1 **Title: Effect of fidaxomicin versus vancomycin on susceptibility to intestinal**
2 **colonization with vancomycin-resistant enterococci and *Klebsiella pneumoniae* in**
3 **mice**
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ABSTRACT

Use of oral vancomycin or metronidazole for treatment of *Clostridium difficile* infection (CDI) may promote colonization by healthcare-associated pathogens due to disruption of the intestinal microbiota. Because the macrocyclic antibiotic fidaxomicin causes less alteration of the intestinal microbiota than vancomycin, we hypothesized that it would not lead to a loss of colonization resistance to vancomycin-resistant enterococci (VRE) and extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP). Mice (8 per group) received orogastric saline, vancomycin or fidaxomicin daily for 5 days at doses resulting in stool concentrations in mice similar to those measured in humans. The mice were challenged with 10^5 colony-forming units (CFU) of orogastric VRE or ESBL-KP on day 2 of treatment and concentrations of the pathogens in stool were monitored. The impact of drug exposure on the microbiome was measured by cultures, real-time polymerase chain reaction for selected anaerobic bacteria, and by deep sequencing. In comparison to saline controls, oral vancomycin promoted establishment of high-density colonization by VRE and ESBL-KP in stool (8-10 $\log_{10}$ CFU/g; $P<0.001$), whereas fidaxomicin did not (<4 $\log_{10}$ CFU; $P>0.5$). Vancomycin treatment resulted in significant reductions in enterococci, *Bacteroides* spp., and *Clostridium leptum*, whereas the population of aerobic and facultative Gram-negative bacilli increased; deep sequencing analysis demonstrated suppression of Firmicutes and expansion of Proteobacteria during vancomycin treatment. Fidaxomicin did not cause significant alteration of the microbiota. In summary, in contrast to vancomycin, fidaxomicin treatment caused minimal disruption of the intestinal microbiota and did not render the microbiota susceptible to VRE and ESBL-KP colonization.

Oral vancomycin and oral metronidazole are the most commonly used antibiotics for treatment of *Clostridium difficile* infection (CDI). One limitation of these agents is that they are non-selective (i.e., they inhibit normal anaerobic intestinal microbiota in addition to *C. difficile)* (1-4). For example, oral vancomycin treatment may result in suppression of *Bacteroides*/*Prevotella*, *Clostridium coccoides*, and *Clostridium leptum* group organisms in stool (2-3). Inhibition of the anaerobic microbiota by vancomycin and metronidazole during CDI treatment may contribute to recurrences of CDI and to colonization by healthcare-associated pathogens such as vancomycin-resistant enterococci (VRE) (4-5).

Fidaxomicin is a macrocycle antibiotic approved by the Federal Drug Administration for treatment of CDI (1). In comparison to vancomycin, fidaxomicin causes minimal disruption of the anaerobic microbiota and in clinical studies was associated with fewer recurrences of CDI and less frequent acquisition of VRE and *Candida* spp. during CDI treatment (1,6). Given the relative sparing of the microbiota during fidaxomicin treatment, we hypothesized that this agent would not lead to a loss of colonization resistance to VRE and extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP). Here, we used a mouse model to compare the effect of fidaxomicin versus vancomycin on establishment of intestinal colonization by VRE and ESBL-KP.

MATERIALS AND METHODS

The pathogens studied. *E. faecium* C68 is a previously described VanB-type clinical VRE isolate (7). *K. pneumoniae* P62 is a clinical isolate that produces an SHV type

extended-spectrum β-lactamase (ESBL). Both organisms have been used in previous mouse model studies (7-8).

Susceptibility testing. Broth dilution minimum inhibitory concentrations (MICs) of the test antibiotics for the test organisms were determined using standard methods for susceptibility testing of aerobic bacteria (9).

Quantification of stool pathogens. Fresh stool specimens were processed as described elsewhere (7-8). In order to quantify VRE and ESBL-KP, diluted samples were plated onto Enterococcosel agar (Becton Dickinson, Cockeysville, MD) containing vancomycin 20 µg/mL and MacConkey agar (Becton Dickinson) containing ceftazidime 10 µg/mL, respectively. The plates were incubated in room air at 37 ºC for 48 hours, and the number of colony-forming units (CFU) of each pathogen per gram of sample was calculated.

Antibiotic dose selection. Dose finding experiments were run to determine the amount of vancomycin and fidaxomicin needed to be dosed to result in stool concentrations in mice similar to those measured in humans (i.e., 1,000 to 2,000 µg/gm of vancomycin and 1,000 to 3,000 µg/gm of fidaxomicin in stool) (10-12 and Merck data on file). Mice (5 per group) received a single oral administration of vancomycin or fidaxomicin. Fecal pellets were collected within 3 intervals of 0-4, 4-8 and 8-24h after dosing. Fecal levels of vancomycin, fidaxomicin and OP-1118 were measured by LC-MS and confirmed using satellite animals dosed at 1.125 mg/day or 37.5 mg/kg for vancomycin and 0.9 mg/day or 30 mg/kg and 2.3 mg/day or 75 mg/kg for fidaxomicin. These dosing regimens resulted in measured maximal fecal peak level of 1826 ug/g of vancomycin and 920 ug/g and 1600 ug/g of fidaxomicin+OP-1118 for the 30 mg/kg and 75 mg/kg

fidaxomicin doses, respectively. For the majority of experiments, the lower dose of fidaxomicin was used based upon the fact that the human dose of fidaxomicin is 80% of the usual daily dose of vancomycin (i.e., 400 mg per day versus 500 mg per day). Additional experiments were conducted using the higher dose of fidaxomicin because this dose resulted in a measured peak concentration that was equivalent to the peak concentration of vancomycin and that was equivalent to concentrations measured in humans receiving fidaxomicin (10).

