Impact of Oral Fidaxomicin Administration on the Intestinal Microbiota and Susceptibility to *Clostridium difficile* Colonization in Mice

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ABSTRACT *Clostridium difficile* infection (CDI), a common cause of hospital-acquired infections, typically occurs after disruption of the normal gut microbiome by broad-spectrum antibiotics. Fidaxomicin is a narrow-spectrum antibiotic that demonstrates a reduced impact on the normal gut microbiota and is approved for the treatment of CDI. To further explore the benefits of this property, we used a murine model to examine the effects of fidaxomicin versus vancomycin on gut microbiota and susceptibility to *C. difficile* colonization while tracking microbiota recovery over time. Mice were exposed to fidaxomicin or vancomycin by oral gavage for 3 days and subsequently challenged with *C. difficile* spores at predetermined time points up to 21 days postexposure to antibiotics. Fecal samples were subsequently collected for analysis. Twenty-four hours postchallenge, mice were euthanized and the colon contents harvested. The microbiota was characterized using 16S rRNA gene sequencing. All fidaxomicin-exposed mice (except for one at day 8) were resistant to *C. difficile* colonization. However, 9 of 15 vancomycin-exposed mice were susceptible to *C. difficile* colonization until day 12. All vancomycin-exposed mice recovered colonization resistance by day 16. Bacterial diversity was similar prior to antibiotic exposure in both arms and decreased substantially after exposure. A shift in taxonomic structure and composition occurred after both exposures; however, the shift was greater in vancomycin-exposed than in fidaxomicin-exposed mice. In summary, compared with vancomycin, fidaxomicin exposure had less impact on microbiota composition, promoted faster microbial recovery, and had less impact on the loss of *C. difficile* colonization resistance.

KEYWORDS *Clostridium difficile*, diarrhea, fidaxomicin, recurrences, vancomycin

Clostridium difficile infection (CDI) remains a leading cause of hospital-acquired diarrhea (1, 2) and is one of the most common causes of hospital-acquired infections (3). The incidence of CDI has increased over the last decade, more than doubling in the United States (1, 4).

The highest incidence of CDI occurs after the normal gut microbiome has been disrupted by broad-spectrum antibiotic treatment for other infections (5). Meta-analyses have identified clindamycin, cephalosporin, and fluoroquinolone antibiotic use to be the greatest risk factor of CDI (6–8). A lack of bacterial community richness and diversity after antibiotic treatment allows colonization and overgrowth of pathogenic species, such as *C. difficile* (9).

After CDI has resolved, posttreatment recurrences can occur, and the rate of recurrence after standard antibiotic treatment is rising (10–13). CDI recurs in 20 to 25% of patients after the first episode (14, 15).

Following an initial recurrence, ~38 to 65% of patients experience further recur-
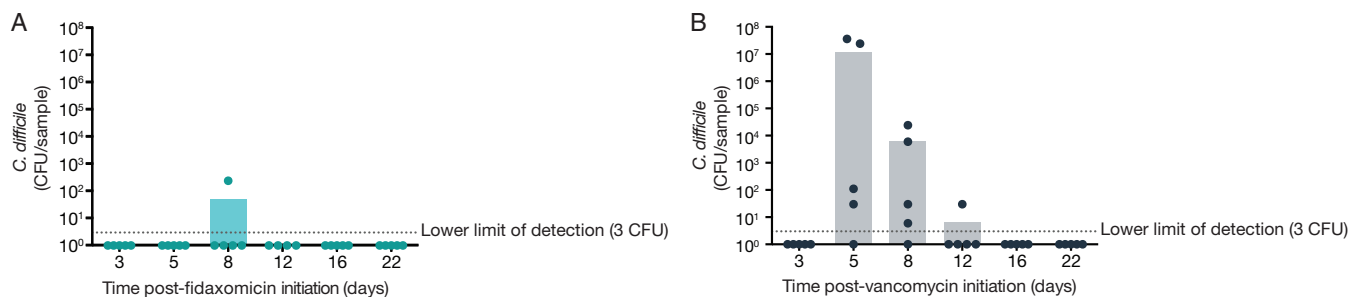
FIG 1 Susceptibility to *C. difficile* colonization following antibiotic regimens. (A) Response to *C. difficile* challenge in fidaxomicin-exposed mice. (B) Response to *C. difficile* challenge in vancomycin-exposed mice. Dots represent data from individual mice; bars represent mean values.

