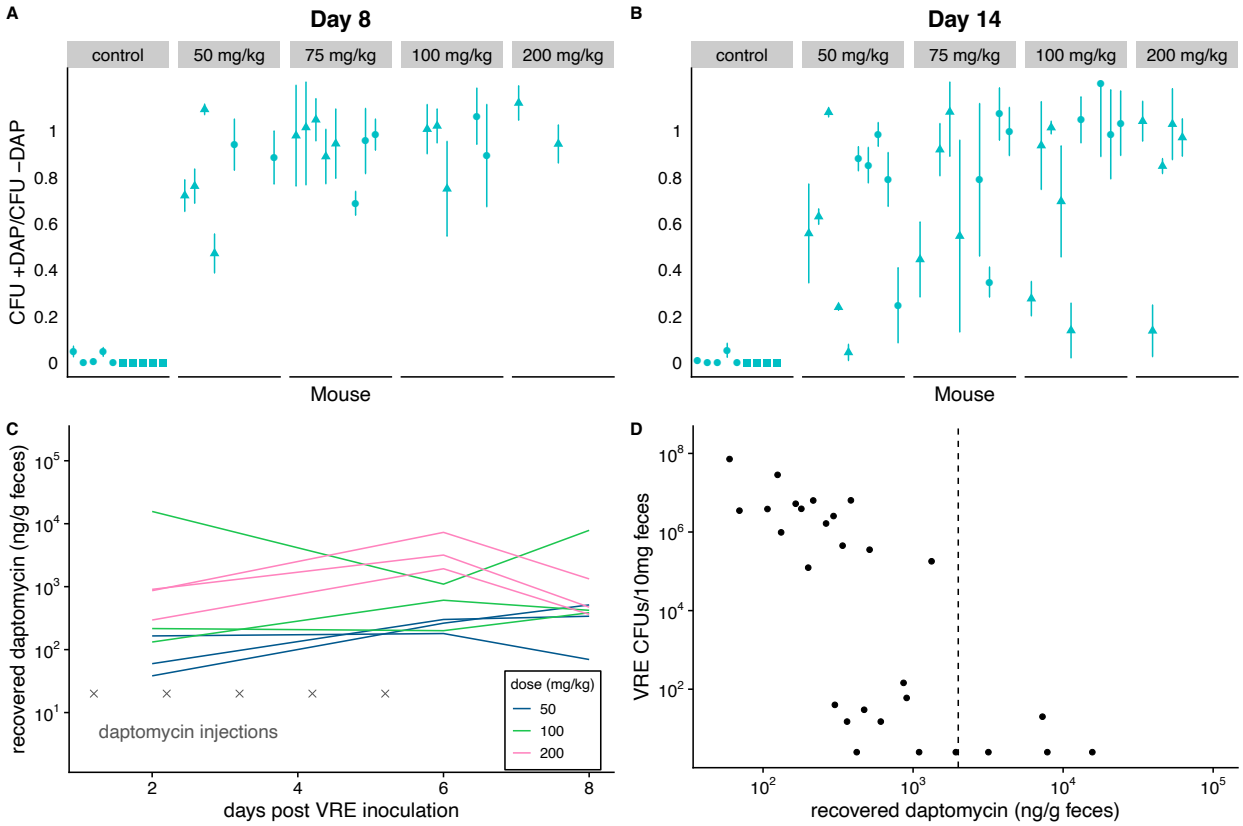

Fig. 2. Subcutaneous daptomycin treatment selects for resistance in the GI tract. **(A-B)** Mouse fecal suspensions were plated on Enterococcus-selective plates with daptomycin (+ DAP) and without daptomycin (- DAP) at Day 8 **(A)** and Day 14 **(B)**. Each point represents the mean of triplicate measures from a single mouse sample with 95% CI. Mice were treated with daptomycin for 5 days (triangles) or 10 days (squares) at the doses listed at times denoted (gray crosses; N = 5 mice per treatment). Samples with VR *E. faecium* density < 3 x 10^3 CFU/10 mg feces were not plated for this assay due to insufficient bacterial density. **(C)** Recovered fecal daptomycin measured by LC-MS for a subset of mice. Each line tracks daptomycin measurements from a single mouse sampled at Days 2, 6, and 8. **(D)** For the subset of fecal samples analyzed by LC-MS, fecal daptomycin plotted against fecal VR *E. faecium* densities (samples from all available treatments and time points plotted together). Dotted line indicates the MIC of the susceptible strain BL00239-1 (MIC=2 µg/mL or 2 µg/g).