Effect of the antibiotics on intestinal microbiota. The Animal Care Committee of the Cleveland Veterans Affairs Medical Center approved the experimental protocol. Initial experiments were conducted to assess the effects of treatment with the test antibiotics or saline on the intestinal microbiota of mice. Female CF-1 mice (6 per group) weighing ~30 g (Harlan Sprague-Dawley, Indianapolis, IN) were housed in individual cages. Mice received daily oroesophageal instillation of the test antibiotics (0.2-mL total volume) for 5 days using a stainless steel feeding tube (Perfektum, Popper & Sons, New Hyde Park, NY).

Quantitative culture of stool microbiota. Stool samples were collected at baseline, on days 2 and 5 of treatment, and 3, 5, and 10 days after treatment for evaluation of the effect of the antibiotics on the microbiota. Quantitative cultures for facultative and aerobic Gram-negative bacilli and enterococci were performed by plating serially-diluted specimens onto MacConkey agar (Difco Laboratories, Detroit) and Enterococcosel agar (Becton Dickinson), respectively.

Deep sequencing analysis of stool microbiota. Deep sequencing analysis was completed for mice treated with vancomycin and the lower dose of fidaxomicin. Fecal

bacterial DNA was extracted from ~500 mg of feces using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing and analysis was carried out by Second Genome (San Bruno, CA). To enrich the samples for the bacterial 16S V4 rDNA region, DNA was polymerase chain reaction (PCR)-amplified using fusion primers designed against surrounding conserved regions which are tailed with sequences to incorporate Illumina (San Diego, CA) adapter and indexing barcodes. After Illumina library construction, amplicons were sequenced using a MiSeq benchtop sequencer instrument (Illumina). Using QIIME and custom scripts, sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes. Resulting sequences were searched against the Greengenes reference database of 16S sequences, clustered at 97% by uclust (closed-reference OTU picking). The longest sequence from each Operation Taxonomic Unit (OTU) thus formed was used as the OTU representative sequence, and assigned taxonomic classification via MOTHUR's Bayesian classifier, trained against the Greengenes database clustered at 98%. Principal Coordinate Analysis (PCoA) using weighted Unifrac as the distance metric was carried out to visualize complex relationships between samples. A Permutation based multivariate analysis of variance test using distance metrics as implemented in the Adonis function in the vegan package for R was used to assess whole microbiome differences among groups (13-14). Bar plot representations were generated to show the top 8 microbial groups at the phylum level.

Analysis of *Bacteroides* spp. and *Clostridium leptum* by real-time PCR (qPCR). **qPCR** analysis was completed for mice treated with vancomycin and the lower dose of fidaxomicin. To determine the effect of antibiotic treatment on the concentration of

Bacteroides spp. and *C. leptum*, a representative Firmicutes organism, qPCR was performed using the methods and primers of Louie et al. (2). Fecal bacterial DNA was extracted from 100 mg of feces using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified template DNA from *Bacteroides fragilis* and *C. leptum* was used for melting curve analysis and to generate standard curves for each primer set using 10-fold serial dilutions of DNA ranging from 10 to 10^{-6} ng. qPCR was performed using the CFX96 detection system (Biorad, Hercules, CA). Amplification and detection were conducted in 96-well plates with SYBR Green 2x qPCR Master Mix (BioRad). Each sample was run in triplicate in a final volume of 20 µl containing a final concentration of 0.3 µM of each primer and 5 µL of 2-ng/µL template DNA using the following parameters: 1 cycle at 94 °C for 5 minutes, followed by 49 cycles at 94 °C for 20 seconds, 56 °C–58 °C for 20 seconds, and 72 °C for 20 seconds.

Effect of the antibiotics on establishment of colonization by VRE and ESBL-KP. To assess the effects of treatment on initial establishment of colonization, mice (8 per group) received oroesophageal instillation of 10,000 CFU of VRE or ESBL-KP on day 2 of 5 of daily treatment with vancomycin or the lower dose of fidaxomicin or saline as described previously. The concentration of VRE and ESBL-KP in stool was measured on day 5 of antibiotic treatment and 3, 5, and 10 days after completion of antibiotics.

Effect of the higher dose of fidaxomicin (75 mg/kg) on the microbiota and establishment of colonization by VRE and ESBL-KP. To assess the impact of the higher dose of fidaxomicin on the microbiota, quantitative cultures for facultative and aerobic Gram-negative bacilli and enterococci were performed as described previously

for mice treated with fidaxomicin or saline for 5 days. To assess the effect of the higher dose of fidaxomicin on establishment of colonization by VRE and ESBL-KP, mice (8 per group) treated for 5 days with oral saline, fidaxomicin 2.3 mg/day (75 mg/kg), clindamycin 1.4 mg/day, or fidaxomicin plus clindamycin received 10,000 CFU of oral VRE or ESBL-KP on day 2 of treatment. The concentration of VRE and ESBL-KP in stool was measured at baseline and 3 and 6 days after pathogen inoculation. The purpose of including a group receiving fidaxomicin plus clindamycin was to assess whether fidaxomicin has sufficient inhibitory activity to prevent clindamycin-associated promotion of VRE overgrowth (7).