rence episodes (16, 17). The primary cause of recurrence is linked to nonoptimal recovery of the protective healthy microbiota (18, 19). In mice, exposure to antibiotics changes the gut microbiome, with a reduction in the predominant bacterial phyla, including *Bacteroidetes* and *Firmicutes*, and an increase in *Proteobacteria*, which make up only 2% of the healthy gut microbiome (20). Similarly, analysis of fecal microbiota composition from patients with CDI shows decreased microbially diverse populations in those with recurrent CDI versus in those presenting with an initial incident of recurrence (21). Thus, the recovery of a healthy and diverse microbiome after antibiotic treatment may reduce CDI recurrence (19).

For many years, two drugs were used primarily for CDI treatment, metronidazole and vancomycin (22). Metronidazole is a nitroimidazole antibiotic that inhibits nucleic acid synthesis by disrupting the DNA of microbial cells (23). Metronidazole is highly active against Gram-negative anaerobic bacteria, such as *Bacteroides fragilis*, and Gram-positive anaerobic bacteria, including *C. difficile*. It is considered appropriate for use in mild-to-moderate first-time cases of CDI (22, 24). Vancomycin is a large hydrophilic antibiotic used to treat Gram-positive bacteria; it is used in both mild-to-moderate and more severe cases of CDI (22). While these antibiotics suppress the growth and proliferation of *C. difficile*, they also disrupt the resident bacterial population in the colon that provides a barrier to CDI (25).

Fidaxomicin is a narrow-spectrum first-in-class macrolide antibacterial drug indicated for use in adults (≥18 years of age) for the treatment of *C. difficile*-associated diarrhea (CDAD). Fidaxomicin has been shown to have little disruptive effect on the major Gram-negative and Gram-positive species of patients with CDI, including *B. fragilis*, clostridial clusters XIVa and IV, and *Bifidobacterium* spp., meaning that commensal gut bacteria are preserved *in vivo* upon treatment (26, 27).

To further explore the preservation of commensal bacteria by fidaxomicin and the associated lack of disruption of natural resistance against CDI, we utilized a murine model. The aims of this study were 3-fold: to evaluate the effect of a 3-day treatment with either vancomycin or fidaxomicin on the normal murine gut microbiota, to determine whether susceptibility to *C. difficile* colonization increased posttreatment, and to assess the recovery of the microbiome over time postexposure initiation.

RESULTS

Microbiological outcomes. In general, a reduced loss of microbiota-mediated *C. difficile* colonization resistance was observed from the fidaxomicin-exposed mice compared with vancomycin-exposed mice (Fig. 1). On day 3, all fidaxomicin- and vancomycin-exposed mice had *C. difficile* CFU numbers below the lower limit of quantification, likely due to insufficient time to grow or to inhibitory levels of antibiotic remaining in the gastrointestinal tract.