We ran two additional experiments to further investigate the competitive dynamics between this susceptible and resistant pair (BL00239-1 & BL00239-1-R) in the presence and absence of daptomycin treatment. First, we tested whether susceptible bacteria competitively suppressed resistant bacteria in the GI tract. We inoculated mice with either a mixture of 10^8 CFU susceptible + 10^3 CFU resistant VR *E. faecium*, or with a resistant-only inoculum at one of two inoculum sizes (10^8 CFU or 10^3 CFU). Mice received 5 days of subcutaneous daptomycin injections at 100 mg/kg or control saline injections. Shedding of resistant and susceptible bacteria were quantified at time points throughout the experiment by plating (Figure 3A). In the absence of daptomycin treatment, the daptomycin susceptible strain remained the most prevalent in mixed populations. When mixed populations were exposed to daptomycin, resistance increased to high frequency in three populations, and the population size fell dramatically in the remaining two populations. In mice inoculated with only 10^3 CFU resistant bacteria, the resistant clone was able to grow to high numbers with or without daptomycin. These data were consistent with the competitive suppression of the resistant strain by the susceptible strain in the absence of daptomycin treatment, and competitive release of the resistant strain during daptomycin treatment (Day et al., 2015; Wargo et al., 2007).

Next, we tested whether the frequency of daptomycin-resistant VR *E. faecium* would decrease over time in the absence of daptomycin treatment, potentially indicating that daptomycin resistance comes at a fitness cost. We inoculated mice with a 1:5 mixture of daptomycin resistant and susceptible VR *E. faecium*. Mice received no daptomycin treatment. After 14 days, the proportion of resistant bacteria had declined in all mice, consistent with a competitive disadvantage (fitness cost) to the daptomycin-resistance mutation (Figure 3B).

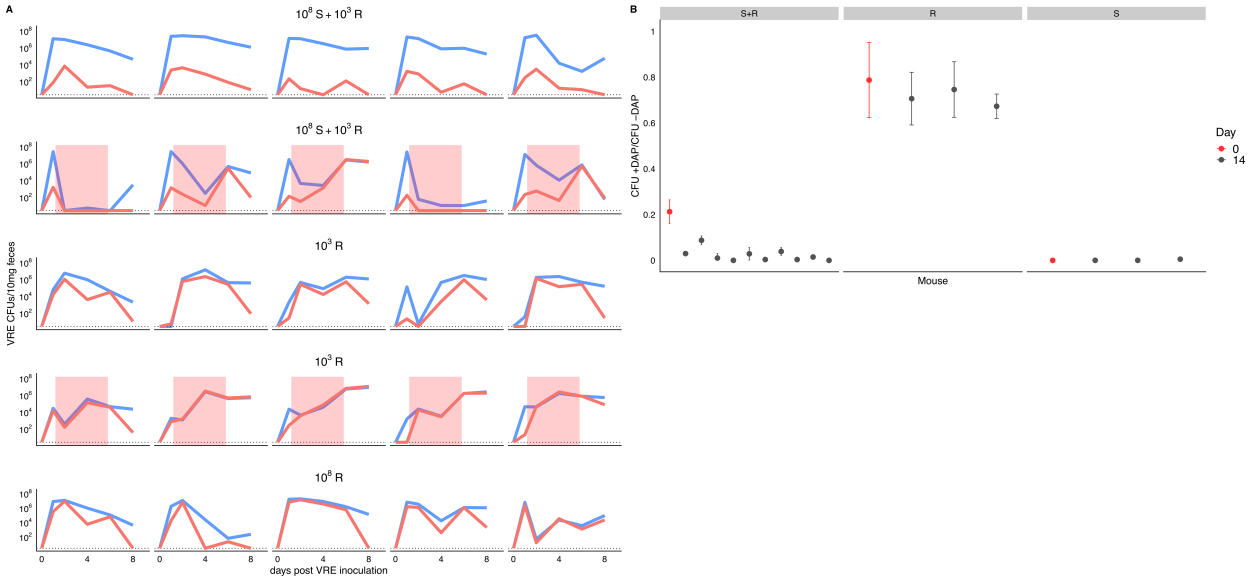

Fig. 3. Competitive dynamics between daptomycin resistant and susceptible VR *E. faecium*. **(A)** Each panel shows VR *E. faecium* counts on plates with daptomycin (+DAP, red line) and without daptomycin (-DAP, blue line) for a single mouse over time. Labels show initial inocula. Red shading indicates days of daptomycin treatment (100 mg/kg daily subcutaneous injections). **(B)** In a second experiment, mice were inoculated with a mix of susceptible and resistant bacteria (S+R, 20% R), resistant bacteria only (R), or susceptible bacteria only (S). Mice received no drug treatment. At Day 14, fecal suspensions were plated on plates with daptomycin (+DAP) and without daptomycin (-DAP) to determine whether the resistant strain had decreased in frequency. The starting inoculum dose is shown in red, and Day 14 samples from each of 10 mice are shown in gray (mean of triplicate measures with 95% CI). Note that resistant bacteria do not form colonies at 100% efficiency on +DAP plates.