Statistical analysis. One-way analysis of variance (ANOVA) was performed to compare concentrations of organisms among the treatment groups. *P*-values were adjusted for multiple comparisons using the Scheffe correction. Computations were performed with the use of Stata (version 5.0, Stata, College Station, Texas) and Origin (Version 9, OriginLab, Northampton, MA).

RESULTS

Susceptibility testing. MICs for ESBL-KP were >256 µg/mL for vancomycin, metronidazole, and fidaxomicin. MICs for VRE were 256, >256, and 2 µg/mL for vancomycin, metronidazole, and fidaxomicin, respectively.

Effect of the antibiotics on indigenous enterococci and facultative Gram-negative bacilli by quantitative culture. Figure 1 shows the effect of antibiotic treatment on the concentrations of enterococci (A) and aerobic and facultative Gram-negative bacilli (B) by culture. Vancomycin significantly reduced levels of enterococci during treatment, whereas fidaxomicin did not. Levels of enterococci returned to baseline concentrations

by 3 days after discontinuation of vancomycin. In comparison to saline controls, vancomycin exposure resulted in a 4 log increase in Gram-negative bacilli, whereas fidaxomicin did not. By 10 days after discontinuation of vancomycin, levels of Gram-negative bacilli were not significantly elevated in comparison to baseline levels.

Effect of the antibiotics on indigenous microbiota by deep sequencing and qPCR. Figure 2 shows the relative proportions of different bacterial phyla on day 5 of antibiotic exposure in comparison to the saline control group, including the summed total for each treatment group and data for individual mice. In control mice, Bacteroidetes and Firmicutes were predominant, with Proteobacteria making up only less than 2% of the indigenous microbiota. Fidaxomicin exposure was associated with a reduction in Firmicutes from ~40% to ~20% with no increase in Proteobacteria. In contrast, vancomycin treatment was associated with suppression of Firmicutes from ~40% to less than 10% of the microbiota and expansion of Proteobacteria.

Figure 3 shows the relative proportions of the different taxa in the vancomycin and fidaxomicin groups before, during and after treatment. For the vancomycin group, there was an increased proportion of Proteobacteria at baseline in comparison to the other groups that was attributable to the presence of 1 outlier mouse; however, the differences between the groups at baseline were not statistically significant. For the vancomycin group, the proportion of Firmicutes increased from the end of treatment (day 5) to 10 days post treatment (day 15), while the proportion of Proteobacteria decreased.

Real-time PCR analysis demonstrated that vancomycin significantly reduced the concentrations of *Bacteroides* spp.(8.7 versus 5.6 $\log_{10}$CFU/g stool) and *C. leptum* (6.2

versus 5.6 $\log_{10}$CFU/g stool) on day 5 of treatment (P<0.001 for each comparison), whereas fidaxomicin did not (P>0.5).

Effect of antibiotic exposure on establishment of colonization by VRE and ESBL-KP. Figure 4 shows the effect of exposure to vancomycin and the lower dose of fidaxomicin on establishment of colonization by VRE (A) and ESBL-KP (B). In comparison to controls, oral vancomycin promoted overgrowth of both pathogens (P<0.001), whereas fidaxomicin did not promote overgrowth of either pathogen. None of the control or fidaxomicin-treated mice had detectable VRE at any time point.

Effect of the higher dose of fidaxomicin (75 mg/kg) on the microbiota and establishment of colonization by VRE and ESBL-KP. In comparison to saline controls, the higher dose of fidaxomicin significantly reduced concentrations of enterococci on day 5 of treatment (4.3 versus 6.1 $\log_{10}$CFU/g stool; P<0.01), with levels returning to baseline by 3 days after treatment. Concentrations of aerobic and facultative Gram-negative bacilli did not differ between the fidaxomicin-treated mice and saline controls at any time point. As shown in Figure 5, in comparison to saline controls, the higher dose of fidaxomicin did not promote overgrowth of VRE when challenged with oral VRE during treatment, whereas clindamycin alone or in combination with fidaxomicin did (P<0.001); the concentrations of VRE were significantly higher in the clindamycin versus the clindamycin plus fidaxomicin group (P<0.01). In comparison to saline controls, the higher dose of fidaxomicin also did not promote overgrowth of ESBL-KP (peak concentration, 3.8 and 3.9 $\log_{10}$CFU/g stool; P=1).

DISCUSSION

In contrast to oral vancomycin, we found that oral fidaxomicin did not promote overgrowth of VRE and ESBL-KP in mice. Vancomycin promoted overgrowth of aerobic and facultative Gram-negative bacilli, whereas fidaxomicin did not. By deep sequencing analysis, vancomycin treatment resulted in marked suppression of Firmicutes and expansion of Proteobacteria, whereas fidaxomicin was associated with only a minor reduction in Firmicutes with no increase in Proteobacteria. By qPCR analysis, vancomycin suppressed levels of *Bacteroides* spp., and *Clostridium leptum*, whereas fidaxomicin did not. These findings add to the body of literature suggesting that the relative preservation of the intestinal microbiota during fidaxomicin treatment may be beneficial in reducing the risk for acquisition and overgrowth of healthcare-associated pathogens during CDI treatment.

