Check for updates

Microbiome Resilience despite a Profound Loss of Minority Microbiota following Clindamycin Challenge in Humanized Gnotobiotic Mice

Lin Liu,[a]* Mariana E. Kirst,[a] Lisa Zhao,[a] Eric Li,[a] (ID) Gary P. Wang[a,b]

[a]Division of Infectious Diseases and Global Medicine, Department of Medicine, University of Florida College of Medicine, Gainesville, Florida, USA
[b]North Florida/South Georgia Veterans Health System, Gainesville, Florida, USA

ABSTRACT Antibiotics are known to induce gut dysbiosis and increase the risk of antibiotic resistance. While antibiotic exposure is a known risk factor leading to compromised colonization resistance against enteric pathogens such as *Clostridioides difficile*, the extent and consequences of antibiotic perturbation on the human gut microbiome remain poorly understood. Human studies on impacts of antibiotics are complicated by the tremendous variability of gut microbiome among individuals, even between identical twins. Furthermore, antibiotic challenge experiments cannot be replicated in human subjects for a given gut microbiome. Here, we transplanted feces from three unrelated human donors into groups of identical germfree (GF) Swiss-Webster mice, and examined the temporal responses of the transplanted microbiome to oral clindamycin challenge in gnotobiotic isolators over 7 weeks. Analysis of 177 longitudinal fecal samples revealed that 59% to 81% of human microbiota established a stable configuration rapidly and stably in GF mice. Microbiome responses to clindamycin challenge was highly reproducible and microbiome-dependent. A short course of clindamycin was sufficient to induce a profound loss (~one third) of the microbiota by disproportionally eliminating minority members of the transplanted microbiota. However, it was inadequate to disrupt the global microbial community structure or function, which rebounded rapidly to resemble its pre-treatment state after clindamycin discontinuation. Furthermore, the response of individual microbes was community-dependent. Taken together, these results suggest that the overall gut microbiome structure is resilient to antibiotic perturbation, the functional consequences of which warrant further investigation.

IMPORTANCE Antibiotics cause imbalance of gut microbiota, which in turn increase our susceptibility to gastrointestinal infections. However, how antibiotics disrupt gut bacterial communities is not well understood, and exposing healthy volunteers to unnecessary antibiotics for research purposes carries clinical and ethical concerns. In this study, we used genetically identical mice transplanted with the same human gut microbiota to control for both genetic and environmental variables. We found that a short course of oral clindamycin was sufficient to eliminate one third of the gut bacteria by disproportionally eliminating minority members of the transplanted microbiota, but it was inadequate to disrupt the overall microbial community structure and function, which rebounded rapidly to its pre-treatment state. These results suggest that gut microbiome is highly resilient to antibiotic challenge and degradation of the human gut ecosystem may require repeated or prolonged antibiotic exposure.

KEYWORDS clindamycin, gut dysbiosis, humanized gnotobiotic mice, microbiome, resilience

Antibiotics are known to alter gut microbiota, select drug resistant organisms, and decrease colonization resistance against enteric pathogens. The impact of antibiotics on colonization resistance is particularly relevant in *Clostridioides difficile* infection, a leading cause of hospital-acquired infections responsible for half a million cases and 29,000 deaths in the United States annually (1, 2). Exposure to antibiotics is a major risk factor leading to compromised colonization resistance against *C. difficile*, and thus a detailed understanding of the impact of antibiotics on gut microbiome is critically important. With increasing appreciation of gut-brain, gut-liver, and gut-immune axes, antibiotic-induced perturbation of gut microbiota may have a far-reaching impact on host physiology and immune functions (3–6). Thus, to improve health outcomes, the therapeutic effects of antibiotics and the associated unintended consequences and perturbation on the gut microbiome will require a thorough investigation.

The impacts of antibiotics on gut microbiota depend on the antibiotic class, dose, duration of exposure, pharmacological action, and the spectrum of the targeted bacteria (7, 8). Microbiome composition generally experiences drastic changes following antibiotic administration. In some studies, the majority of the microbiota returns to its pre-exposure level within 2 to 4 weeks (9, 10). In others, it remains altered despite discontinuation of antibiotics (follow-up duration ranging from 14 days to 24 months) (11–13). Whether repeated use of antibiotics leads to permanent alterations of the microbiome is unclear, but seems likely given the elevated risk of *C. difficile* colitis associated with frequent antimicrobial therapy. In-depth investigation of the impacts of antibiotic exposure is thus essential to better understand this process. However, such investigations in human subjects are hampered by the tremendous variability of gut microbiome among individuals, even between identical twins, because both genetic and environmental factors play an important role in shaping the microbiome (14–17). In addition, exposing healthy volunteers to unnecessary antibiotics for research purposes carries ethical and clinical concerns. Therefore, germfree (GF) mice colonized with human fecal microbiota is an ideal model for investigating the role of human-associated microbiota in host physiology and pathology (18–22). Humanized mice in a controlled gnotobiotic environment provide the ability to control both genetic and environmental variables to allow for systematic investigations of the effect of antibiotics on microbiome composition, structure, and function.

In this study, we generated three groups of gnotobiotic mice harboring different human microbiota by transplanting fecal samples from three healthy volunteers into GF mice. We then challenged the humanized mice in gnotobiotic isolators with clindamycin, an antibiotic frequently associated with *C. difficile* infection, and examined the impact of oral clindamycin on human-associated gut microbiota.

RESULTS

Transplantation of human fecal microbiota into GF mice. To evaluate the reproducibility of human fecal microbiota transplantation without environmental contamination, we generated three sets of ex-GF mice with distinct human microbiota (groups B, C, and D) (Fig. 1). Fecal samples from three unrelated human donors were transplanted into a set of otherwise identical GF male Swiss-Webster mice (*n* = 12 in each group). The animals were maintained in three separate gnotobiotic isolators throughout the experiment. A fourth group (group A) was orally gavaged with reduced sterile phosphate-buffered saline (PBS) to serve as a control. The control mice remained GF over the duration (7 weeks) of the entire experiment, as assessed by standard culture and 16S rRNA PCR of fecal samples.