In vitro characterization of cholestyramine as a potential adjuvant

If an orally-administered adjuvant could reduce daptomycin activity in the GI tract, this could prevent the emergence of daptomycin-resistant *E. faecium* in gut, potentially reducing transmission of resistant bacteria without impacting the effectiveness of intravenous daptomycin therapy. We identified cholestyramine, an FDA-approved bile-acid sequestrant, as a potential adjuvant for daptomycin therapy. Cholestyramine is a high-molecular weight anion exchange resin that binds with bile acids, forming an insoluble complex that is excreted in the feces (Jacobson et al., 2007). Cholestyramine is known to interact with a number of co-administered drugs through the same mechanism, reducing their bioactivity (Jacobson et al., 2007). We hypothesized that cholestyramine would bind daptomycin based on their chemical structures.

In vitro tests were consistent with cholestyramine binding daptomycin. Daptomycin solutions were incubated with cholestyramine, and then the cholestyramine was removed by centrifugation. The resulting supernatants were analyzed for changes in daptomycin concentration and activity. Daptomycin concentrations can be measured directly by ultraviolet (UV) absorbance at 364 nm (Figure 4A). Daptomycin concentrations were reduced in supernatants after incubation with cholestyramine in a dose-dependent manner (Figure 4B). Additionally, daptomycin solutions incubated with cholestyramine had reduced antibiotic activity against *E. faecium* in broth microdilution (Figure 4C). Together, these results were consistent with cholestyramine removing daptomycin from solution, supporting cholestyramine as a candidate adjuvant for daptomycin therapy.