Because fidaxomicin has minimal activity against Gram-negative bacilli, the lack of promotion of overgrowth of indigenous Gram-negative bacilli and ESBL-KP is attributable entirely to relative preservation of the intestinal microbiota. However, fidaxomicin does have activity against enterococci (MIC for VRE test strain, 2 µg/mL). Therefore, lack of promotion of VRE overgrowth could be attributable to inhibitory activity against enterococci. The fact that fidaxomicin did not completely prevent overgrowth of VRE induced by disruption of the microbiota by clindamycin, it is likely that the reduced VRE expansion is due to both inhibitory activity and relative preservation of the microbiota.

Our findings for fidaxomicin and vancomycin are consistent with previous studies (4-6). Fidaxomicin treatment of CDI was associated with infrequent acquisition of VRE and *Candida* spp. colonization in comparison to oral vancomycin (6). Fidaxomicin may

represent a good alternative to metronidazole use when vancomycin is not being considered. The finding that fidaxomicin exposure did not promote colonization by ESBL-KP is significant given the increasing importance of emerging multi-resistant Gram-negative pathogens (15).

Our study has some limitations. The study was conducted using a mouse model with healthy mice. Additional studies will be required to confirm that the findings are applicable to patients with CDI. We studied only one strain each of VRE and *K. pneumoniae*. However, we have previously shown that multiple VRE and *K. pneumoniae* strains gave similar results in the mouse model (7-8). We studied only one species of antimicrobial-resistant Gram-negative bacilli. Future studies are needed that include other species such as *Acinetobacter* spp. Although the lower dose of fidaxomicin was 80% of the vancomycin dose (i.e., the same the ratio as in human dosing), the fecal concentration of fidaxomicin plus OP-1118 measured in mouse fecal pellets was lower than levels measured in human feces (10) and lower than the fecal concentration of vancomycin in mice. The lower fecal fidaxomicin levels measured in mice could potentially be due to lower technical extraction and recovery of fidaxomicin and OP-1118 from mouse versus human samples or due to differences between excretion or metabolism of the drug in mice and humans. The higher dose of fidaxomicin did result in a measured fecal fidaxomicin concentration that was similar to the concentration of vancomycin, and the higher dose did not promote colonization by VRE or ESBL-KP. Finally, we did not include metronidazole in our evaluation. However, Lewis et al. (16) recently demonstrated that oral metronidazole promotes colonization by VRE and

antibiotic-resistant Gram-negative bacilli in mice, although to a lesser degree than oral vancomycin.

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Figure legends

FIG. 1. Effect of antibiotic treatment on the concentrations of enterococci (A) and aerobic and facultative Gram-negative bacilli (B) in stool by culture. Mice received daily oral antibiotic treatment for 5 days. Error bars represent standard error. *$P<0.05$

FIG. 2. Comparison of the stool microbiota of mice by 16S deep sequencing analysis after 5 days of antibiotic treatment. The relative abundances of the major bacterial phyla are shown. Numbers indicate data for individual mice in each group. FDX stands for fidaxomicin, UNT for Untreated (Control) and VAN for vancomycin.