As expected, transplantation of human fecal microbiota reduced the cecum of GF animals compared with control gavage. To assess temporal variation and stability of human gut microbiota, fecal pellets were collected longitudinally and a subset of fecal samples analyzed by 16S rRNA sequencing. A total of ~7.3 million 16S rRNA sequence reads with a total of 610 bacterial phylotypes (averaging 41,500 reads per sample). Rarefaction analysis showed that the sequencing depth was sufficient and further
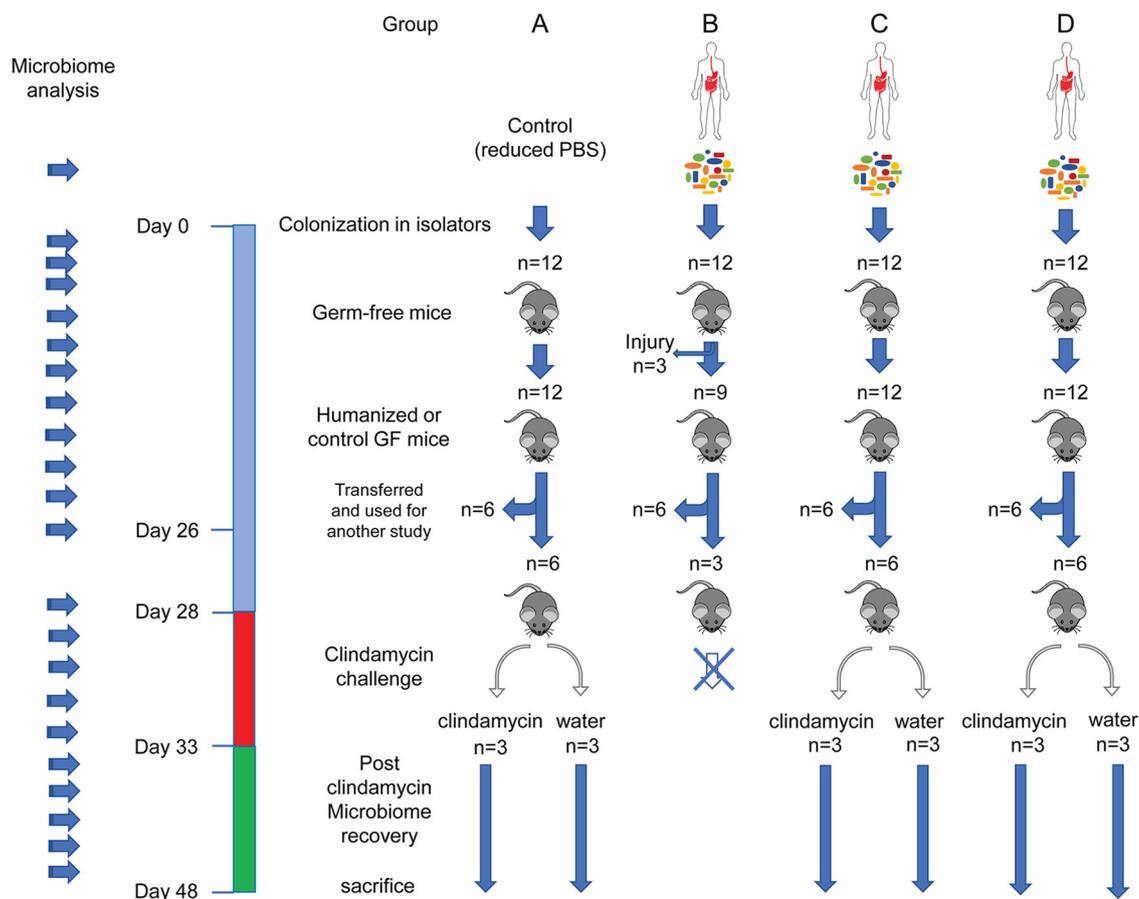
FIG 1 Study design. Swiss-Webster germfree (GF) mice were gavaged with fecal samples from three unrelated healthy human donors or reduced PBS as mock control, and maintained in four different isolators. After 4 weeks, two groups of ex-GF mice and one group of GF mice were challenged with clindamycin or given sterile drinking water as controls (n = 3 per group) for 5 days. Murine fecal samples were collected longitudinally throughout the experiment and stored in a −20°C freezer for 16S rRNA analysis.

sampling would likely yield few additional operational taxonomic units (OTUs) (23). Good's coverage estimates approached 1.00 for all samples (0.9853 to 0.9996).

Human microbiota established a stable microbiome configuration rapidly in GF mice. We first examined the assemblage and temporal variation of human fecal microbiota transplanted in the murine gut (Fig. 2A). Unifrac analysis showed that donor microbiota underwent an initial brief (~3 days) and dynamic transition, then achieved a stable microbiome configuration within 7 days in the murine gastrointestinal tract. The tight clustering of microbial communities from day 3 through day 20 for each donor group demonstrates reproducibility and stability of the transplanted human microbiota in GF mice. In addition, the clear separation of the transplanted microbiota according to donor microbiota showed that the establishment of the final microbiome configuration in the murine gut was donor microbiome-dependent. Furthermore, the microbial community structure in mice was distinguishable from that of their respective donor microbiota, suggesting that the assemblage and structure of gut microbiome may be species-dependent, although factors such as diets could have also contributed to these differences. Comparison of individual bacterial taxa between donor and murine microbiomes showed that 59% to 81% of human-associated bacterial phylotypes (OTUs) were successfully transplanted in mice and that the transplanted microbiota in mice had lower diversity than the donor microbiota (Fig. 2B). However, there were detectable differences in alpha diversity between groups.

Clindamycin challenge decreased species richness but not biodiversity of gut microbiota. Prior to antibiotics challenge experiments, one animal from each cage (i.e., six mice from each group) was removed and used for a separate study (Fig. 1). In
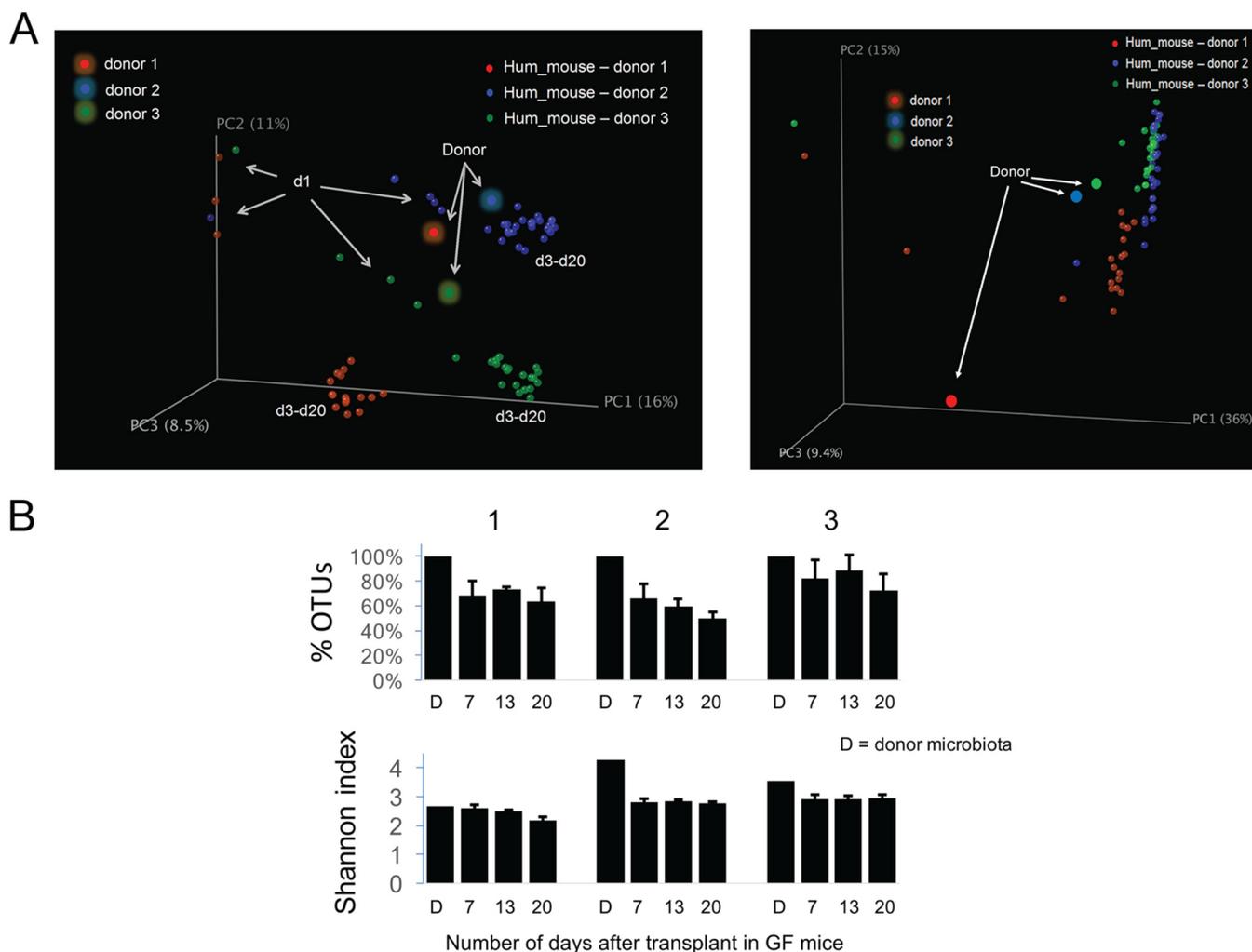
FIG 2 Unifrac analysis of fecal microbiota from human donors and recipient mice. Human fecal samples from three unrelated healthy volunteers (highlighted dots) were gavaged into male Swiss Webster GF mice in three separate isolators (*n* = 12 mice per isolator) and fecal samples were collected from each animal longitudinally. Temporal variation in fecal microbiota was examined using 16S rRNA analysis (four to five time points from three animals per group were analyzed). (A) Unweighted (left) and weighted (right) Unifrac was used to generate distances among all microbiome samples. Scatterplots were then generated using principal coordinate analysis (PCA). The percentage of variation explained by each principal coordinate (PC) is indicated on the axes. Each point represents a microbial community. d1, day 1 postcolonization; d3 to d20, day 3 through day 20; each color represents a unique donor inoculum. (B) Microbial richness (% OTUs) and diversity (Shannon Index) were compared between donor human microbiota (D) and transplanted microbiota established in GF mice.

