

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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14 Running head: Fidaxomicin and vancomycin and VRE and Klebsiella colonization

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24 **ABSTRACT**

25 Use of oral vancomycin or metronidazole for treatment of *Clostridium difficile*
26 infection (CDI) may promote colonization by healthcare-associated pathogens due to
27 disruption of the intestinal microbiota. Because the macrocyclic antibiotic fidaxomicin
28 causes less alteration of the intestinal microbiota than vancomycin, we hypothesized that
29 it would not lead to a loss of colonization resistance to vancomycin-resistant enterococci
30 (VRE) and extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* (ESBL-
31 KP). Mice (8 per group) received orogastric saline, vancomycin or fidaxomicin daily for
32 5 days at doses resulting in stool concentrations in mice similar to those measured in
33 humans. The mice were challenged with 10^5 colony-forming units (CFU) of orogastric
34 VRE or ESBL-KP on day 2 of treatment and concentrations of the pathogens in stool
35 were monitored. The impact of drug exposure on the microbiome was measured by
36 cultures, real-time polymerase chain reaction for selected anaerobic bacteria, and by deep
37 sequencing. In comparison to saline controls, oral vancomycin promoted establishment
38 of high-density colonization by VRE and ESBL-KP in stool (8-10 \log_{10} CFU/g;
39 $P < 0.001$), whereas fidaxomicin did not ($< 4 \log_{10}$ CFU; $P > 0.5$). Vancomycin treatment
40 resulted in significant reductions in enterococci, *Bacteroides* spp., and *Clostridium*
41 *leptum*, whereas the population of aerobic and facultative Gram-negative bacilli
42 increased; deep sequencing analysis demonstrated suppression of Firmicutes and
43 expansion of Proteobacteria during vancomycin treatment. Fidaxomicin did not cause
44 significant alteration of the microbiota. In summary, in contrast to vancomycin,
45 fidaxomicin treatment caused minimal disruption of the intestinal microbiota and did not
46 render the microbiota susceptible to VRE and ESBL-KP colonization.

47

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

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

67 MATERIALS AND METHODS

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

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

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

75 **Quantification of stool pathogens.** Fresh stool specimens were processed as
76 described elsewhere (7-8). In order to quantify VRE and ESBL-KP, diluted samples
77 were plated onto Enterococcosel agar (Becton Dickinson, Cockeysville, MD) containing
78 vancomycin 20 $\mu\text{g}/\text{mL}$ and MacConkey agar (Becton Dickinson) containing ceftazidime
79 10 $\mu\text{g}/\text{mL}$, respectively. The plates were incubated in room air at 37 $^{\circ}\text{C}$ for 48 hours, and
80 the number of colony-forming units (CFU) of each pathogen per gram of sample was
81 calculated.

82 **Antibiotic dose selection.** Dose finding experiments were run to determine the amount
83 of vancomycin and fidaxomicin needed to be dosed to result in stool concentrations in
84 mice similar to those measured in humans (i.e., 1,000 to 2,000 $\mu\text{g}/\text{gm}$ of vancomycin and
85 1,000 to 3,000 $\mu\text{g}/\text{gm}$ of fidaxomicin in stool) (10-12 and Merck data on file). Mice (5
86 per group) received a single oral administration of vancomycin or fidaxomicin. Fecal
87 pellets were collected within 3 intervals of 0-4, 4-8 and 8-24h after dosing. Fecal levels
88 of vancomycin, fidaxomicin and OP-1118 were measured by LC-MS and confirmed
89 using satellite animals dosed at 1.125 mg/day or 37.5 mg/kg for vancomycin and 0.9
90 mg/day or 30 mg/kg and 2.3 mg/day or 75 mg/kg for fidaxomicin. These dosing
91 regimens resulted in measured maximal fecal peak level of 1826 $\mu\text{g}/\text{g}$ of vancomycin and
92 920 $\mu\text{g}/\text{g}$ and 1600 $\mu\text{g}/\text{g}$ of fidaxomicin+OP-1118 for the 30 mg/kg and 75 mg/kg

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

100 **Effect of the antibiotics on intestinal microbiota.** The Animal Care Committee of
101 the Cleveland Veterans Affairs Medical Center approved the experimental protocol.
102 Initial experiments were conducted to assess the effects of treatment with the test
103 antibiotics or saline on the intestinal microbiota of mice. Female CF-1 mice (6 per
104 group) weighing ~30 g (Harlan Sprague-Dawley, Indianapolis, IN) were housed in
105 individual cages. Mice received daily oroesophageal instillation of the test antibiotics
106 (0.2-mL total volume) for 5 days using a stainless steel feeding tube (Perfektum, Popper
107 & Sons, New Hyde Park, NY).