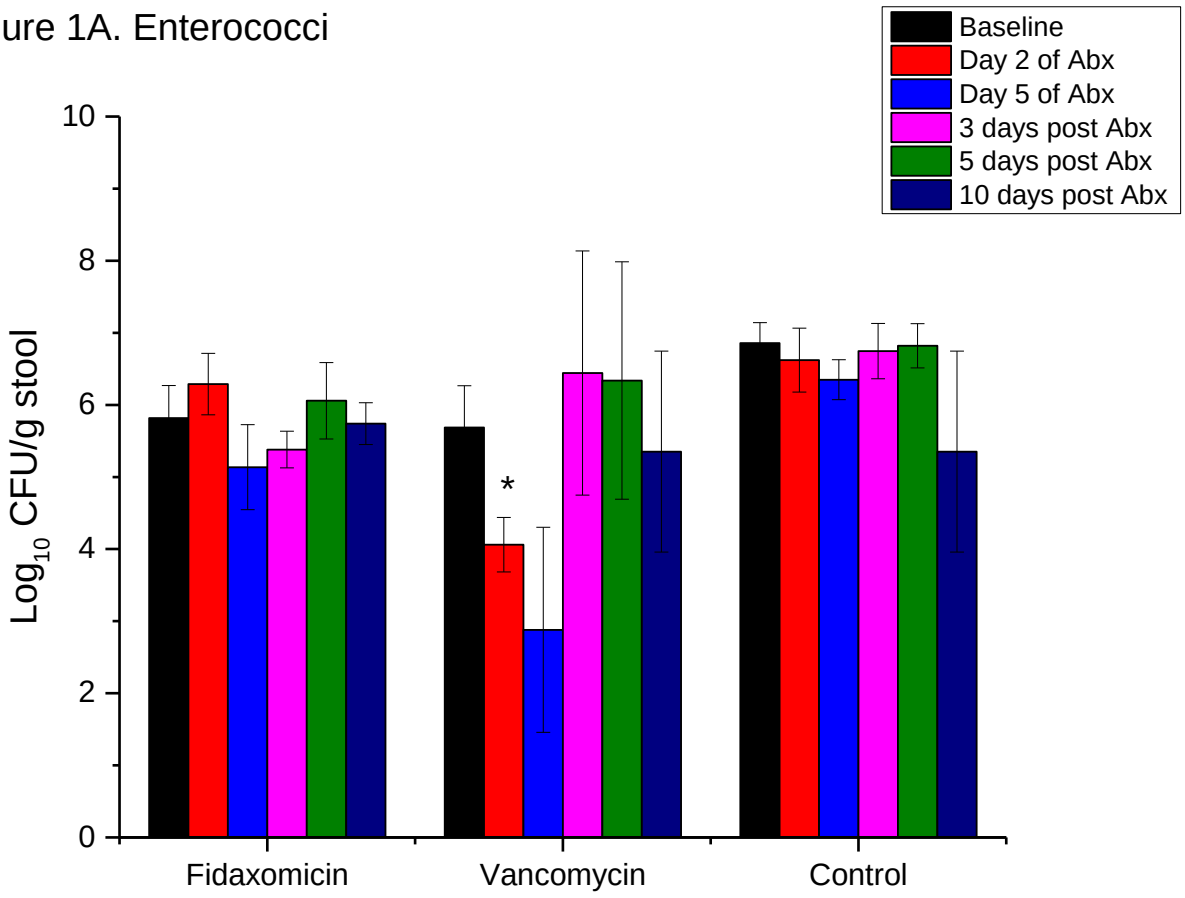
FIG. 3. Comparison of the stool microbiota of mice by 16S deep sequencing analysis before, during, and after treatment with oral fidaxomicin or vancomycin. Mice received daily oral antibiotic treatment for 5 days (Day 0 to Day 5). Numbers indicate day of sample collection: day 0, prior to treatment; day 5, after 5 days of antibiotic treatment; day 10, 5 days after last antibiotic dose; day 15, 10 days after last antibiotic dose. The relative abundances of the major bacterial phyla are shown as a composite of 5 total mice in each group at each time point. FDX stands for fidaxomicin, UNT for Untreated (Control) and VAN for vancomycin.

FIG. 4. Effect of antibiotic treatment on establishment of colonization by vancomycin-resistant enterococci (VRE) (A) and extended-spectrum β-lactamase producing *Klebsiella pneumonia* (ESBL-KP) (B) in mice. Mice received daily oral antibiotic treatment for 5

days. The pathogens were administered orally on day 2 of antibiotic treatment. Error bars represent standard error.

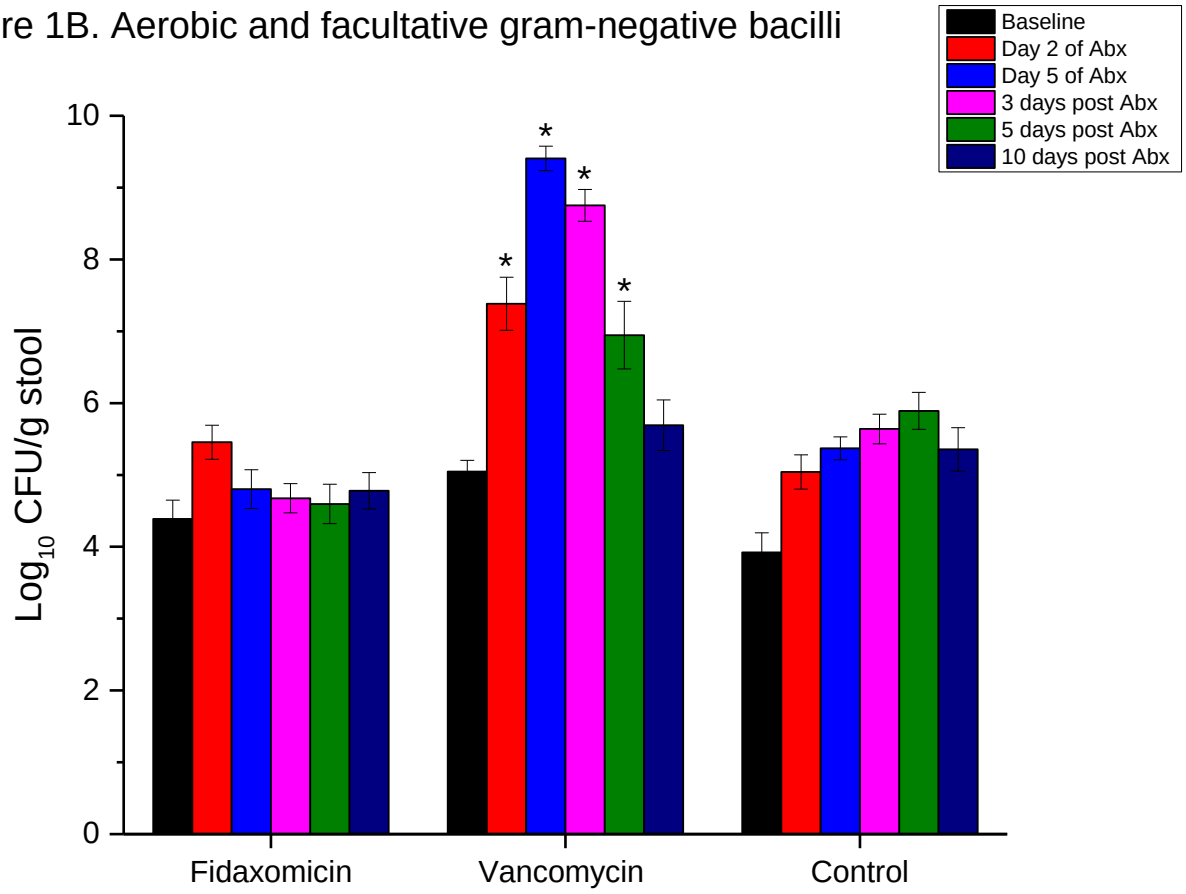
FIG. 5. Effect of antibiotic treatment on establishment of colonization by vancomycin-resistant enterococci (VRE) in mice. Mice received daily oral antibiotic treatment for 5 days. The pathogens were administered orally on day 2 of antibiotic treatment. Error bars represent standard error.