addition, three animals from group B were sacrificed due to suspected gavage injuries, and thus group B was not included in the clindamycin challenge study. For the remaining animals (*n* = 6 for each group), three were treated with clindamycin in drinking water and three animals received mock treatment with sterile water. To examine the impact of antibiotics on gut microbiota in the absence of environmental microbial contamination, all animals remained in gnotobiotic isolators and fecal pellets were collected longitudinally for 16S rRNA sequence analysis. GF mice (group A, GF gavage control) was also treated with clindamycin or mock treatment (*n* = 3/group). Standard culture and 16S rRNA PCR of their fecal samples showed no contamination of environmental microbes throughout the experiment. No diarrhea or changes in food intake or body weight were observed in any of the animals throughout the experiment. Longitudinal 16S rRNA analysis demonstrated that both species richness (observed OTU numbers) and biodiversity (Shannon entropy) decreased rapidly and sharply following clindamycin treatment (*P* < 0.01; compared with controls) (Fig. 3). After clindamycin was removed on day 5, OTU numbers and Shannon indices gradually increased and plateaued at ~12 days, and remained stable until the end of the experiment (day
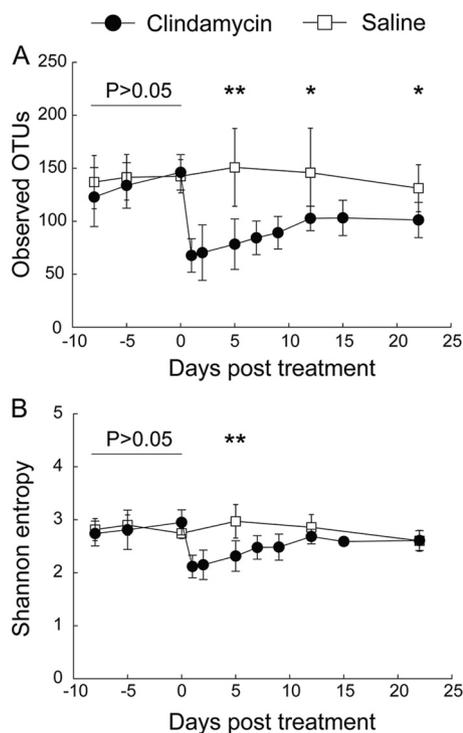
FIG 3 Changes in gut microbial diversity and richness in response to oral clindamycin challenge. (A) Species richness (observed OTUs) and (B) Shannon diversity (entropy) of fecal microbiota before and after 5 days of clindamycin or mock treatment are shown on the y-axes. Mean values are compared between the clindamycin group (solid circles) and the saline controls (empty squares). No statistically significant difference was observed in OTU numbers or Shannon entropy between the two groups at the three time points prior to clindamycin treatment ($P > 0.05$). A single asterisk indicates a P value of <0.05 at the corresponding time points, and double asterisks indicate a P value of <0.01.

21). Although microbial diversity rebounded to the pre-treatment level by the end of the experiment (Fig. 3B), species richness did not. No temporal change in OTU numbers or Shannon entropy was detected in the mock challenge (sterile water) group.

Clindamycin rapidly induced gut dysbiosis in mice. To further evaluate the microbial population structure impacted the most by clindamycin challenge, we utilized the Unifrac approach. We posited that because changes in weighted Unifrac analysis are driven predominantly by abundant taxa and unweighted Unifrac is sensitive to changes in all members of the community including minority species, comparison of weighted and unweighted analysis should provide insight into the relative effects of clindamycin on dominant and minority microbial populations. In weighted Unifrac analysis, microbial communities before and after clindamycin challenge clustered tightly, indicating little impact of clindamycin on dominant members of the microbiota (Fig. 4). Using PICRUSt (a bioinformatics tool to predict gene functions based on 16S rRNA information) (24), comparison of the predicted functional metagenome before and after clindamycin challenge showed only 25 to 42 of 328 (8% to 13%) gene categories differentially abundant with an LDA score of >2, indicating only minor impact of clindamycin on the overall microbiome function. In contrast, unweighted Unifrac analysis revealed a clear separation between communities before and immediately after clindamycin challenge, followed by a gradual rebound of microbiome after discontinuation of clindamycin to resemble, but not cluster with its pretreatment state (Fig. 4). These changes were reproducible between mice within each group, and the same pattern of microbiome evolution was observed in the two groups of mice that harbored two different donor human microbiota. No temporal change in microbial communities was observed in the control groups.

Clindamycin eradicated a large number of minority taxa from the gut microbiota. The species richness and Unifrac analysis (Fig. 3 and 4) suggest that a certain number