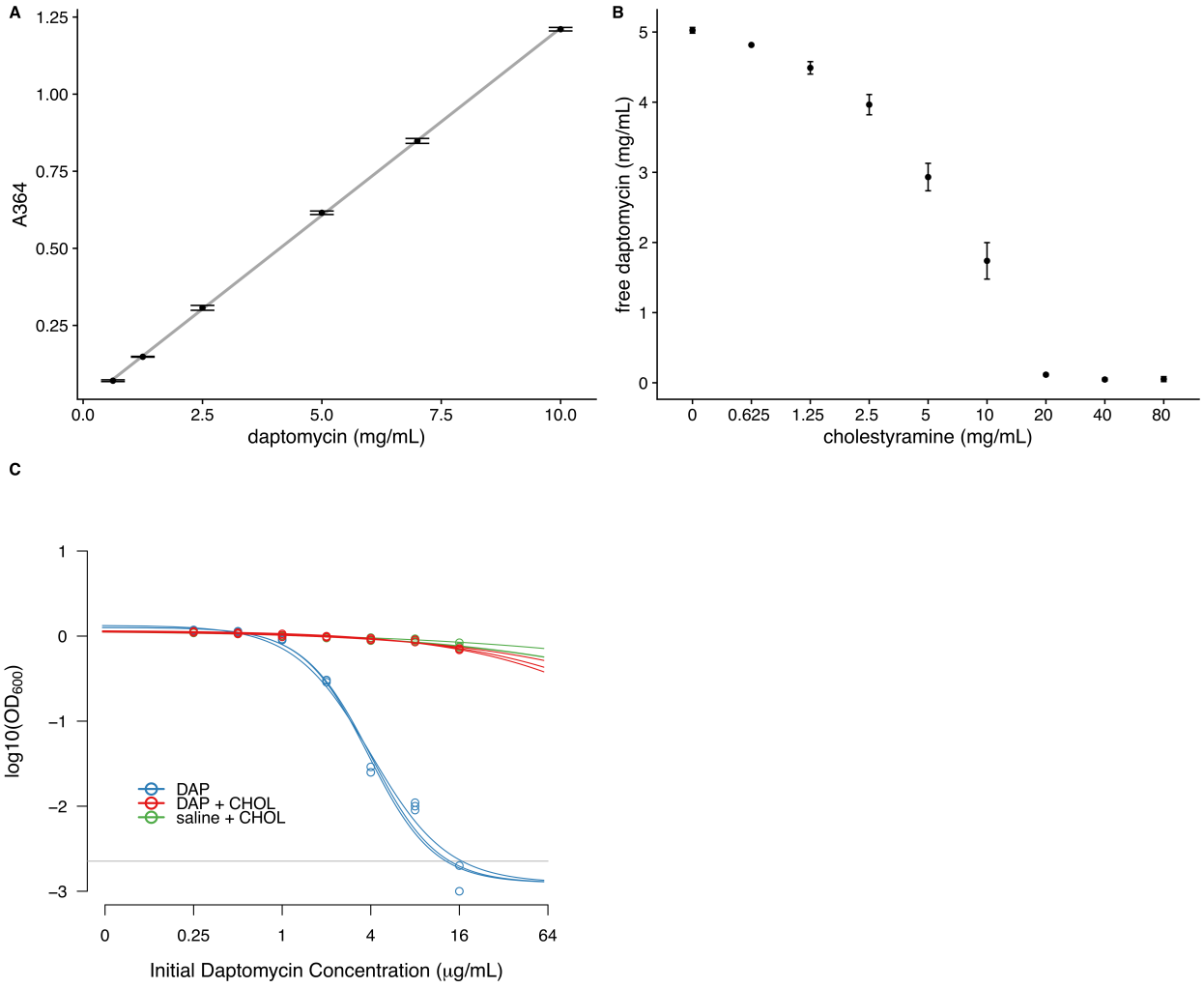

Fig. 4. Cholestyramine captures daptomycin *in vitro* **(A)** Calibration curve showing that daptomycin concentration can be measured by UV absorption at 364 nm. Mean and 95% CI shown (N=3 per concentration tested). **(B)** Daptomycin concentration was reduced in solutions treated with cholestyramine. Mean and 95% CI shown (N=3 per concentration tested). **(C)** Daptomycin solutions treated with cholestyramine had reduced biological activity against VRE in broth microdilutions (N=3 per antibiotic treatment). Bacterial densities (OD_{600}) following growth in the presence of daptomycin (DAP), daptomycin solution treated with cholestyramine (DAP + CHOL), or saline solution treated with cholestyramine (saline + CHOL) are shown. Concentrations are shown as the initial concentration of daptomycin in solution prior to cholestyramine treatment. Saline controls were constant across all listed concentrations. Horizontal line shows detection threshold.

Adjunctive cholestyramine therapy prevents emergence of daptomycin-resistance

We conducted three experiments to test whether adjunctive therapy with cholestyramine could prevent the emergence of daptomycin-resistant VR *E. faecium* in the mouse GI tract. In each experiment, mice were orally inoculated with a 1:20 mixture of daptomycin resistant and susceptible VR *E. faecium* and then treated with subcutaneous daptomycin injections for 5 days. Densities of total VR *E. faecium* and daptomycin-resistant VR *E. faecium* were determined by plating (Figure 5). The experiments tested the evolutionary impact of oral cholestyramine in three different combinations of mouse and VR *E. faecium* strains 1) Swiss-Webster mice with *E. faecium* strains BL00239-1 & BL00239-1-R (Figure 5A-B), 2) C57/B6 mice with *E. faecium* strains BL00239-1 & BL00239-1-R (Figure 5C-D), and 3) Swiss-Webster mice with *E. faecium* strains PR00708-14 & PR00708-14-R (Figure 5E-F). Data from these experiments were analyzed together, with a block effect included in the models. At Days 8 and 14, the cholestyramine-supplemented diet reduced the proportion of daptomycin-resistant VR *E. faecium* in daptomycin-treated mice (mixed effects negative binomial regression, diet*dose interaction, $p < 0.05$).