Figure 1A. Enterococci



*P<0.05

Figure 1B. Aerobic and facultative gram-negative bacilli



*P<0.05

Figure 2. Deep sequencing day 5

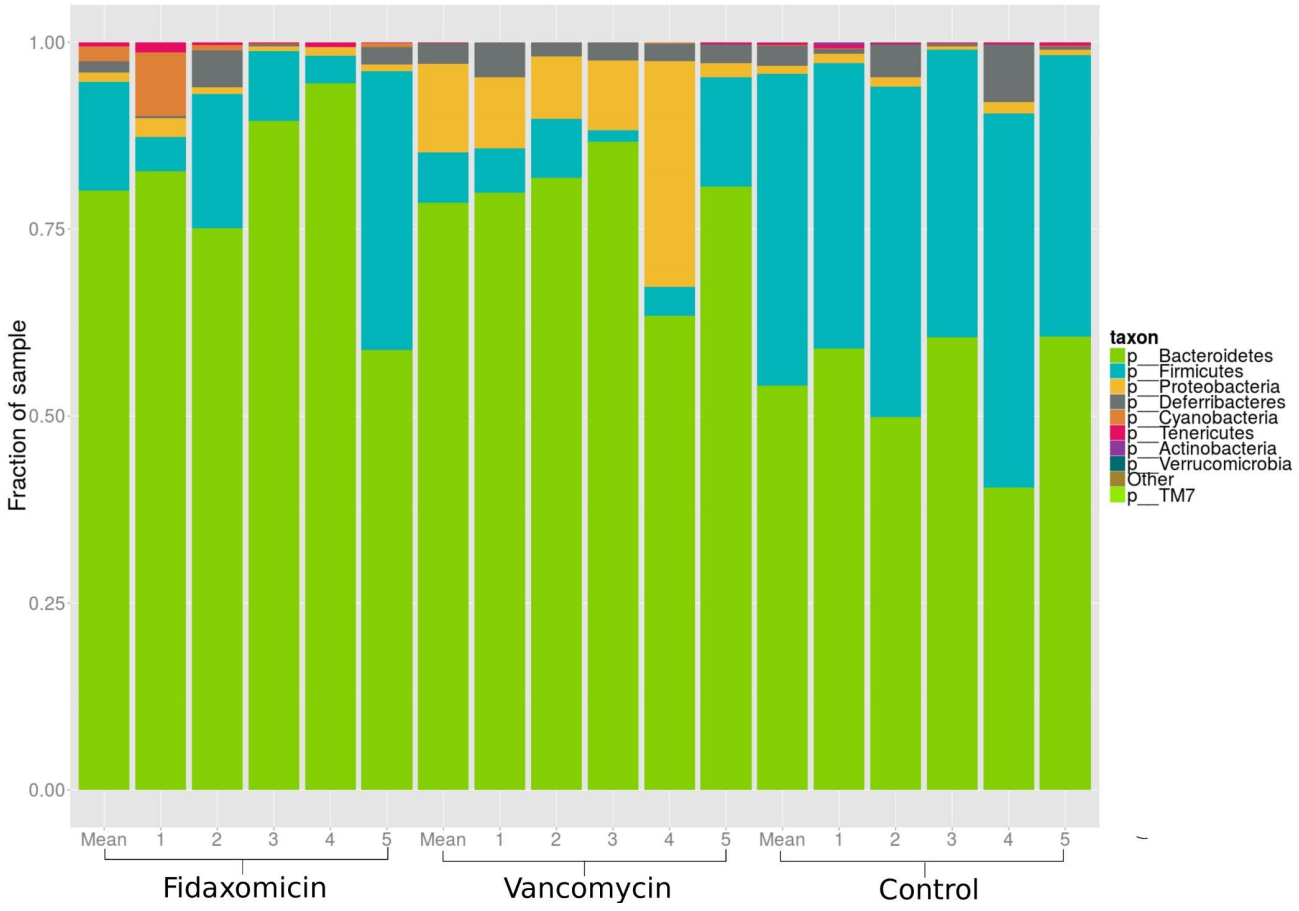


Figure 3. Deep sequencing before during and after vancomycin and fidaxomicin

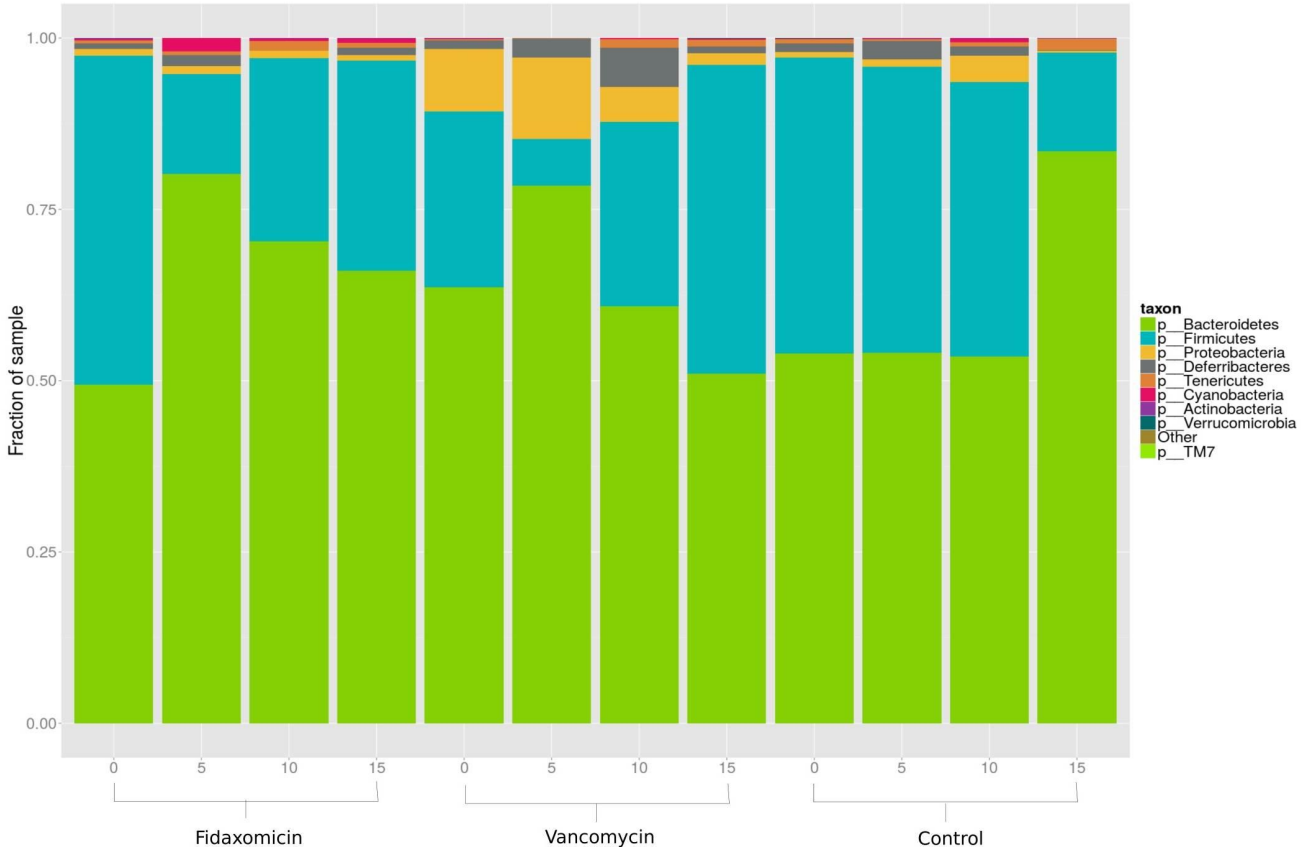


Figure 4A. VRE