**Unweighted
Unifrac**

**Weighted
Unifrac**
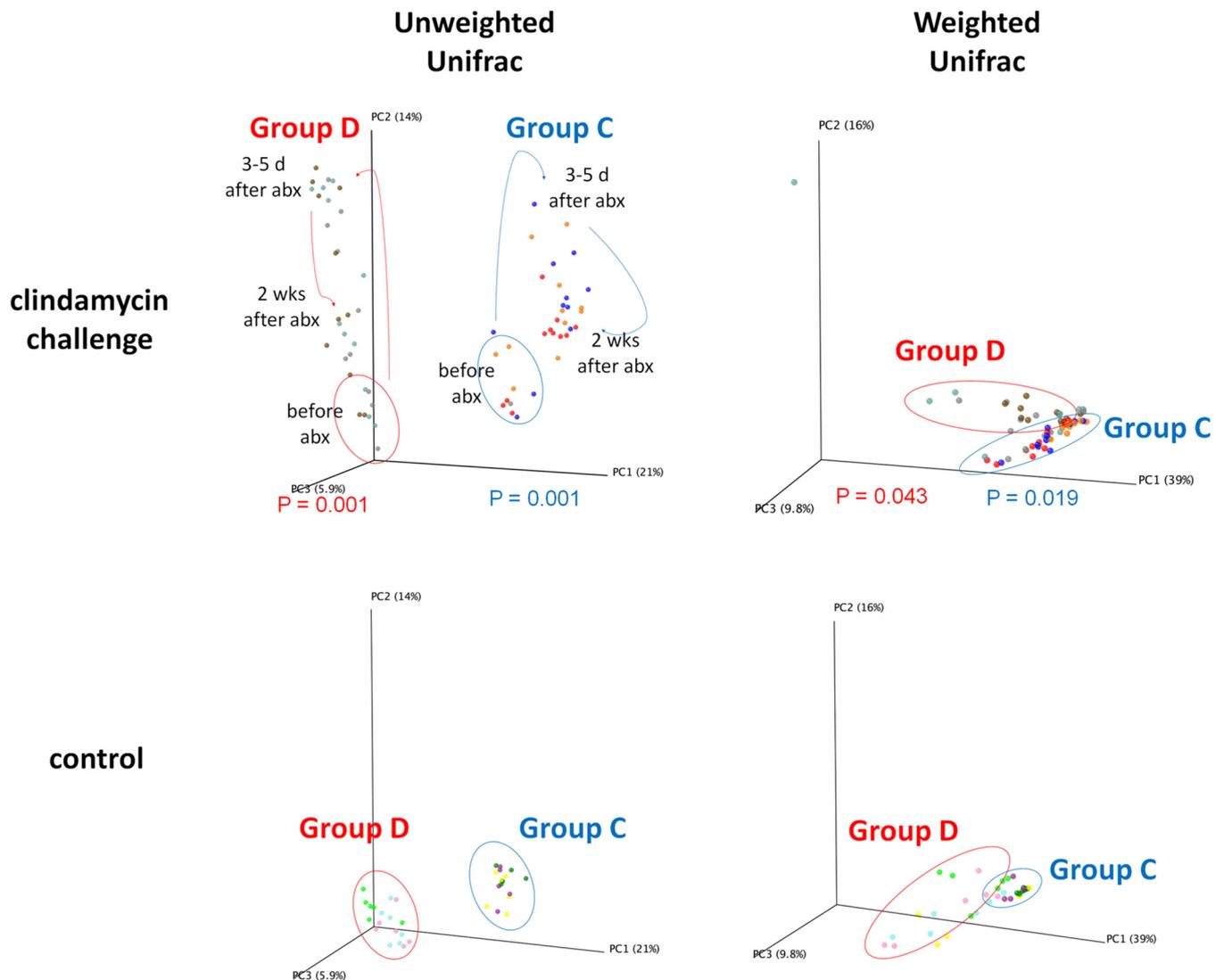
FIG 4 A single course of oral clindamycin induced a rapid and reproducible disturbance in gut microbiota. Ex-GF mice harboring human gut microbiota were treated with oral clindamycin for 5 days and longitudinal fecal microbiota compared using 16S rRNA sequence analysis. Weighted (right) and unweighted (left) analyses are shown as scatterplots using Unifrac principal coordinate analysis (PCA). The percentage of variation explained by each principal coordinate (PC) is indicated on the corresponding axes. Each color point represents a microbial community and samples from the same animal are shown using the same color dots. In unweighted Unifrac (top left), microbial communities before clindamycin treatment, 3 to 5 days after clindamycin, and 2 weeks after clindamycin clustered separately. In contrast, weighted Unifrac (top left) showed tighter clustering of microbial communities before and after clindamycin challenge. Circles and arrows in blue and red represent microbial communities from two different donor microbiome, C and D, respectively. Fecal microbiome before and after clindamycin challenge were compared and the statistical significance of the microbiome difference before and after clindamycin was determined using permutational multivariate analysis of variance (PERMANOVA) and the *P* values for group C and D are shown in blue and red, respectively. Ex-GF control mice treated with sterile water showed no appreciable changes in gut microbiome throughout the experiment (bottom left and right).

of minority taxa in the transplanted microbiota was eradicated by clindamycin. To further examine the fate of these minority taxa, we applied the following criteria to define species eliminated by clindamycin: (i) it must be present at baseline (i.e., day 0, just prior to clindamycin challenge) in at least two (of three) mice within groups; (ii) it must be present in at least one other pre-treatment time points (8 or 5 days before clindamycin challenge); (iii) it must be absent at the final time point (day 22); and (iv) it must also be absent in at least one of the two time points prior to day 22 (i.e., day 12 and 15, demonstrating persistent absence). Using these criteria, 52 species in microbiome community C and 43 species in community D were eliminated by clindamycin, constituting 62% (52/84) and 43% (43/100) of the species that were suppressed by clindamycin, and 39% (52/134) and 29% (43/148), or approximately one third, of all species at
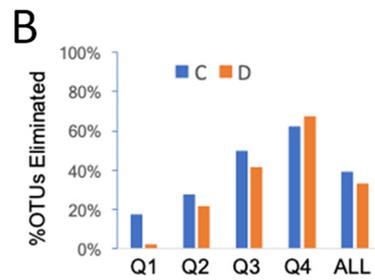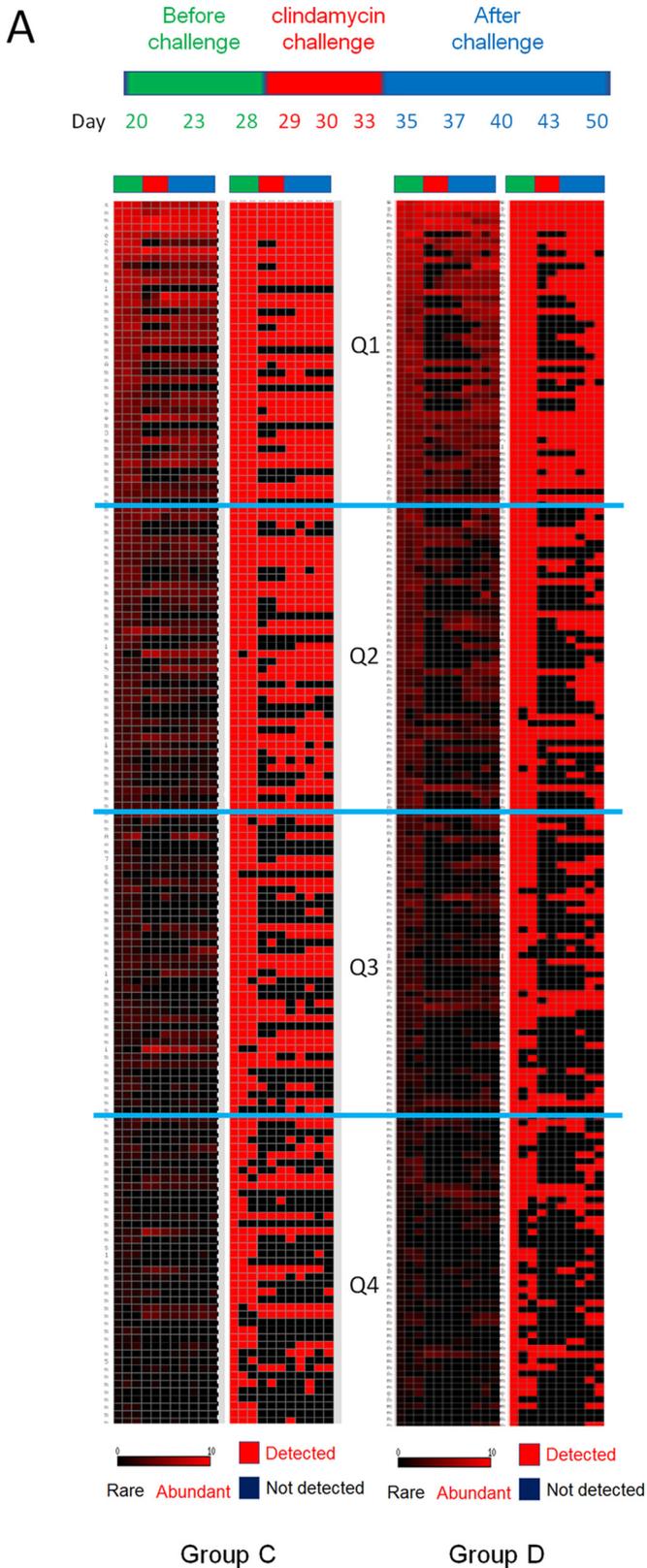
FIG 5 Clindamycin challenge disproportionally eliminates minority members of the transplanted microbiota. Two groups of humanized mice (group C and group D, see Fig. 1) were challenged with 5 days of clindamycin, and longitudinal fecal microbiome were analyzed. (A) For each group (C and D), two heatmaps are shown. The heatmap on the left shows the relative abundance of OTUs (red: high abundance; black: low abundance), and the heatmap on the right indicates OTUs detected (red) or not detected (black). Each column represents samples from a specific time point (from left to right, before