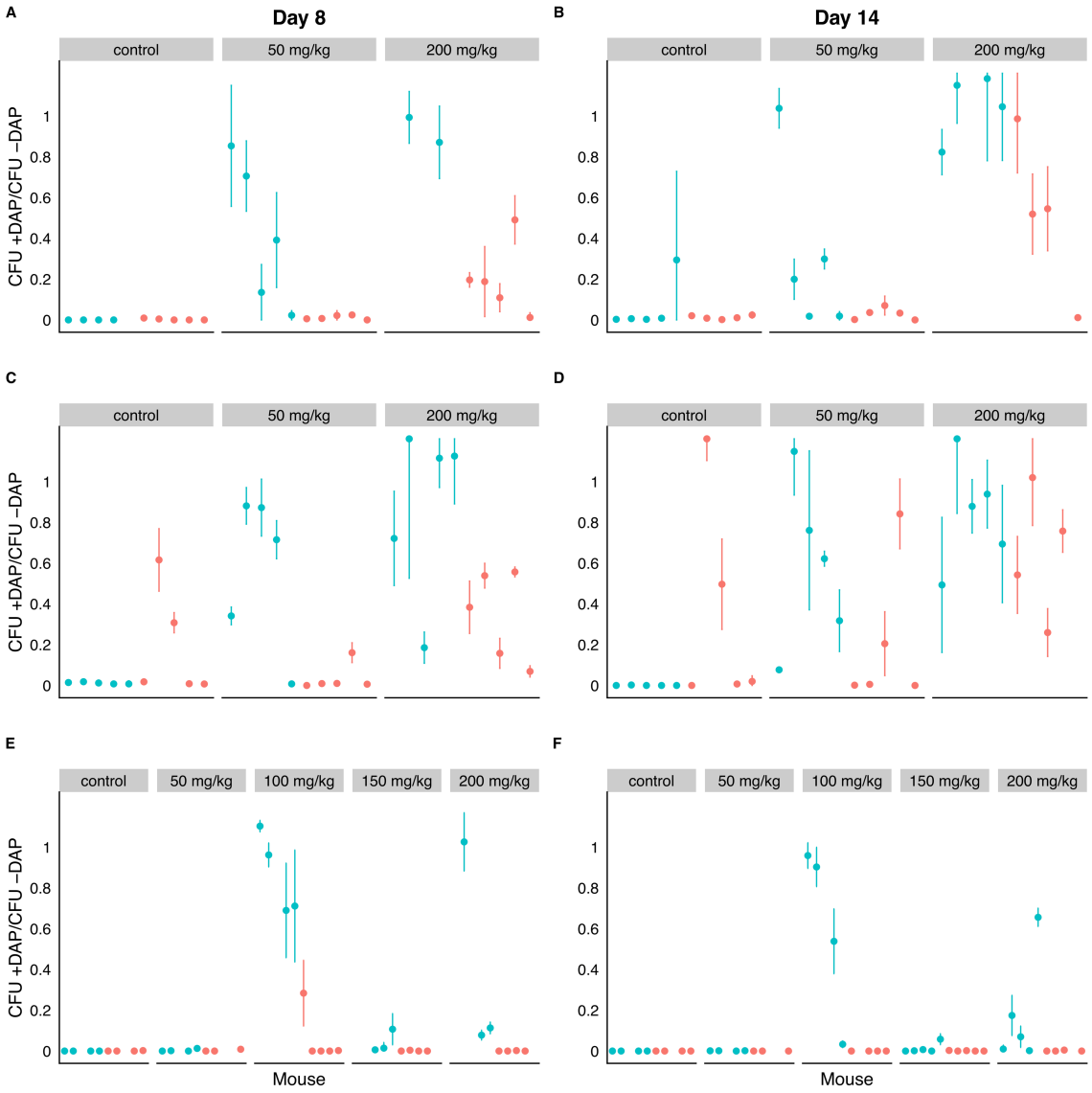

Fig. 5. Adjunctive cholestyramine prevents emergence of daptomycin resistance in GI tract. Mouse fecal suspensions were plated on Enterococcus-selective plates with daptomycin (+ DAP) and without daptomycin (- DAP) at Day 8 and Day 14. Each point represents the mean of triplicate measures from a single mouse sample with 95% CI. Blue points represent mice fed on a standard diet and red points represent mice fed a cholestyramine-supplemented diet. Mice were treated with daptomycin or saline (controls) for 5 days at the doses listed (N = 5 mice per treatment). Samples with VR *E. faecium* density < 3 x 10^3 CFU/10 mg feces were not plated for this assay due to insufficient bacterial density. Data for three experiments are shown. Values >1 are consistent with sampling variation. **(A-B)** Swiss-Webster mice colonized with strains BL00239-1 + BL00239-1-R. **(C-D)** C57/B6 mice colonized with strains BL00239-1 + BL00239-1-R. **(E-F)** Swiss-Webster mice colonized with strains PR00708-14 & PR00708-14-R.

We also quantified absolute densities of daptomycin-resistant and susceptible VR *E. faecium* over time by plating samples from Days 0, 1, 2, 4, 6, 8, and 14 (Figure 6). These data showed that the cholestyramine-supplemented diet reduced fecal shedding of daptomycin-resistant VR *E. faecium* in daptomycin-treated mice. Data for experiments 1 and 2, which both used strain BL00239-1, were analyzed together (Figure 6A-B). For strain BL00239-1, shedding of daptomycin-resistant VR *E. faecium* was suppressed by the addition of cholestyramine (diet*dose*day interaction $p < 0.01$), with greatest reduction in shedding of daptomycin-resistant VR *E. faecium* at 50 mg/kg. In that treatment, the cholestyramine diet reduced the total shedding of resistant bacteria (Days 2-14) by an average of 10-fold. The addition of cholestyramine also reduced the shedding of daptomycin-resistant VR *E. faecium* for the second strain (diet*dose*day interaction $p < 0.01$), with the greatest reduction in shedding of daptomycin-resistant VR *E. faecium* at 100 mg/kg (Figure 6C-D). In that treatment, the cholestyramine diet reduced the total shedding of daptomycin-resistant VR *E. faecium* (Days 2-14) by an average of 80-fold.

For both strains, total VR *E. faecium* shedding was influenced by the addition of cholestyramine (diet*dose*day interaction $p < 0.01$ in each case). If we consider only the control treatments, where there is no possibility of cholestyramine protecting against daptomycin killing, the addition of cholestyramine to the diet does not significantly influence total VR *E. faecium* counts alone or in combination with other factors ($p > 0.2$).