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

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

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

136 **Analysis of *Bacteroides* spp. and *Clostridium leptum* by real-time PCR (qPCR).**

137 qPCR analysis was completed for mice treated with vancomycin and the lower dose of
138 fidaxomicin. To determine the effect of antibiotic treatment on the concentration of

139 *Bacteroides* spp. and *C. leptum*, a representative Firmicutes organism, qPCR was
140 performed using the methods and primers of Louie et al. (2). Fecal bacterial DNA was
141 extracted from 100 mg of feces using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden,
142 Germany) according to the manufacturer's instructions. Purified template DNA from
143 *Bacteroides fragilis* and *C. leptum* was used for melting curve analysis and to generate
144 standard curves for each primer set using 10-fold serial dilutions of DNA ranging from
145 10 to 10⁻⁶ ng. qPCR was performed using the CFX96 detection system (Biorad,
146 Hercules, CA). Amplification and detection were conducted in 96-well plates with
147 SYBR Green 2x qPCR Master Mix (BioRad). Each sample was run in triplicate in a final
148 volume of 20 μ L containing a final concentration of 0.3 μ M of each primer and 5 μ L of 2-
149 ng/ μ L template DNA using the following parameters: 1 cycle at 94 °C for 5 minutes,
150 followed by 49 cycles at 94 °C for 20 seconds, 56 °C–58 °C for 20 seconds, and 72 °C
151 for 20 seconds.

152 **Effect of the antibiotics on establishment of colonization by VRE and ESBL-KP.**

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

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

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

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

176 **RESULTS**

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

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

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

189 **Effect of the antibiotics on indigenous microbiota by deep sequencing and qPCR.**

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

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

205 Real-time PCR analysis demonstrated that vancomycin significantly reduced the
206 concentrations of *Bacteroides* spp.(8.7 versus 5.6 log₁₀CFU/g stool) and *C. leptum* (6.2

207 versus 5.6 log₁₀CFU/g stool) on day 5 of treatment ($P<0.001$ for each comparison),
208 whereas fidaxomicin did not ($P>0.5$).

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

215 **Effect of the higher dose of fidaxomicin (75 mg/kg) on the microbiota and**
216 **establishment of colonization by VRE and ESBL-KP.** In comparison to saline
217 controls, the higher dose of fidaxomicin significantly reduced concentrations of
218 enterococci on day 5 of treatment (4.3 versus 6.1 log₁₀CFU/g stool; $P<0.01$), with levels
219 returning to baseline by 3 days after treatment. Concentrations of aerobic and facultative
220 Gram-negative bacilli did not differ between the fidaxomicin-treated mice and saline
221 controls at any time point. As shown in Figure 5, in comparison to saline controls, the
222 higher dose of fidaxomicin did not promote overgrowth of VRE when challenged with
223 oral VRE during treatment, whereas clindamycin alone or in combination with
224 fidaxomicin did ($P<0.001$); the concentrations of VRE were significantly higher in the
225 clindamycin versus the clindamycin plus fidaxomicin group ($P<0.01$). In comparison to
226 saline controls, the higher dose of fidaxomicin also did not promote overgrowth of
227 ESBL-KP (peak concentration, 3.8 and 3.9 log₁₀CFU/g stool; $P=1$).

228 **DISCUSSION**

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

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

249 Our findings for fidaxomicin and vancomycin are consistent with previous studies
250 (4-6). Fidaxomicin treatment of CDI was associated with infrequent acquisition of VRE
251 and *Candida* spp. colonization in comparison to oral vancomycin (6). Fidaxomicin may

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

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

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

275 vancomycin.

276 **ACKNOWLEDGMENT**

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337 **Figure legends**

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

341

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

346

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

355

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

359 days. The pathogens were administered orally on day 2 of antibiotic treatment. Error

360 bars represent standard error.