On study days 5, 8, 12, 16, and 22, all fidaxomicin-exposed mice, except for one on day 8, were still resistant to *C. difficile* colonization. Vancomycin-exposed mice lost resistance to *C. difficile* colonization between days 5 and 12; 4 out of 5 mice were susceptible to *C. difficile* challenge on days 5 and 8, and one out of five mice was still
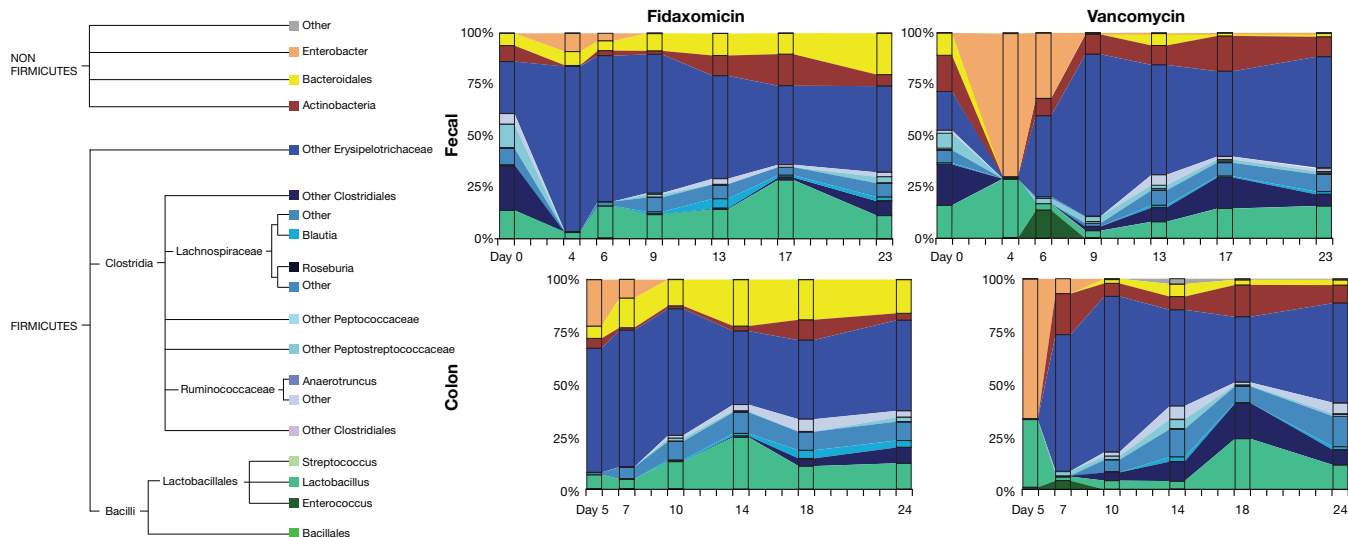
FIG 2 Fecal and colon microbiota composition following antibiotic regimens. Taxonomy is described to the most detailed level possible.

susceptible to challenge by day 12. All mice in the vancomycin group had recovered colonization resistance by day 16 (2 weeks postcessation of antibiotics).

Impact on intestinal microbiota and diversity. Fecal bacterial diversity was similar before antibiotic exposure in both arms but decreased substantially after exposure (Fig. 2; see also Table S1 in the supplemental material). A shift in taxonomic structure and composition occurred after exposure to both antibiotics (Fig. 3). Fecal samples from mice exposed to either of the antibiotics showed an increase in *Enterobacteriales* and a decrease in *Clostridiales* immediately after antibiotic cessation. This shift was greater in vancomycin-exposed than in fidaxomicin-exposed mice. Immediately after antibiotic cessation, fecal (assessed on day 3) and colonic (assessed on day 4) samples showed that operational taxonomic units (OTUs) for *Clostridiales* in both exposure arms had increased. *Enterobacteriales* started to decrease after the cessation of fidaxomicin exposure; however, the trend toward recovery was delayed in vancomycin-exposed mice. Exposure to either antibiotic resulted in a reduction in the richness and evenness of the bacterial community, as evidenced by the Shannon diversity index scores calculated from fecal and colonic samples. This difference was less pronounced in mice exposed to fidaxomicin than in mice exposed to vancomycin (Fig. 4). During the recovery of the microbiota following the administration of either antibiotic, there was a lack of expansion of *Enterococcus* OTUs and a rapid expansion of various other orders of bacteria. Principal coordinates were generated to explain maximum aggregate variance of samples in each exposure arm. The coordinates indicated that the early vancomycin samples clustered separately from the majority of other samples (Fig. 5). To evaluate the similarity in composition between samples, Jaccard distances were generated, and statistically significant differences in the structure of the fecal ($P < 0.01$, F-statistic $= 3.41$) and colon ($P < 0.01$, F-statistic $= 2.95$) microbiomes were observed after fidaxomicin and vancomycin treatments.