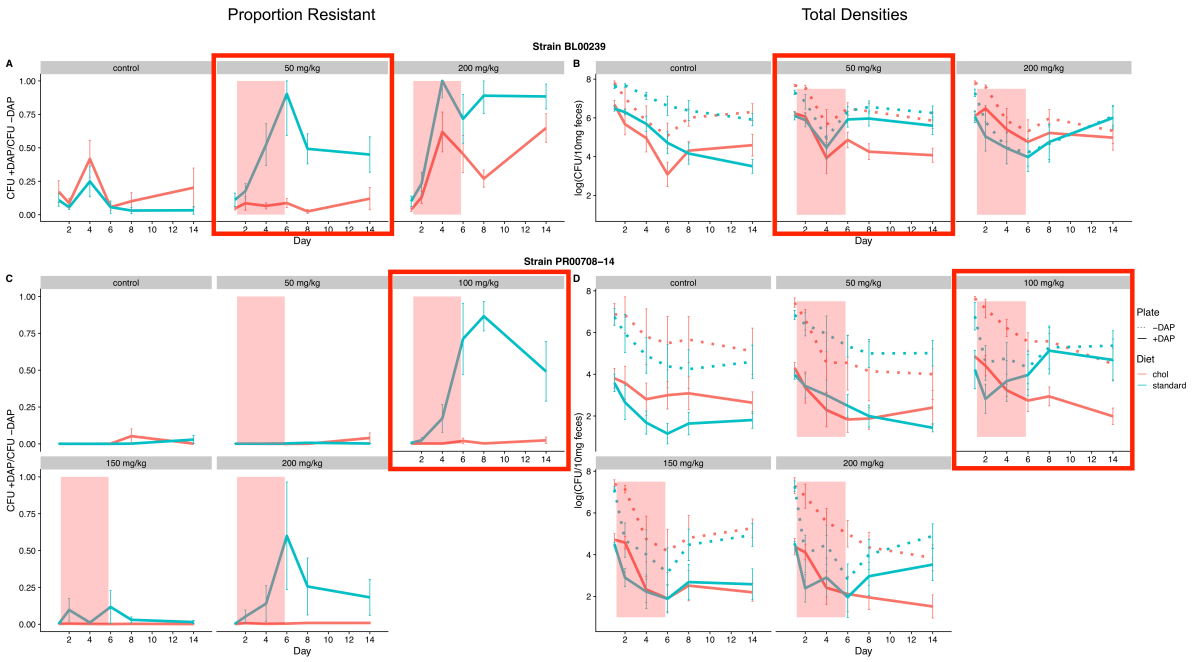

Fig. 6. Adjunctive cholestyramine prevents emergence of daptomycin resistance in GI tract. **(A)** The proportion of fecal VR *E. faecium* that were daptomycin-resistant over time for mice colonized with strains BL00239-1 + BL00239-1-R. Proportions were determined by plating on agar with daptomycin (+DAP) and without (-DAP). Data shown were combined from two experiments (total N = 10 per treatment, mean + SEM shown). Daptomycin doses are listed in panel titles. The pink shaded region indicates days of daptomycin therapy. The highlighted panels show the dose where resistance emergence was most effectively suppressed. (**B)** Total VR *E. faecium* densities (R + S) corresponding to data shown in Panel A. **(C)** The proportion of fecal VR *E. faecium* that were daptomycin-resistant over time for mice colonized with strains PR00708-14 & PR00708-14-R (total n = 5 per treatment, mean + SEM shown). (**D)** Total VR *E. faecium* densities (R+S) corresponding to data shown in Panel C.

Discussion

Here we have shown proof of concept for an adjunctive therapy approach to prevent the emergence of daptomycin-resistant *E. faecium* in the GI tract during daptomycin therapy. Ideally, this approach would allow clinicians to treat bloodstream infections with intravenous daptomycin without fueling the hospital transmission of multidrug-resistant bacteria. This would be a novel approach because the desired outcome is reduced resistance evolution and reduced transmission of resistant pathogens. With some optimization, this adjunctive approach could be implemented in hospitals. Cholestyramine is an inexpensive, FDA-approved drug with few side

effects (Beckett and Wilhite, 2015; Jacobson et al., 2007). Cholestyramine has been used clinically for over 50 years, including in hospital patient populations that would be targets for adjunctive cholestyramine therapy (Guyton and Goldberg, 2009). Cholestyramine does have potential to interfere with other orally administered drugs, which could be a risk considered on a patient by patient basis (Scaldaferri et al., 2013). This drug-drug interaction potential is managed in practice by administering oral drugs an hour before or 4 to 6 hours after cholestyramine administration. Because the effects and risks of cholestyramine are well understood, testing cholestyramine as an adjuvant with daptomycin in human trials is appealingly low risk to patient health while offering large potential gains to hospital infection control.