baseline in communities C and D, respectively (Fig. 5 and supplemental figure and data). The majority of these species were members of the *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae* families, which accounted for 94% and 84% of all species eliminated by clindamycin in communities C and D, respectively. Interestingly, these minority species constituted only 0.01% to 0.59% of the total microbial community abundance (proportions based on sequence reads) at baseline in community C, and 0.01% to 3.37% in community D, thus consistent with the results from weighted and unweighted Unifrac analysis (Fig. 4).

Thus, together with the observed changes in alpha diversity indices (Fig. 3) and microbiome structure (Fig. 4), these data suggest that a short course of oral clindamycin challenge was sufficient to induce a profound loss of transplanted microbiota by disproportionally eliminating minority members of the microbiota, but was inadequate to perturb the global microbial community structure (and possibly function), thus supporting the hypothesis that gut microbiome is resilient to a single short course of antibiotic perturbation.

Community-dependent response of individual microbes to clindamycin challenge. Given that a subset of taxa may be shared between two different human microbiota, we asked if a shared microbe residing in two different communities would respond similarly to clindamycin challenge. We identified a total of 30 bacterial taxa that were shared between the two groups of mice harboring two different microbiota communities (supplementary data). For each taxon, we quantified the changes in relative abundance before and after clindamycin challenge and compared their responses in the two communities (Fig. 6). Surprisingly, while 57% (17/30) of the shared taxa exhibited concordant responses to clindamycin (Fig. 6A; the shared species were suppressed (I) or enriched (II) in both mice groups), 43% (13/30) had discordant responses (pattern III and IV). For example, microbial species was suppressed in one community but remained largely unaffected in the other in response pattern III. In pattern IV, species were suppressed in one community but enriched in the other. For most shared species, the impact of clindamycin was transient and the organisms rebounded to a level close to its pretreatment state. However, several species remained persistently suppressed or enriched through the end of our experiment. For example, *Lachnospiraceae* OTU 1342 (pattern I) was persistently suppressed in group C mice (Fig. 6B). *Ruminococcaceae* OTU 1931 (pattern IV) was persistently enriched in group C but suppressed in group D mice.

As expected for gut-associated microbes, most of the shared taxa (Fig. 7) were members of the *Firmicutes* and *Bacteroidetes* phyla. Consistent with clindamycin's spectrum of antimicrobial activity, many of the *Firmicutes* species were suppressed (response pattern I) and no shared *Firmicutes* species became more abundant (pattern II) in both groups. For *Bacteroidetes* species, differential response to clindamycin challenge (patterns I to IV) was observed, consistent with their increasing resistance to clindamycin. In contrast, all shared *Proteobacteria* species (*E. coli*, *Cronobacter*, *Enterobacter*, *and Klebsiella* spp.), which are usually minority constituents of the gut microbiota, were enriched following clindamycin challenge, which could be due to the suppression of *Firmicutes* species and/or a result of clindamycin insensitivity among aerobic Gram-negative bacteria.

DISCUSSION

In recent decades, intense efforts have focused on the mechanisms and effects of antibiotics on specific strains of bacteria. It is only in recent years that interests in the field have expanded to understand the global impacts and clinical consequences of antimicrobial

FIG 5 Legend (Continued)
clindamycin challenge: day 20, 23, 28; during clindamycin challenge: day 29, 30, 33; after challenge: day 35, 37. 40, 43, and 50; see Fig. 1). Each row is an OTU, ordered from top to bottom by the relative abundance of the OTU on day 20. OTUs were binned by quartiles (Q1 to Q4). Q1 includes top 25% OTUs in relative abundance on day 20, whereas Q4 includes the bottom 25% OTUs representing minority or low abundance OTUs. (B) The proportion of OTUs eliminated by clindamycin challenge in each quartile. A high percentage of OTUs in Q4 (i.e., minority OTUs) were eliminated by clindamycin challenge, and a low proportion of OTUs in Q1 were eliminated. Overall, approximately one third of OTUs were eliminated ("ALL").
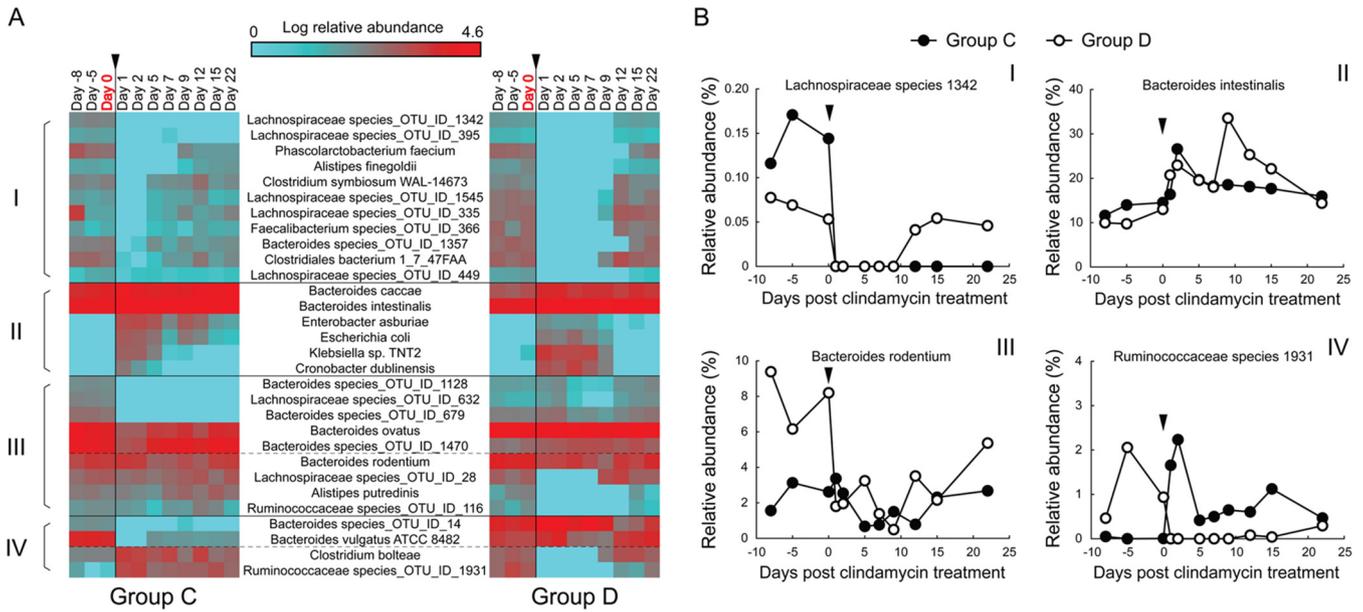
FIG 6 Community-dependent response of gut-associated microbes to clindamycin challenge. (A) Heat map of relative abundance of gut-associated microbes showing community-dependent, individualized response to clindamycin challenge. Mean values were used for both groups. Relative abundance is expressed as $\log_{10}$ (percent relative abundance x 100,000) for better visualization of minority species. The log value scale is shown on top. Post-treatment days are indicated above the heat map with pre-treatment baseline bolded in red and the black arrowhead indicating the start of 5-day clindamycin treatment. Four major response patterns were observed: (I) suppressed in both mice groups; (II) enriched in both groups; (III) suppressed in one group but fluctuating or no significant change in the other; and (IV) suppressed in one group but enriched in the other. The taxonomy of each bacterial species is shown in-between the heat maps. (B) Temporal changes of the relative abundance of a representative species from each group are shown. Black arrowheads indicate the start of clindamycin challenge.