DISCUSSION

In this study, the influence of fidaxomicin or vancomycin on susceptibility to *C. difficile* gut colonization and microbially diverse populations was evaluated in a murine model. All mice but one exposed to fidaxomicin remained resistant to *C. difficile* colonization postexposure to antibiotics, which correlated with reduced disruption and rapid recovery of the intestinal microbiota. Exposure to either fidaxomicin or vancomycin resulted in a loss of microbially diverse populations and a shift in the structure and composition of the microbiota.

The magnitude and specificity of the effect on microbiota for each treatment
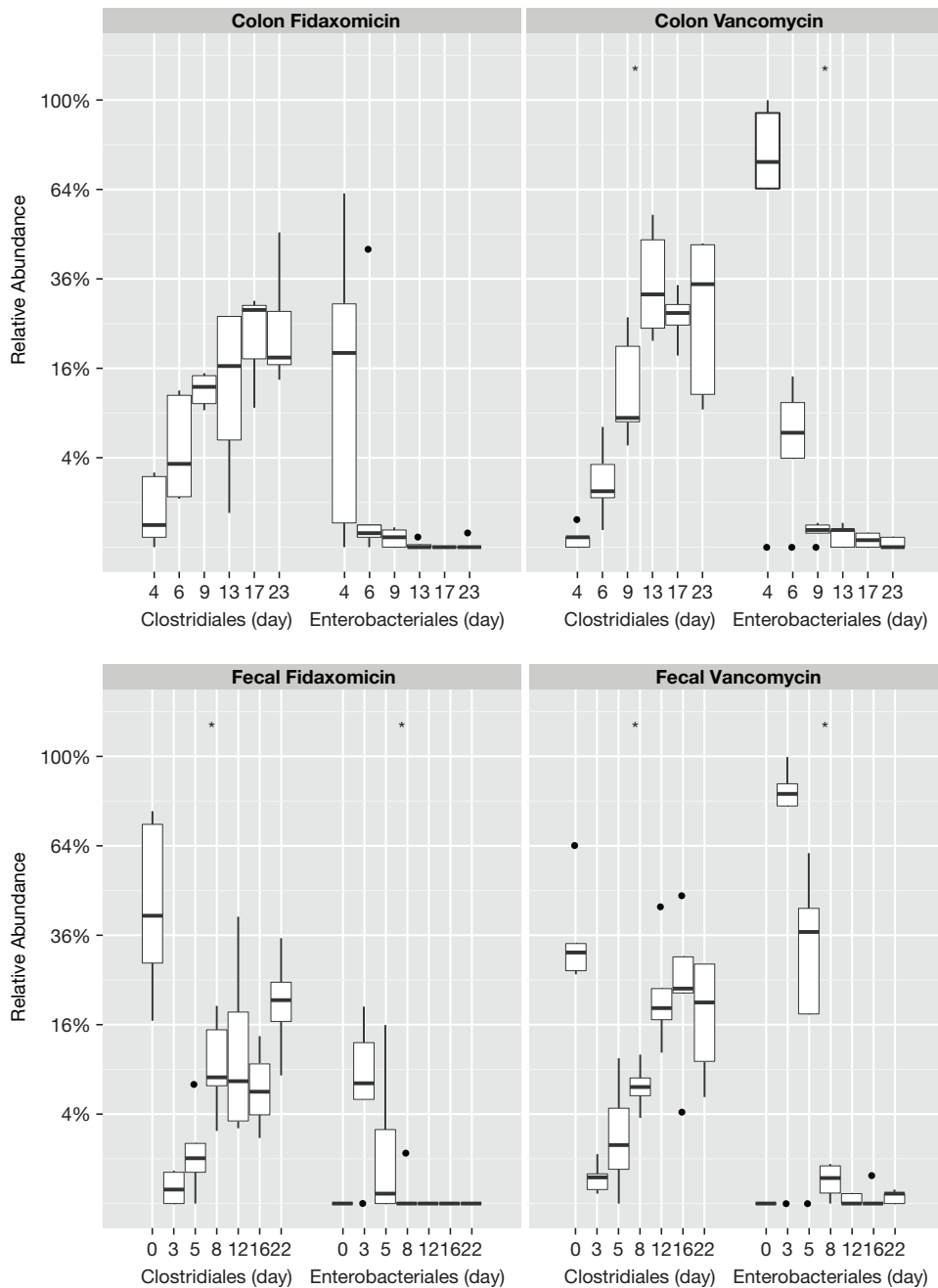
FIG 3 Tukey box-and-whisker plot showing fecal and colon taxonomic abundance following antibiotic regimens. Boxes represent Q1/Q3 limits; whiskers are ±1.5 standard deviation (SD). Dots represent data points outside the ±1.5 standard deviation (SD), and the horizontal lines are the statistical medians.