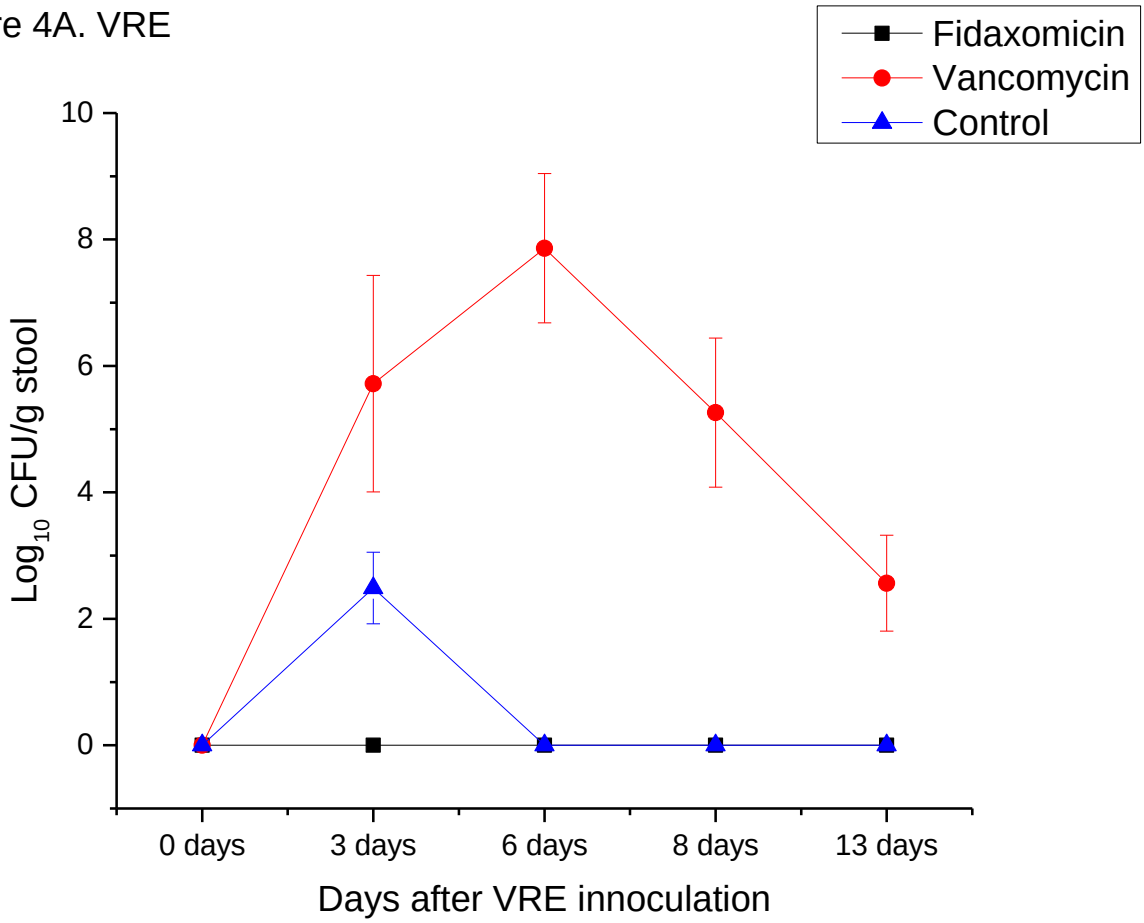


Figure 4B. ESBL-KP

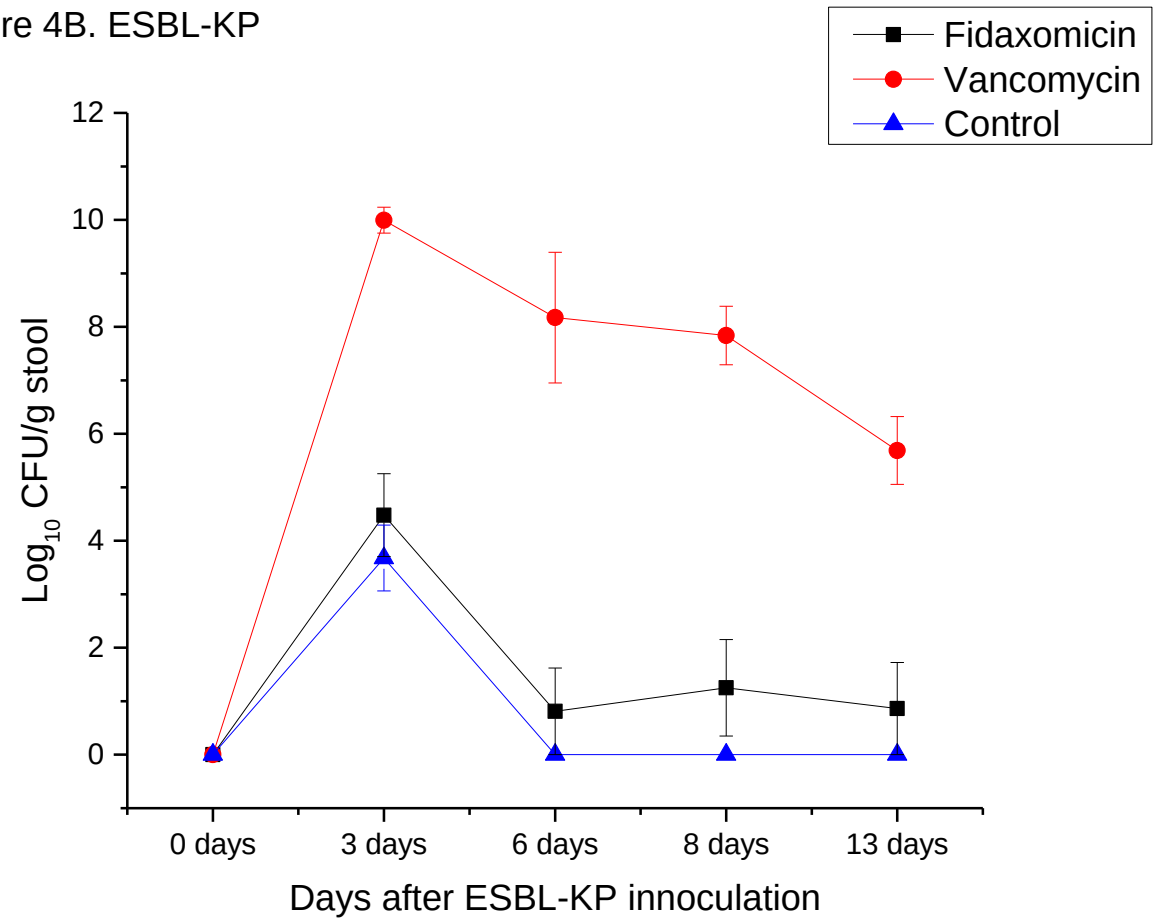


Figure 5. VRE inoculation during treatment