361

362 FIG. 5. Effect of antibiotic treatment on establishment of colonization by vancomycin-

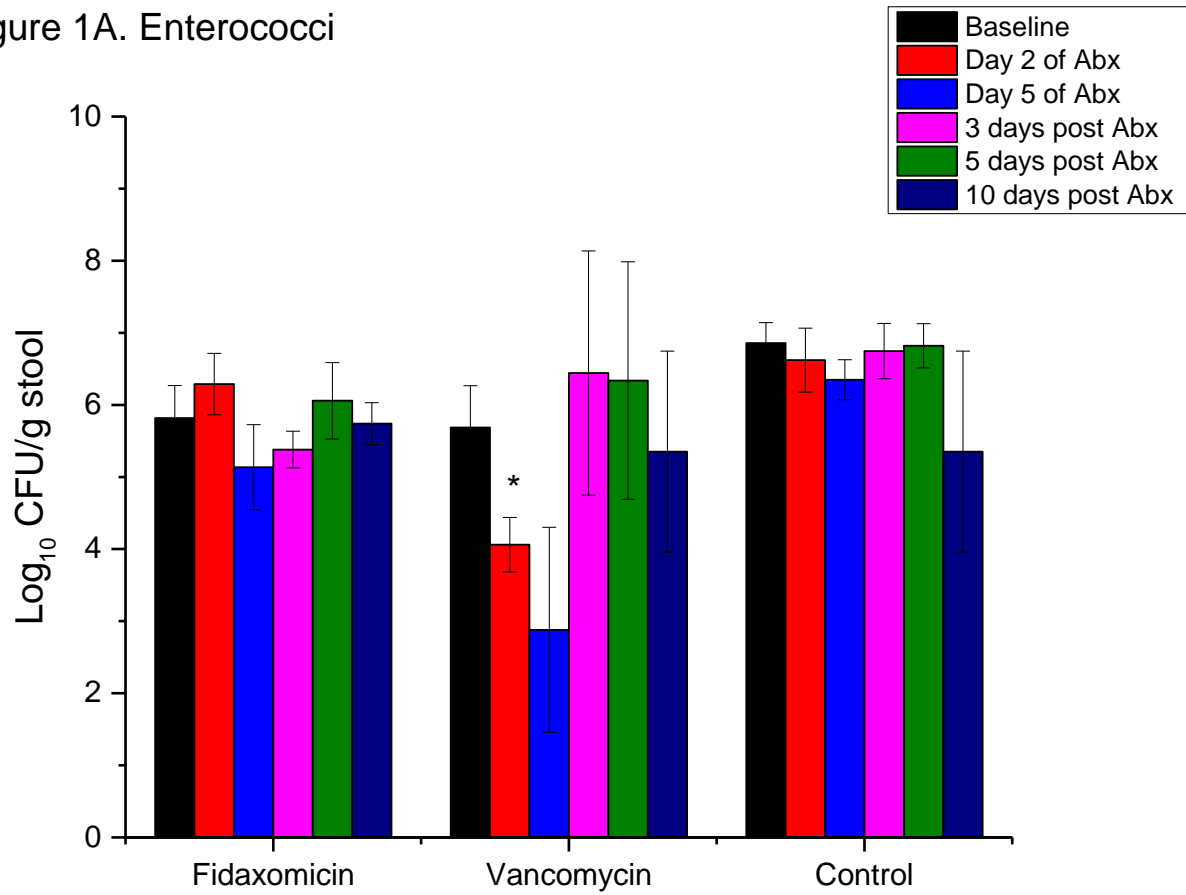
363 resistant enterococci (VRE) in mice. Mice received daily oral antibiotic treatment for 5

364 days. The pathogens were administered orally on day 2 of antibiotic treatment. Error

365 bars represent standard error.