differed; for example, in mice exposed to vancomycin, a larger percentage of the microbiota present after exposure in both colon and fecal samples was of the enterococcal clade than in the gut microbiota of animals exposed to fidaxomicin. This observation is in agreement with previous fidaxomicin animal studies in which exposure to fidaxomicin led to minimal microbiota dysbiosis and did not promote colonization by vancomycin-resistant enterococci and *Klebsiella pneumoniae*, whereas exposure to vancomycin did promote colonization by these bacteria (20, 28).

The lesser impact of fidaxomicin on the microbiota correlates with prior observations in patients with CDI (26). The link between the reduced susceptibility to *C. difficile* challenge and exposure to fidaxomicin versus vancomycin supports the observations in
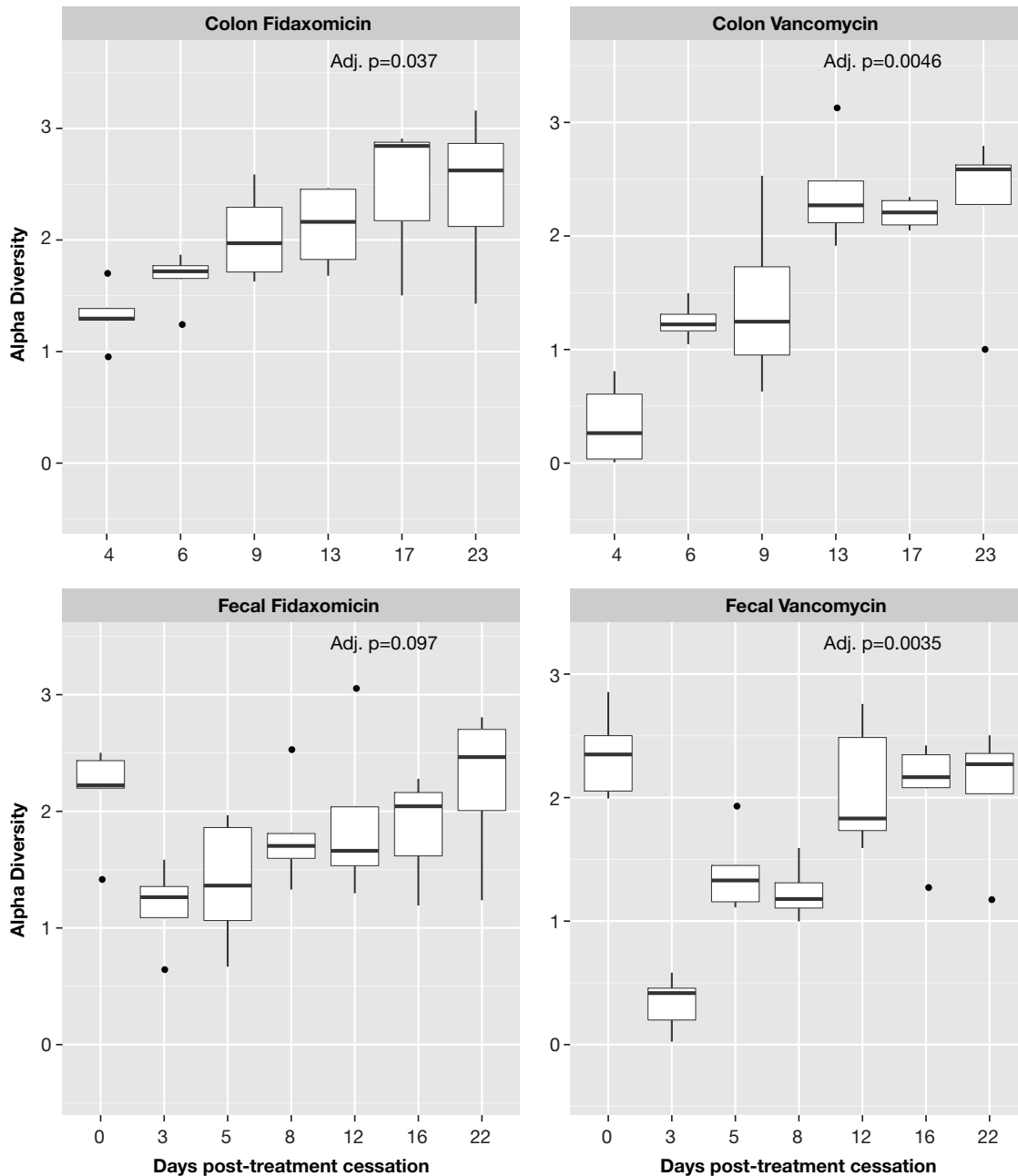
FIG 4 Tukey box-and-whisker plot showing biodiversity of fecal and colon microbiota following antibiotic regimens (Shannon diversity index scores). Shannon diversity indices of fecal and colon bacterial communities collected at successive time points following antibiotic regimen, calculated from 16S rRNA gene sequence analyses. Boxes represent Q1/Q3 limits; whiskers are ± 1.5 standard deviation (SD). Dots represent data points outside the ± 1.5 SD, and the horizontal lines are the statistical medians. Adj. p, adjusted P value.