In addition to binding antibiotics, cholestyramine sequesters bile acids in the intestine, which alters the GI tract environment. This means that cholestyramine treatment could indirectly affect bacteria in the GI tract through mechanisms other than direct interactions with drugs. For example, exposure to bile acids has implications for *E. faecium* phenotypes. Secondary bile acids trigger a morphotype switch in *Enterococcus* that facilitates intestinal colonization and biofilm formation (McKenney et al., 2019). In our experiments, we did not observe differences in colonization efficiency or VR *E. faecium* counts in mice treated with cholestyramine alone. In mouse models, cholestyramine has also been shown to reduce bile acid-mediated resistance to *Clostridium difficile* infection, which is a possible risk associated with cholestyramine treatment (Buffie et al., 2015).

Here we tested cholestyramine as an adjuvant to reduce selection for antibiotic resistance in the GI tract, but other adjuvants could be used or designed for the same purpose. At least two other drugs that reduce antibiotic activity in the GI tract are currently in development. DAV-132, a formulation of activated charcoal encased in zinc-pectinate beads, was shown to site-specifically bind antimicrobials in the gut in a stage I clinical trial (de Gunzburg et al., 2018, 2015). DAV-132 has been shown to reduce fecal concentrations of antibiotics by 99% without affecting plasma concentrations (Burdet et al., 2017; de Gunzburg et al., 2018, 2015; Khoder et al., 2010). Activated charcoal could adsorb antibiotics in the gut, and could be an effective adjuvant for a broad range of antibiotic classes. Adjuvants with specific activity against particular antibiotics could also be developed. This has been successfully demonstrated with orally-administered β-lactamases given with intravenous β-lactam antibiotics. β-lactamases enzymatically inactivate β-lactams. Under the name SYN-004, this β-lactamase treatment

advanced to clinical trials in human subjects (Kaleko et al., 2016; Kokai-Kun et al., 2017; Pitout, 2009; Tarkkanen et al., 2009). Data from clinical trials show the drugs successfully inactivate β-lactams in the digestive tract without adversely affecting levels of antibiotic in plasma (Kaleko et al., 2016; Kokai-Kun et al., 2017; Pitout, 2009; Tarkkanen et al., 2009). These drugs have been developed with the goal of preventing infection with *C. difficile* after antibiotic therapy, but they could likely also prevent the emergence of antibiotic resistance in the GI tract. The orally-administered β-lactamase SYN-006 mitigated the enrichment of genes associated with antibiotic resistance in the microbiomes of pigs treated with a carbapenem antibiotic (Connelly et al., 2019). So far as we are aware, there is no direct experimental evidence analogous to ours that those drugs can prevent resistance emergence in colonizing opportunistic pathogens, but it seems likely they could.

Adjunctive therapies like the one proposed here could help manage resistance evolution in other important pathogens listed by Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) as major threats (CDC, 2019; WHO, 2017). Like VRE, many opportunistic pathogens experience substantial antibiotic exposure when they are not the targets of treatment. Opportunistic pathogens like *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacter cloacae* colonize the gut asymptomatically, where they can be unintentionally exposed to antibiotics (Morley et al., 2019). According to one estimate, over 90% of the total antimicrobial exposure experienced by *K. pneumoniae* occurs when *K. pneumoniae* was not the target of treatment (Tedijanto et al., 2018). For *H. influenzae*, *E. coli*, and *Staphylococcus aureus* over 80% of total exposure to antibiotics was estimated to occur when the bacteria were bystanders (Tedijanto et al., 2018). These colonizing populations are sources for infections, so selection for resistance in bystander populations can contribute to rising rates of resistant infections (Morley et al., 2019). Adjunctive strategies that allow intravenous antibiotics to reach target sites while reducing off-target exposure could help stem the spread of resistant pathogens listed by the CDC as urgent or serious threats (CDC, 2019), including carbapenem-resistant Enterobacteriaceae (CRE), extended-spectrum beta-lactamase producing Enterobacteriaceae, vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and drug resistant *Staphylococcus aureus*. The strategy presented here focuses on inactivating antibiotic in the gastrointestinal tract, the likely source of the bulk of the antimicrobial resistance in pathogens listed as top threats by the CDC, but similar

strategies could be developed to shield microbiota at other sites, such as the skin and respiratory tract.

Materials and Methods

Mice and bacterial strains

Unless otherwise specified, mice in all experiments were female Swiss Webster. In one experiment, inbred female C57/B6 mice were used. Mice were fed a standard diet (5001 Laboratory Rodent Diet) or a standard diet supplemented with 2% w/w cholestyramine resin. All mice were housed individually during experiments.