therapy on the overall host microbiota. In the present study, we transplanted human fecal microbial communities into groups of otherwise identical GF mice and examined the responses of distinct human microbiome in identical murine host to oral clindamycin challenge in the absence of environmental contamination. We showed that a single, short course of oral clindamycin induced potentially irreversible eradication of gut microflora *in vivo*, eliminating predominantly minority populations of the commensal gut microbiota (Fig. 3 and 5). However, the overall microbiome rebounded rapidly to resemble its pre-challenge state, suggesting a high resilience of community structure to antibiotic perturbation. Using genetically identical mice harboring the same transplanted microbiome, we also demonstrated that microbiome responses to clindamycin challenge is highly reproducible and is microbiome-dependent.

A particular area of clinical importance is antibiotic-induced loss of colonization resistance to pathogenic species (25, 26), which include *Clostridioides difficile* and *Salmonella* infection during and following antibiotic therapy. It has been widely appreciated that antibiotic disruption of the host microbiota increases the susceptibility to infection, but a detailed understanding of its impact on microbiome has been complicated by genetic and microbiome differences among people in human studies, and the difficulty in controlling for microbiome and environmental variables in traditional animal models. Collins et al. have shown in a humanized mouse model that pretreatment with a 5-antibiotic cocktail increased susceptibility of experimental mice to *C. difficile* infection (22). In our study using similar humanized mice, oral clindamycin eliminated a large proportion of minority species from the microbiome. However, dominant species remained relatively unperturbed or experienced only transient alterations in abundance. Interestingly, the same bacterial species in different microbial environments could have differential responses to clindamycin (Fig. 6A, B; response patterns III and IV), and different species within the same phylum could display differential responses to the same antibiotic challenge. These findings raise the question of whether microbial responses to antibiotics are determined by the inherent susceptibility of their respective species, or the microbial communities in which they reside. In
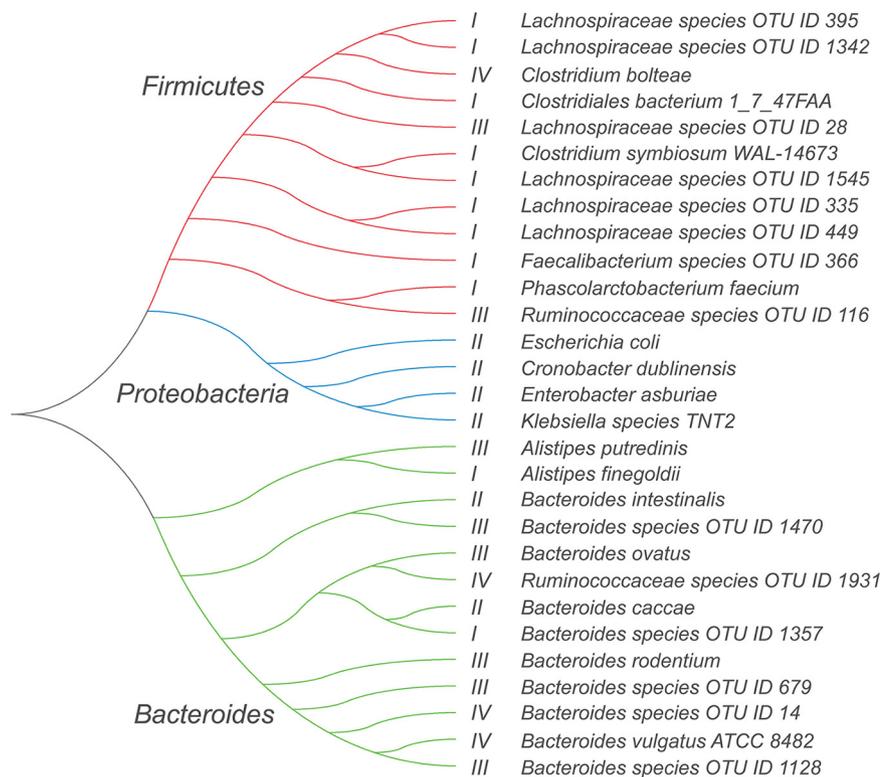
FIG 7 Differential responses of Firmicutes, Bacteroidetes, and Proteobacteria species to clindamycin challenge. A maximum likelihood tree was constructed using representative 16S rRNA sequences of the 30 shared bacterial species from Fig. 6. The response patterns (I through IV) to clindamycin challenge for each species as shown in Fig. 5 is indicated. The three major phyla are depicted in red (Firmicutes), blue (Proteobacteria), and green (Bacteroidetes) branches.

the latter case, which component would play a more important role? As shown in Fig. 6A, the number of species that responded differently to clindamycin is comparable to the number of species that responded similarly (13 versus 17), suggesting that specific microbial communities could shape the response pattern of individual species to antibiotics. Horizontal gene transfer is known to be an important mechanism for the acquisition of antibiotic resistance in bacteria (27, 28), which may involve transformation, conjugation, and transduction. Thus, different antibiotic resistance genes may be transferred between bacteria in different microbial communities, potentially resulting in antibiotic resistance profiles that are community-dependent. Such a hypothesis warrants further investigation, as it potentially generates an added level of complexity related to environment-bacteria interactions and their potential impacts on antibiotic susceptibility.

Among the species that were eradicated in both communities (Fig. 3A, 6, and 7), many belonged to the *Bacteroidaceae* and *Lachnospiraceae* families, consistent with previous studies showing clindamycin profoundly eliminating anaerobic species (29). As the majority of gut microbiome are difficult to cultivate, the functions of these minority species and their short- and long-term impacts on host physiology remain unknown. However, clindamycin-induced perturbation of the gut microbiota is known to reduce colonization resistance against opportunistic pathogens such as *C. difficile*. Given that *C. difficile* infection can develop both during and after antibiotic therapy, perturbation of both dominant and minority species may play a role in host susceptibility to *C. difficile* infection. In our study, the most significant effect of clindamycin on gut microbiota is the permanent loss of minority species with a transient suppression on overall abundance. While one can envision how perturbation of dominant species creates a niche for *C. difficile* to establish colonization in the host, the impact of

minority species on susceptibility to *C. difficile* is more perplexing. A previous study using conventional mice showed significant perturbation of gut microbiota after a single dose of clindamycin, which conferred long-lasting susceptibility to *C. difficile* infection (30). Our study employed a similar experimental design but in a more controlled environment. Such a long-lasting effect observed in the previous study could be a consequence of the loss of minority species. Buffie et al. proposed a mechanism for competitive inhibition of *C. difficile* by phylogenetically related *Clostridium scindens* (a member of the *Lachnospiraceae* family) through the production of secondary bile salts (31). A similar inhibitory mechanism has been reported for *Staphylococcus* where lugdunin, a bio-product of *S. lugdunensis*, inhibits colonization of *S. aureus* (32). Interestingly, both *C. scindens* and *S. lugdunensis* were minority species in their respective microbial communities. Thus, these studies suggest that minority species in a microbiome may be important and defining their role in host physiology may be critical for understanding disease pathogenesis.