366

Figure 1A. Enterococci



*P<0.05

Figure 1B. Aerobic and facultative gram-negative bacilli

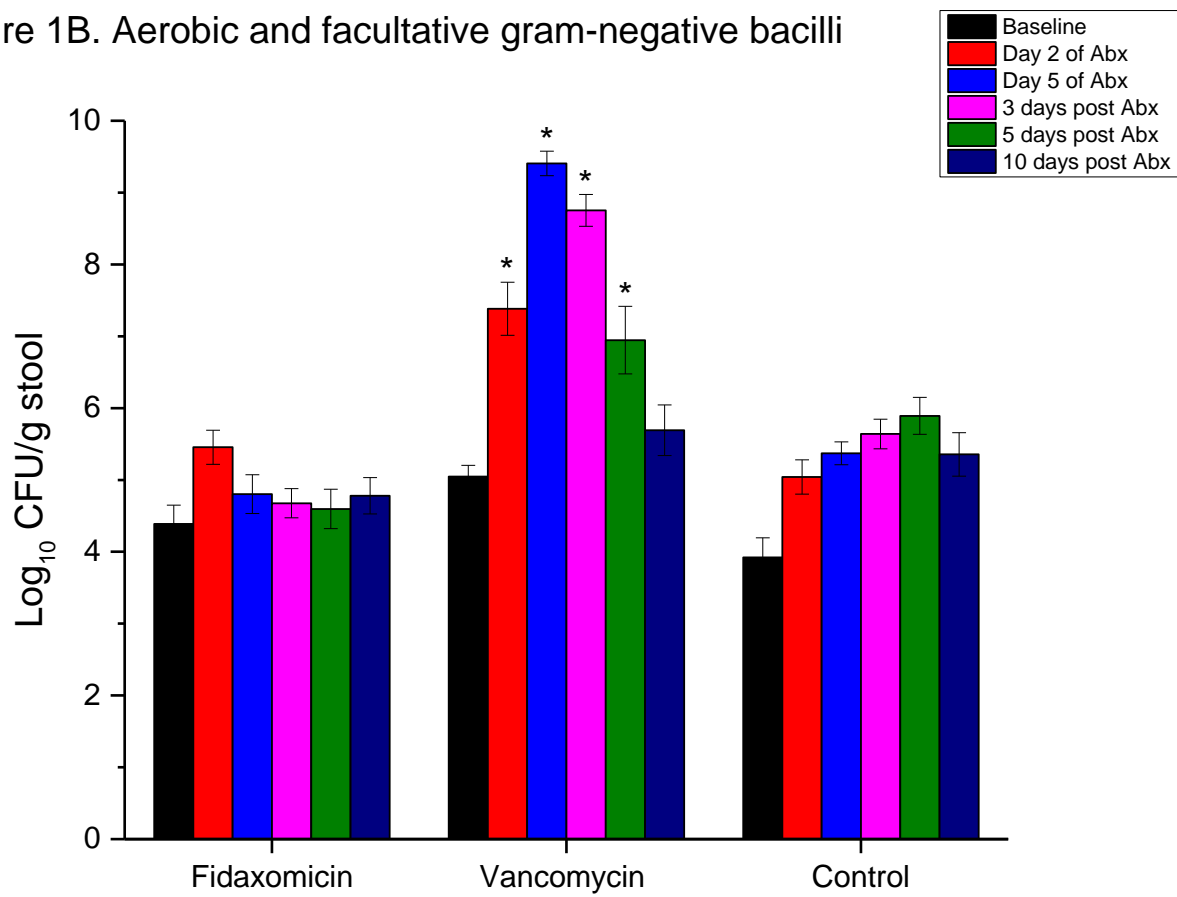