clinical trials where fidaxomicin has demonstrated decreased CDI recurrence rates compared with vancomycin (19, 26, 27).

With regard to the clinical setting, the preservation of species able to restrict *C. difficile* overgrowth upon exposure to fidaxomicin may allow for a faster recovery of the microbiota in patients with CDI, returning the patient to a state supportive of lowering recurrence after successful treatment. A narrow-spectrum antibiotic preserving or supporting recovery of a microbiome that will suppress the growth of colonizing species is important when considering the recent emergence of vancomycin-resistant enterococci, which can cause further problems when treating patients with CDI with vancomycin (20, 28, 29).
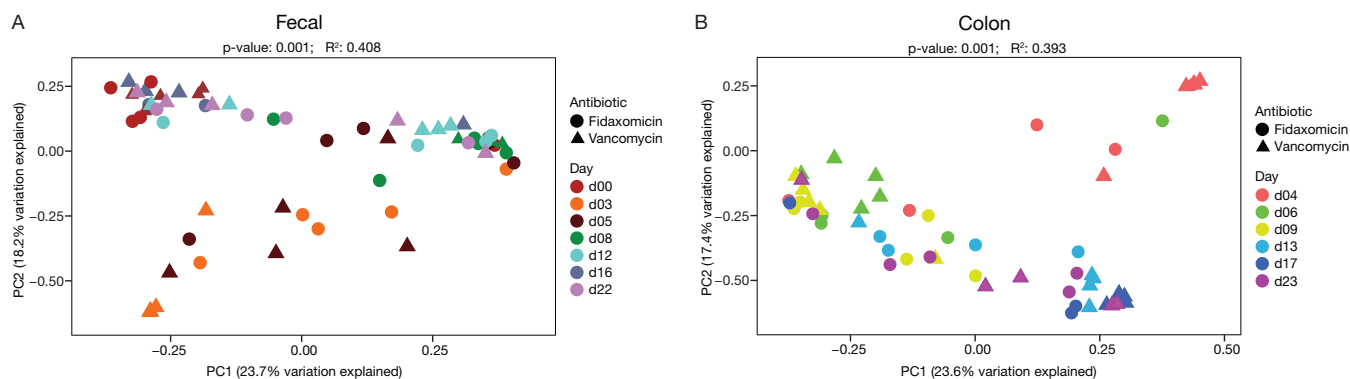
FIG 5 Principal-coordinate analysis plots of Jaccard distances of fecal (A) and colon (B) microbiota generated from 16S rRNA gene sequence analysis following antibiotic regimens. Axis labels indicate the percentage of variance explained by the respective principal coordinate axis. PC1, principal coordinate 1; PC2, principal coordinate 2.