Daptomycin-susceptible VR *E. faecium* strains were isolated from different patients at the University of Michigan Hospital. Strain BL00239-1 was isolated from a bloodstream infection, and strain PR00708-14 was isolated from a perirectal swab.

Daptomycin treatment experiments

All mice were pretreated with ampicillin (0.5 g/L in drinking water) for 7 days before *E. faecium* inoculation. Ampicillin disrupts the natural gut flora and facilitates *Enterococcus* colonization (McKenney et al., 2019). *E. faecium* strains were plated from glycerol stocks and then grown overnight in liquid culture in Brain Heart Infusion broth. Mice were inoculated via oral gavage with 10^8 CFU *E. faecium* suspended in saline. *E. faecium* inoculum counts were confirmed by plating. Following *E. faecium* inoculation, mice were split into individual cages with untreated water and any experimental diets. Daptomycin doses were administered daily starting one day post inoculation via subcutaneous injection or oral gavage. Daptomycin doses were based on an average mouse weight for each experiment. For stool collection, mice were placed in clean plastic cups, and fresh stool was collected using a sterile toothpick. Stool samples were suspended in PBS (25 uL PBS/mg stool) and frozen with glycerol at -80ºC for subsequent analysis. Preliminary experiments were performed to determine the timing of experimental conditions (Supp. Fig. 2).

In vitro tests of daptomycin interaction with cholestyramine

For measurements of UV absorbance, solutions of 5 mg/mL daptomycin in phosphate-buffered saline (PBS) were combined with various concentrations of cholestyramine. These mixtures were vortexed for 30 seconds, then allowed to incubate for 5 minutes at room temperature. Following incubation, cholestyramine was removed by centrifugation. Supernatants were analyzed for absorbance at 364 nm on a NanoVue Plus Spectrophotometer. A calibration curve was used to determine daptomycin concentrations from A364 values.

For tests of daptomycin bioactivity, solutions of 1 mg/mL daptomycin were incubated with or without 12 mg/mL cholestyramine for 45 minutes at 37ºC with shaking (n=3 per treatment). The cholestyramine was removed by centrifugation, and the supernatant was used in broth microdilutions with *E. faecium*. Saline solution incubated with cholestyramine run as a control had no effect on cell growth.

Analysis of VRE in stool samples

VR *E. faecium* were enumerated by plating diluted fecal suspensions on selective plates (Enterococcosel agar supplemented with 16 ug/mL vancomycin). Plates were incubated at 35ºC for 40-48 hours, and colonies were counted. To quantify the proportion of these bacteria that were daptomycin resistant, fecal suspensions were plated on calcium-supplemented Enterococcosel plates with 16 ug/mL vancomycin and 10 ug/mL daptomycin. Plates were incubated at 35ºC for 40-48 hours, and colonies were counted.

In some experiments, *E. faecium* clones were isolated from fecal samples and analyzed by broth microdilution. Clones were purified by streaking twice on Enterococcosel agar with 16 ug/mL vancomycin, and were then stored in glycerol stocks at -80ºC. Broth microdilutions were performed according to Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). After incubation, cell densities were measured by OD600 absorbance in a plate reader. OD values were fitted to a Hill function curve to determine the computed MIC (MIC_c) as described previously (Kinnear et al., 2020).

Genome sequencing

Whole genomic DNA preparations were submitted to the University of Michigan sequencing core for Illumina library preparation and paired end 125 bp Illumina HiSeq2500. De novo

genome assembly was performed using SPAdes (Bankevich et al., 2012), the core genome was extracted using Roary (Page et al., 2015), and genomes were annotated using Prokka (Seemann, 2014).

Analysis of daptomycin concentrations

Fecal daptomycin concentrations were measured via LC-MS at the University of Michigan Pharmacokinetics Core. A labeled daptomycin-d5 internal standard was used to generate calibration curves.

Statistical analysis

Statistical analyses were run in R v1.2.1335 (Brooks et al., 2017) using the packages 'nlme' (J et al., 2019) and 'glmmTMB' (Brooks, Mollie et al., 2017). Proportions of resistant bacteria were analyzed using mixed effects negative binomial regression. Absolute VRE densities were analyzed using mixed models with an autoregressive error structure as previously described (Pollitt et al., 2012). Models used interpolated values for VRE densities over time. The basic model structure in R was as follows: lme(data, log10(VRE.density)~Daptomycin*Diet*Block*Day, random=~1|Mouse, correlation = corAR1(form = ~Day|Mouse)). Model selection was performed based on AIC value.

List of Supplementary Materials:

Supp. Fig. 1: Dynamics of VRE shedding for experiment shown in Fig. 2

Supp. Fig. 2: Effect of cholestyramine on daptomycin-resistance emergence when cholestyramine is administered one day later

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