* $P < 0.05$

Figure 2. Deep sequencing day 5

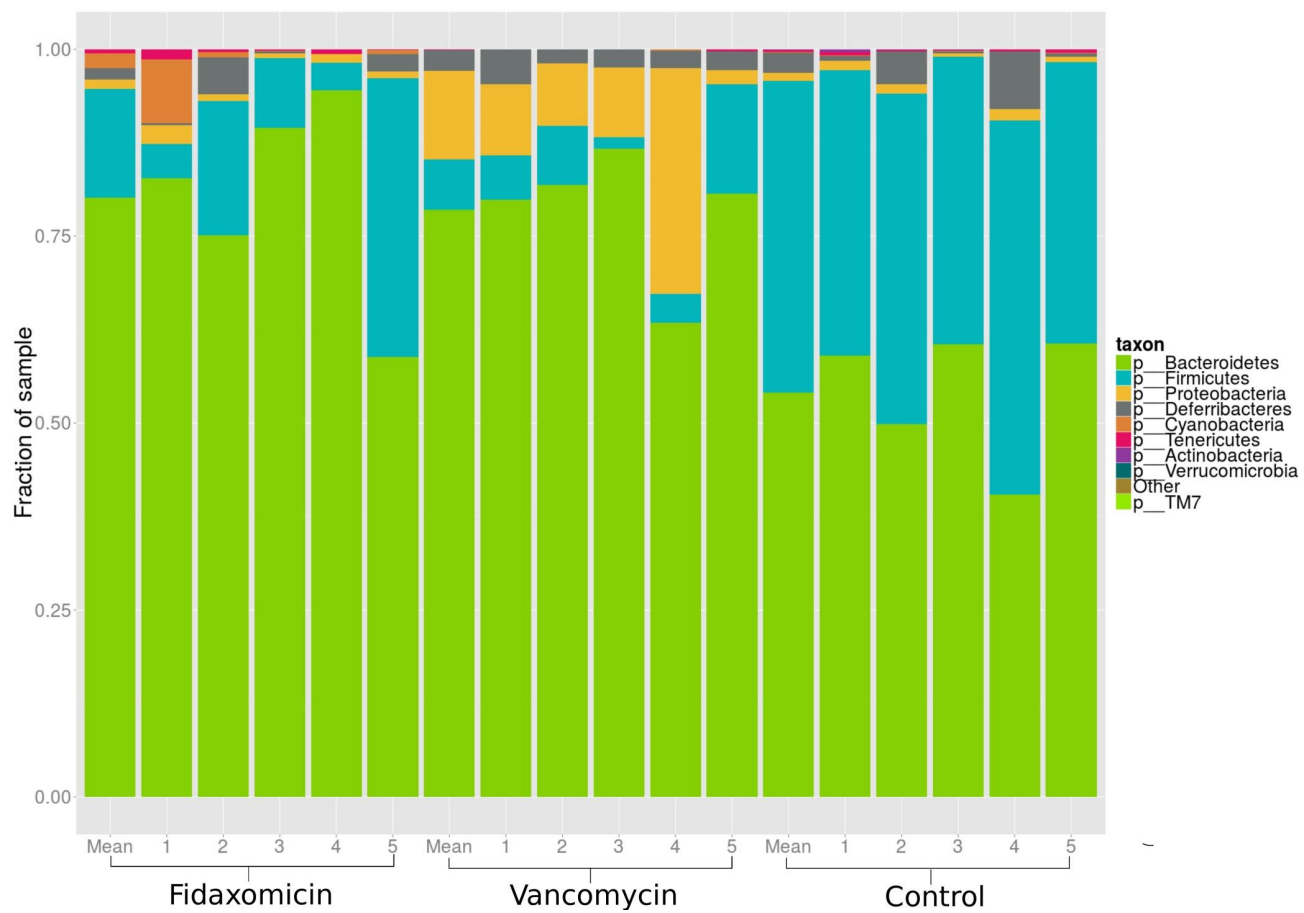


Figure 3. Deep sequencing