A potential limitation of the study was that the concentration of fidaxomicin was not measured following exposure. The lack of impact on colonization resistance in mice, particularly at the earliest time points investigated, could be explained by antibiotics persisting at inhibitory levels in the gut postexposure. Vancomycin can persist in patients with CDI for several days at therapeutic concentrations after the cessation of treatment (30). Similarly, fidaxomicin is known to bind to *C. difficile* spores *in vitro* (31), although this mechanism is unlikely to be applicable to our model, as there were no spores prior to *C. difficile* challenge. Alternatively, residual fidaxomicin in the colon may have a continuing effect on the gut microbiota. An additional limitation of the study was the relatively short treatment duration of fidaxomicin and vancomycin assessed, compared with clinical treatment norms.

While both fidaxomicin and vancomycin alter gut microbiota composition and structure, the gut microbiota in mice exposed to fidaxomicin recovered more quickly than in those exposed to vancomycin. Furthermore, fidaxomicin had a lower impact on the composition and structure of the gut microbiota. Mice exposed to fidaxomicin proved to maintain colonization resistance to subsequent spore challenges, whereas mice exposed to vancomycin and then subsequently challenged with *C. difficile* spores remained susceptible to colonization until day 10.

MATERIALS AND METHODS

Mouse husbandry. All experiments were performed with wild-type female C57BL/6 mice, age 6 to 8 weeks (Jackson Laboratories, ME, USA). The mice were housed in the specific pathogen-free facility at Memorial Sloan Kettering's Animal Resource Center, fed irradiated feed, and provided with acidified water. Cages were changed at least once a week. The experiments were performed in compliance with Memorial Sloan Kettering's institutional guidelines and were approved by its Institutional Animal Care and Use Committee.

Antibiotic administration and study design. A total of 30 mice per experimental arm were exposed to 1.125 mg/day vancomycin (37.5 mg/kg in water) or 0.9 mg/day fidaxomicin (30 mg/kg in Labrasol) by oral gavage (200 μl on days 0, 1, and 2) (Fig. 6). The antibiotics were given at biologically relevant doses, as previously described (20). Each antibiotic arm consisted of 5 independently housed colonies of 6 mice during antibiotic exposure. Following the course of antibiotic exposure, a single mouse from each colony was removed, housed separately, and challenged with 1×10^3 *C. difficile* VPI 10463 (American Type Culture Collection 43255) spores in phosphate-buffered saline by oral gavage on days 3, 5, 8, 12, 16, and 22, as previously described (32). Fecal content, collected from the cage floor, was analyzed on the same day of *C. difficile* challenge. Colonic samples were recovered from sacrificed mice 1 day after challenge on days 4, 6, 9, 13, 17, and 23. Samples were immediately flash-frozen and DNA extracted as previously described (33). The samples (approximately 100 mg) were suspended in 500 μl of extraction buffer (200 mmol/liter Tris [pH 8.0], 200 mmol/liter sodium chloride, 20 mol/liter EDTA), and 200 μl of 20% sodium dodecyl sulfate, 500 μl of phenol-chloroform-isoamyl alcohol (24:24:1), and with 500 μl of 0.1-mm-diameter zirconia/silica beads (BioSpec Products, OK, USA). Bacterial cells were lysed by bead beating (BioSpec Products) for 2 min, and DNA was isolated with 2 rounds of phenol-chloroform-isoamyl alcohol extraction. Postextraction, DNA was precipitated in ethanol and resuspended in 200 μl of Tris-EDTA buffer with 100-μg/ml RNase. The DNA sample was then purified with QIAamp Mini spin columns (Qiagen, MD, USA).
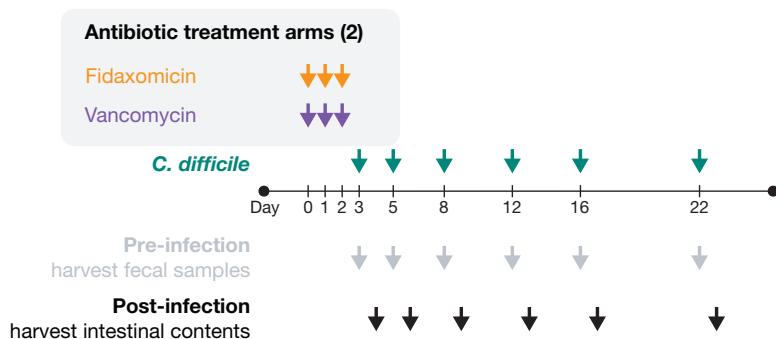
FIG 6 Study design. Mice were exposed to fidaxomicin (30 mg/kg) or vancomycin (37.5 mg/kg) by oral gavage once daily for 3 consecutive days (days 0, 1, and 2); on days 3, 5, 8, 12, 16, and 22 (post-antibiotic exposure initiation), and 1 mouse was removed from each colony, housed separately, and challenged with 1×10^3 *C. difficile* spores of strain VPI 10463 by oral gavage. Twenty-four hours later, the separated mice were sacrificed, and the colon contents were collected.