Antibiotic-induced perturbation of dominant species is of particular interest. In our study, we did not observe a lasting impact of clindamycin on most of the dominant species (Fig. 3B, 5). However, we employed only a short course of clindamycin. It remains to be seen how a longer duration, higher doses, or repeated uses of antibiotics would influence the dominant and minority species in well-controlled gnotobiotic animal models. Thus, additional studies using other commonly used antibiotics are needed to advance our knowledge of antimicrobial impacts on global microbial communities in different body habitats. Together, such efforts will provide invaluable insights into appropriate and judicious use of antibiotics in clinical settings.

Few studies have investigated the effects of antibiotics on gut microbiome using gnotobiotic mice. In one study, a single dose of clindamycin was given to conventional mice and longitudinal changes of gut microbiome composition were examined (30). However, variability in gut microbiome among conventional mice makes it difficult to compare results directly with ours. We transplanted the same microbiome into identical GF mice in isolators to avoid acquisition of environmental species throughout the experiments. Human microbiota associated (HMA) mice are now commonly used in gut microbiome studies related to metabolic and autoimmune diseases including diabetes, obesity, and inflammatory bowel diseases (33). Such a model offers the potential for determining cause-and-effect relationships between microbiota and phenotype, rather than correlations that can be deduced using human subjects (34). Although human microbiota can only be partially reassembled in GF mice (Fig. 2), such a model, when carefully applied and interpreted, remains the best model available to study human microbiota related diseases. One limitation of our study was the small number of human donors, which could potentially contribute to differential responses of bacterial species to antibiotic challenge. Despite this limitation, our results demonstrate a clear concordant response among biological replicates, suggesting a predictable fate of specific bacterial species within specific microbial communities. If a larger number of microbiome donors were used, more subtle trends could have been identified.

This study had limitations. First, the data was limited to the short duration of the experiments after antibiotic challenge. Our data set showed steady recovery of majority species, but a longer duration following antibiotic challenge will provide additional data regarding rebound of minority species long-term. Second, our study employed a 5-day duration of antibiotic challenge that is commonly done in animals studies. The impacts of antibiotic duration on gut microbiota was not specifically examined but would be of great interest in future studies to enable clinical translation. Third, the discordant responses of species to antibiotic challenge could be partially explained by changes of other species within the same community. However, similar trends observed in our study have been reported in other studies, e.g., the enrichment of enterococci after clindamycin treatment, thus suggesting a real trend rather than passive changes (bias). Fourth, it was unfortunate that group B was lost and was not included in the clindamycin challenge, which would have strengthened our work. Finally, the present study focused on

the impact of antibiotics on microbiome composition and structure. The functional impact on host physiology or susceptibility to opportunistic pathogens such as *C. difficile* will require further investigations.

In summary, we showed that a single, short-course clindamycin treatment reproducibly suppressed and eradicated a large number of minority gut microbes in gnotobiotic mice transplanted with human microbiome, but the overall microbial community structure was resilient to such a challenge. These results support the need for a comprehensive evaluation of the impacts of antibiotics on the functions of gut microbiota, which is now known to play an important role in host physiology both in- and outside the GI tract.

MATERIALS AND METHODS

Colonization of human fecal microbiota into GF mice. GF Swiss-Webster mice (5 to 9 weeks old, from Taconic Biosciences, Inc.) were housed in gnotobiotic isolators according to standard protocols employed by Animal Care Service at the University of Florida. To generate mice with humanized gut microbiota, GF mice (*n* = 12 per isolator) were orally gavaged in separate isolators with 100 μL aliquot of human fecal samples prepared from three unrelated volunteers, and a fourth group served as a control and were gavaged with sterile reduced PBS (Fig. 1). Human fecal samples were freshly collected and immediately transferred into an anaerobic chamber, and resuspended in sterile pre-reduced PBS. Fecal pellets were collected from mice longitudinally and gut microbiome was analyzed using 16S rRNA sequencing. The study was approved by University of Florida Institutional Review Board and Institutional Animal Care and Use Committee. Informed consent was obtained from all human volunteers.

Clindamycin challenge of ex-GF mice. To assess the effects of antibiotic perturbation on gut microbiota, ex-GF mice from group C and D plus GF controls from group A were given oral clindamycin (500 μg/mL) in drinking water for 5 days (*n* = 3) or maintained on sterile drinking water (*n* = 3) (Fig. 1). Fecal samples were collected longitudinally over 3 weeks following oral clindamycin challenge and stored at $-20°C$ until microbiome analysis.

Sample preparation and 16S rRNA amplification. Genomic DNA was extracted from each fecal sample by using the Mobio PowerSoil DNA extraction kit (Carlsbad, CA) according to the manufacturer's instructions. For each sample, bacterial 16S rRNA V1-V3 or V3-V5 gene segment was amplified in quadruplicates using primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′), or 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGTCAATTYMTTTRAGTTT-3′). Both the forward and the reverse primers contained universal Illumina adapter sequences, as well as individual unique barcodes between PCR primer sequence and the Illumina adapter sequence to allow multiplex sequencing. Each 20-μL PCR mixture contained 2 μL of the purified DNA template, 1 μL Accuprime PCR buffer II (Invitrogen), 5 μM (each) the forward and the reverse primer, and 1 U of Accuprime *Taq* high fidelity polymerase (Invitrogen). PCR amplification was performed as follows: a denaturation step at 95°C for 30 s followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 68°C for 5 min.

Sample pooling and Illumina sequencing. Triplicates of barcoded PCR products prepared from each sample were pooled and analyzed on a 1% SYBR Safe (Invitrogen, Carlsbad, CA) agarose gel. Gel slices containing amplicons of expected size ($\sim$670 bp) were excised and purified using the Qiagen gel extraction kit (Qiagen, Valencia, CA). Purified PCR products were quantified using a Qubit HS DNA quantification kit (Invitrogen, Carlsbad, CA), pooled with equal molar concentration, and sequenced using the Illumina MiSeq platform.

Bioinformatics analysis of Illumina MiSeq sequences. MiSeq reads were demultiplexed and assigned to each sample based on unique forward and reverse barcode combinations using custom R scripts. Barcode and primer sequences were removed using the following criteria to retain high-quality reads for subsequent analysis: (i) Q-score of $\geq$ 30; (ii) reads that matched PCR primer sequence and paired barcodes with 100% identity; (iii) paired reads with $>=$10 overlapping bases for reads joining. OTUs were formed using a closed-reference OTU picking method where the trimmed reads were clustered against the Silva database version 108 using USEARCH with $\geq$ 97% sequence identity and $\geq$ 50% alignable query criteria. Taxonomic assignments were also based upon the reference sequence collection. Alpha diversity indices were calculated using R version 3.1.0 (23). Unifrac analyses were conducted using Qiime version 1.3.0 (23), and statistical significance was determined using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. PICRUSt was utilized to predict gene functions based on 16S rRNA information (24). Linear discriminant analysis effect size (LEfSe) was used to determine differentially abundant gene categories. Heatmaps were generated using the Matrix2png web interface (http://www.chibi.ubc.ca/matrix2png/). Phylogenetic trees were generated using integrated models in MEGA (Version 6) (35).

Statistical analysis. Student's *t* test was used to calculate significance when normality was satisfied, otherwise Mann-Whitney rank sum test was used. A *P* value of $<$0.05 was considered statistically significant.

Data availability. Nucleotide sequence accession number. Sequence reads have been deposited at DANS archive under (https://doi.org/10.17026/dans-xaz-xwzd).

REFERENCES

1. Ananthakrishnan AN. 2011. Clostridium difficile infection: epidemiology, risk factors and management. Nat Rev Gastroenterol Hepatol 8:17–26. https://doi.org/10.1038/nrgastro.2010.190.

2. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC. 2015. Burden of clostridium difficile infection in the United States. N Engl J Med 372:825–834. https://doi.org/10.1056/NEJMoa1408913.

3. Musso G, Gambino R, Cassader M. 2010. Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded? Diabetes Care 33:2277–2284. https://doi.org/10.2337/dc10-0556.

4. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108:3047–3052. https://doi.org/10.1073/pnas.1010529108.

5. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. 2016. The gut microbiota and host health: a new clinical frontier. Gut 65:330–339. https://doi.org/10.1136/gutjnl-2015-309990.

6. Perera M, Al-Hebshi NN, Perera I, Ipe D, Ulett GC, Speicher DJ, Chen T, Johnson NW. 2018. Inflammatory bacteriome and oral squamous cell carcinoma. J Dent Res 97:725–732. https://doi.org/10.1177/0022034518767118.

7. Perez-Cobas AE, Gosalbes MJ, Friedrichs A, Knecht H, Artacho A, Eismann K, Otto W, Rojo D, Bargiela R, von Bergen M, Neulinger SC, Daumer C, Heinsen FA, Latorre A, Barbas C, Seifert J, dos Santos VM, Ott SJ, Ferrer M, Moya A. 2013. Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut 62:1591–1601. https://doi.org/10.1136/gutjnl-2012-303184.

8. Iizumi T, Battaglia T, Ruiz V, Perez GI. 2017. Gut microbiome and antibiotics. Arch Med Res 48:727–734. https://doi.org/10.1016/j.arcmed.2017.11.004.

9. Becattini S, Taur Y, Pamer EG. 2016. Antibiotic-induced changes in the intestinal microbiota and disease. Trends Mol Med 22:458–478. https://doi.org/10.1016/j.molmed.2016.04.003.

10. Sullivan A, Edlund C, Nord CE. 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis 1:101–114. https://doi.org/10.1016/S1473-3099(01)00066-4.

11. Jernberg C, Sullivan A, Edlund C, Jansson JK. 2005. Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. Appl Environ Microbiol 71:501–506. https://doi.org/10.1128/AEM.71.1.501-506.2005.

12. Rashid M-U, Zaura E, Buijs MJ, Keijser BJF, Crielaard W, Nord CE, Weintraub A. 2015. Determining the long-term effect of antibiotic administration on the human normal intestinal microbiota using culture and pyrosequencing methods. Clin Infect Dis 60 Suppl 2:S77–84. https://doi.org/10.1093/cid/civ137.

13. Lindgren M, Lofmark S, Edlund C, Huovinen P, Jalava J. 2009. Prolonged impact of a one-week course of clindamycin on Enterococcus spp. in human normal microbiota. Scand J Infect Dis 41:215–219. https://doi.org/10.1080/00365540802651897.

14. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. 2009. A core gut microbiome in obese and lean twins. Nature 457:480–484. https://doi.org/10.1038/nature07540.

15. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 1:6ra14. https://doi.org/10.1126/scitranslmed.3000322.

16. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. 2014. Human genetics shape the gut microbiome. Cell 159:789–799. https://doi.org/10.1016/j.cell.2014.09.053.

17. Org E, Parks BW, Joo JWJ, Emert B, Schwartzman W, Kang EY, Mehrabian M, Pan C, Knight R, Gunsalus R, Drake TA, Eskin E, Lusis AJ. 2015. Genetic and environmental control of host-gut microbiota interactions. Genome Res 25:1558–1569. https://doi.org/10.1101/gr.194118.115.

18. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 341:1241214. https://doi.org/10.1126/science.1241214.

19. Grover M, Kashyap PC. 2014. Germ-free mice as a model to study effect of gut microbiota on host physiology. Neurogastroenterol Motil 26:745–748. https://doi.org/10.1111/nmo.12366.

20. Yu F, Han W, Zhan G, Li S, Jiang X, Wang L, Xiang S, Zhu B, Yang L, Luo A, Hua F, Yang C. 2019. Abnormal gut microbiota composition contributes to the development of type 2 diabetes mellitus in db/db mice. Aging (Albany NY) 11:10454–10467. https://doi.org/10.18632/aging.102469.

21. Yu F, Han W, Zhan G, Li S, Xiang S, Zhu B, Jiang X, Yang L, Luo A, Hua F, Yang C. 2019. Abnormal gut microbiota composition contributes to cognitive dysfunction in streptozotocin-induced diabetic mice. Aging (Albany NY) 11:3262–3279. https://doi.org/10.18632/aging.101978.

22. Collins J, Auchtung JM, Schaefer L, Eaton KA, Britton RA. 2015. Humanized microbiota mice as a model of recurrent Clostridium difficile disease. Microbiome 3:35. https://doi.org/10.1186/s40168-015-0097-2.

23. Kirst ME, Li EC, Alfant B, Chi Y-Y, Walker C, Magnusson I, Wang GP. 2015. Dysbiosis and alterations in predicted functions of the subgingival microbiome in chronic periodontitis. Appl Environ Microbiol 81:783–793. https://doi.org/10.1128/AEM.02712-14.

24. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814–821. https://doi.org/10.1038/nbt.2676.

25. Kim S, Covington A, Pamer EG. 2017. The intestinal microbiota: antibiotics, colonization resistance, and enteric pathogens. Immunol Rev 279:90–105. https://doi.org/10.1111/imr.12563.

26. Lewis BB, Buffie CG, Carter RA, Leiner I, Toussaint NC, Miller LC, Gobourne A, Ling L, Pamer EG. 2015. Loss of microbiota-mediated colonization resistance to clostridium difficile infection with oral vancomycin compared with metronidazole. J Infect Dis 212:1656–1665. https://doi.org/10.1093/infdis/jiv256.

27. Modi SR, Collins JJ, Relman DA. 2014. Antibiotics and the gut microbiota. J Clin Invest 124:4212–4218. https://doi.org/10.1172/JCI72333.

28. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA. 2012. The application of ecological theory toward an understanding of the human microbiome. Science 336:1255–1262. https://doi.org/10.1126/science.1224203.

29. Zimmermann P, Curtis N. 2019. The effect of antibiotics on the composition of the intestinal microbiota - a systematic review. J Infect 79:471–489. https://doi.org/10.1016/j.jinf.2019.10.008.

30. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG. 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun 80:62–73. https://doi.org/10.1128/IAI.05496-11.

31. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew M, Viale A, Littmann E, van den Brink MRM, Jenq RR, Taur Y, Sander C, Cross JR, Toussaint NC, Xavier JB, Pamer EG. 2015. Precision microbiome reconstitution restores bile acid mediated resistance to clostridium difficile. Nature 517:205–208. https://doi.org/10.1038/nature13828.

32. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, Burian M, Schilling NA, Slavetinsky C, Marschal M, Willmann M, Kalbacher H, Schittek B, Brötz-Oesterhelt H, Grond S, Peschel A, Krismer B. 2016. Human commensals producing a novel antibiotic impair pathogen colonization. Nature 535:511–516. https://doi.org/10.1038/nature18634.

33. Wrzosek L, Ciocan D, Borentain P, Spatz M, Puchois V, Hugot C, Ferrere G, Mayeur C, Perlemuter G, Cassard A-M. 2018. Transplantation of human microbiota into conventional mice durably reshapes the gut microbiota. Sci Rep 8:6854. https://doi.org/10.1038/s41598-018-25300-3.

34. Arrieta MC, Walter J, Finlay BB. 2016. Human microbiota-associated mice: a model with challenges. Cell Host Microbe 19:575–578. https://doi.org/10.1016/j.chom.2016.04.014.

35. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197.