before during and after vancomycin and fidaxomicin

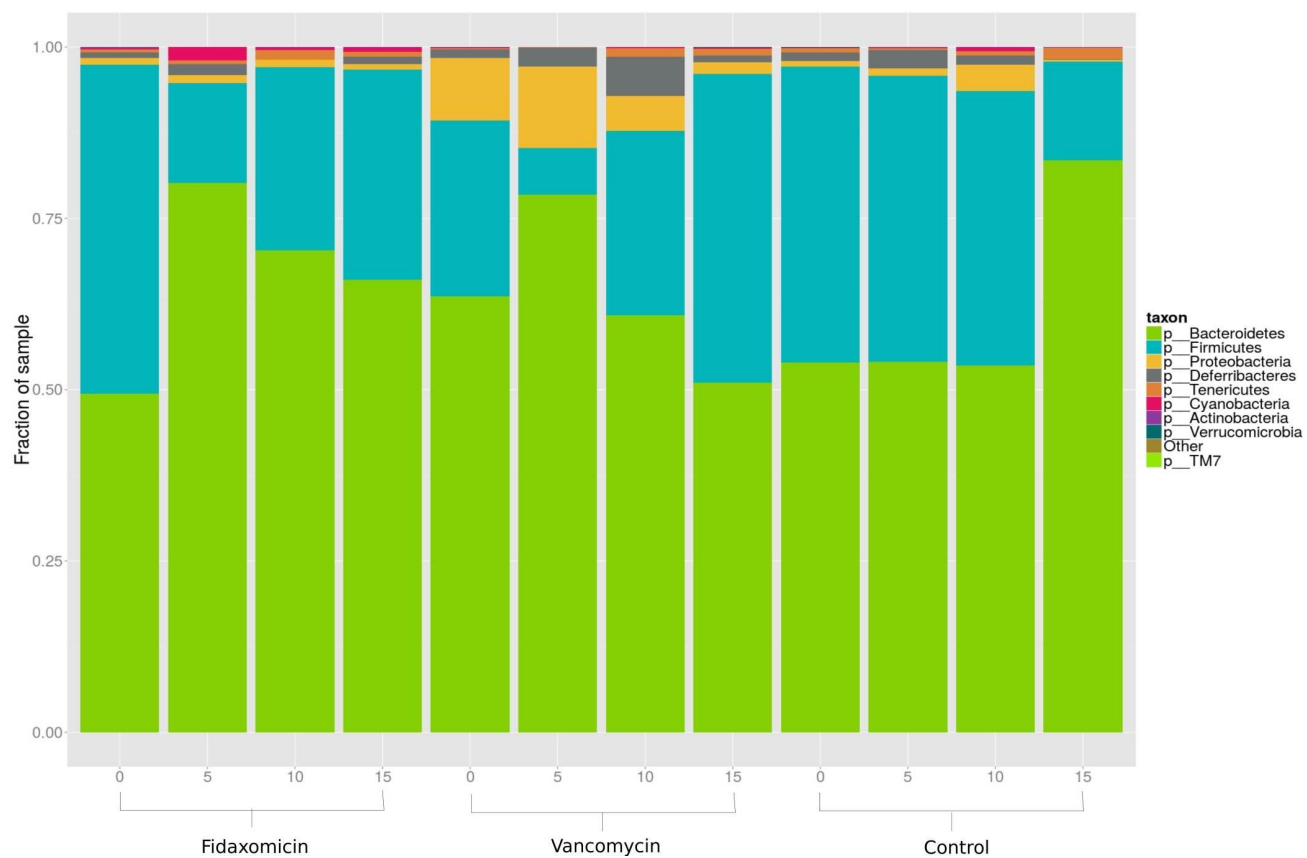


Figure 4A. VRE

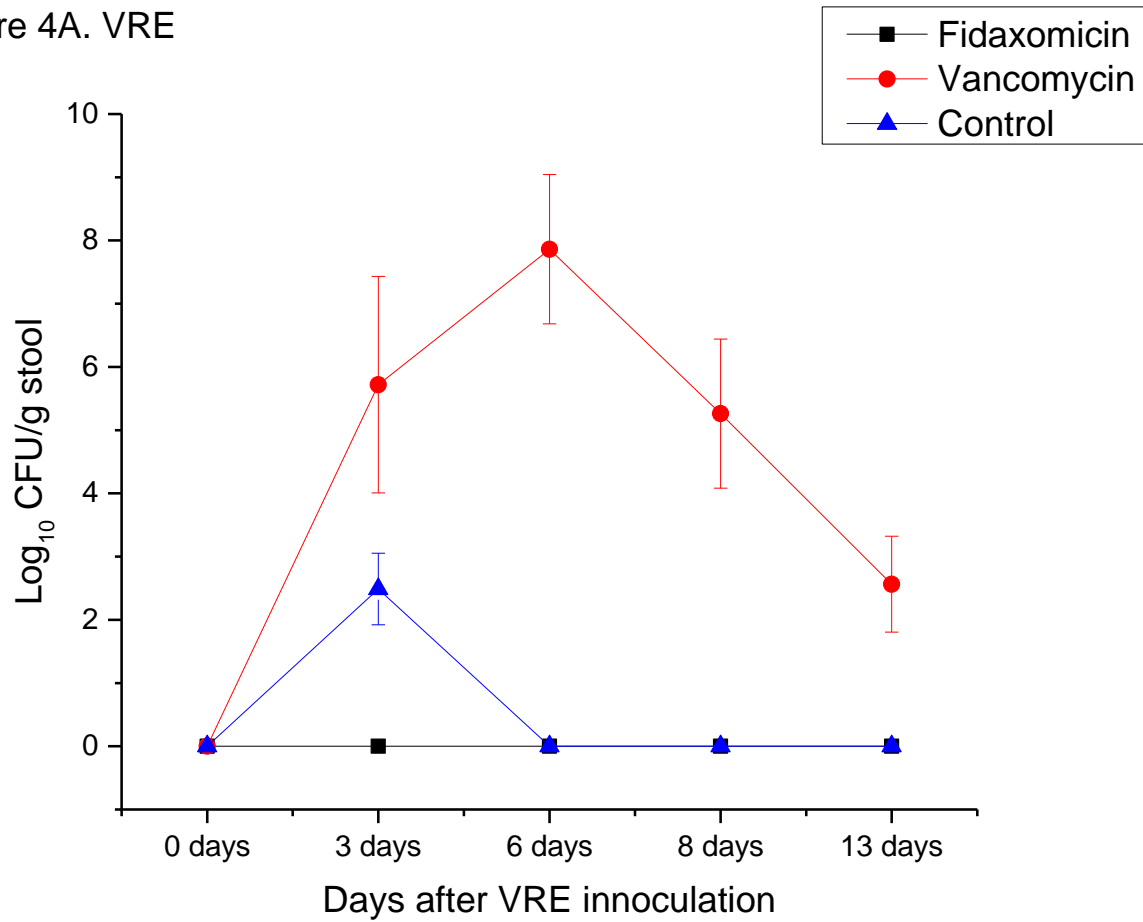