Sample analysis. The susceptibility of mice to *C. difficile* colonization was determined by selective culture of fecal and colonic contents. Colonization resistance was defined as the *C. difficile* CFU count being under the lower limit of detection (<3 CFU). Fecal and colon sample 16S rRNA genes were analyzed via Illumina MiSeq (CA, USA) to determine intestinal microbiota composition. The broad-range bacterial 16S primers 517F (5′-GCCAGCAGCCGCGCTAA-3′) and 798R (5′-AGGGTATCTAATCCT-3′) were used at 0.2-mmol/liter concentrations with the DyNAmo SYBR green quantitative PCR kit (Finnzymes, Thermo Fisher Scientific, MA, USA) quantifying amplifications for library normalization. Sample amplification was compared with standard curves to quantify the 16S rRNA gene copy number. The cycling program was as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 30 s, and finished with 95°C for 15 min, 60°C for 1 min, and 95°C for 15 min (33). The 16S rRNA gene V4-V5 sequences were processed and clustered into OTUs with >97% sequence homology. Amplicons of the V4-V5 region of the 16S rRNA gene were amplified and sequenced with the Illumina MiSeq platform, as previously described (32).

Sequence reads were merged using a minimum overlap of 50 bp and filter of 0.5% expected error. The merged reads were stripped from primer sequences and then oriented identically. The resulting data set was analyzed using the UPARSE pipeline (34). Reads were dereplicated, sorted, and clustered at 97% sequence identity. Singletons were removed from centroid consideration. Centroids or OTUs were aligned to the SILVA version 123 (35) database using USEARCH (36) to determine taxonomies. OTU-based bacterial richness and evenness were estimated by calculating the Shannon diversity index. Jaccard distances were calculated, and principal coordinates for each exposure arm were generated from 16S rRNA gene V4-V5 sequences.

Data and statistical analysis. The OTU table was used for downstream analyses using the Agile Toolkit for Incisive Microbial Analyses (ATIMA) visualization toolkit, which was developed at the Alkek Center for Metagenomics and Microbiome Research. ATIMA is a standalone tool for analyzing and visualizing microbiome data sets built in R (37) combining publicly available packages (i.e., APE and VEGAN) (38) and purpose-written code to import sample data and identify trends in taxa abundance, alpha diversity, and beta diversity with sample metadata. The data set was rarefied to 5,012 reads per sample, and the significance of categorical variables was determined using the nonparametric Kruskal-Wallis test (39) for comparing 3 or more categories. Correlation between 2 continuous variables was determined with R's base "lm" function for linear regression models, where *P* values indicate the probability that the slope of the regression line is zero. Principal-coordinate analysis (PCoA) plots were constructed with Jaccard distances and employed the Monte Carlo permutation test (40) to estimate *P* values. All *P* values were adjusted for multiple comparisons with the Benjamini-Hochberg false-discovery rate (FDR) algorithm (41).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .02112-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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