Figure 4B. ESBL-KP

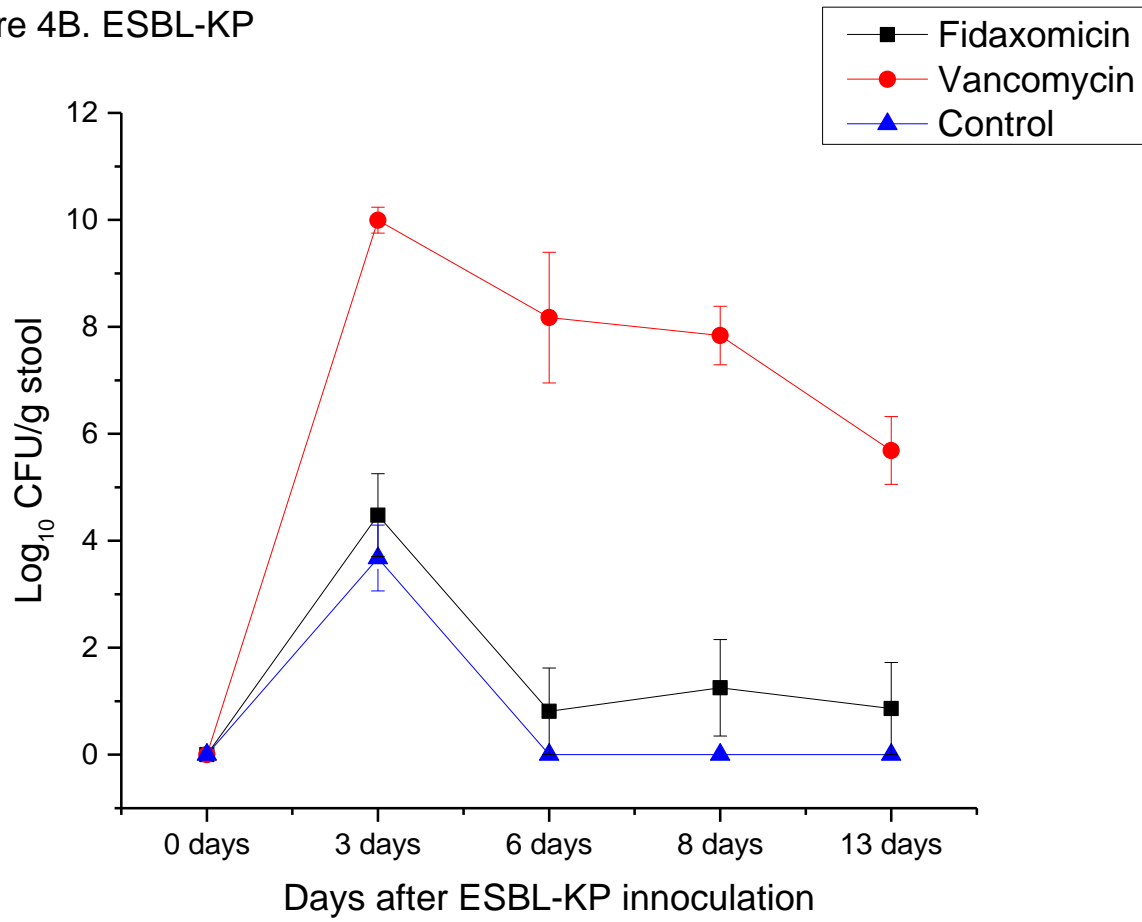


Figure 5. VRE inoculation during treatment

