



# Impact of Amoxicillin-Clavulanate followed by Autologous Fecal Microbiota Transplantation on Fecal Microbiome Structure and Metabolic Potential

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**ABSTRACT** Strategies to prevent multidrug-resistant organism (MDRO) infections are scarce, but autologous fecal microbiota transplantation (autoFMT) may limit gastrointestinal MDRO expansion. AutoFMT involves banking one's feces during a healthy state for later use in restoring gut microbiota following perturbation. This pilot study evaluated the effect of autoFMT on gastrointestinal microbiome taxonomic composition, resistance gene content, and metabolic capacity after exposure to amoxicillin-clavulanic acid (Amox-Clav). Ten healthy participants were enrolled. All received 5 days of Amox-Clav. Half were randomized to autoFMT, derived from stool collected pre-antimicrobial exposure, by enema, and half to saline enema. Participants submitted stool samples pre- and post-Amox-Clav and enema and during a 90-day follow-up period. Shotgun metagenomic sequencing revealed taxonomic composition, resistance gene content, and metabolic capacity. Amox-Clav significantly altered gut taxonomic composition in all participants ( $n = 10$ ,  $P < 0.01$ ); however, only three participants exhibited major changes at the phylum level following exposure. In the cohort as a whole, beta-lactamase genes were enriched following Amox-Clav ( $P < 0.05$ ), and predicted metabolic capacity was significantly altered ( $P < 0.01$ ). Species composition, metabolic capacity, and beta-lactamase abundance returned to pre-antimicrobial exposure state 7 days after either autoFMT or saline enema ( $P > 0.05$ , compared to enrollment). Alterations to microbial metabolic capacity occurred following antimicrobial exposure even in participants without substantial taxonomic disruption, potentially creating open niches for pathogen colonization. Our findings suggest that metabolic potential is an important consideration for complete assessment of antimicrobial impact on the microbiome. AutoFMT was well tolerated and may have contributed to phylogenetic recovery. (This study has been registered at ClinicalTrials.gov under identifier NCT02046525.)

**IMPORTANCE** The spread of multidrug resistance among pathogenic organisms threatens the efficacy of antimicrobial treatment options. The human gut serves as a reservoir for many drug-resistant organisms and their resistance genes, and perturbation of the gut microbiome by antimicrobial exposure can open metabolic niches to resistant pathogens. Once established in the gut, antimicrobial-resistant bacteria can persist even after antimicrobial exposure ceases. Strategies to prevent

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 We found that after antibiotics, metabolic capacity was perturbed even in cases where bacterial phyla remained unchanged and that stool banking and redelivery (autoFMT) was safe and well tolerated. @bulowcr @volatilebug

multidrug-resistant organism (MDRO) infections are scarce, but autologous fecal microbiota transplantation (autoFMT) may limit gastrointestinal MDRO expansion. AutoFMT involves banking one's feces during a healthy state for later use in restoring gut microbiota following perturbation. This pilot study evaluated the effect of amoxicillin-clavulanic acid (Amox-Clav) exposure and autoFMT on gastrointestinal microbiome taxonomic composition, resistance gene content, and metabolic capacity. Importantly, we found that metabolic capacity was perturbed even in cases where gross phylogeny remained unchanged and that autoFMT was safe and well tolerated.

**KEYWORDS** antimicrobial resistance, fecal microbiota transplantation, metagenomics, microbiome, multidrug resistance

The spread of multidrug resistance among pathogenic organisms has rendered many treatment options ineffective. The World Health Organization has described the situation as the dawn of a postantimicrobial era (1). The human gut serves as a reservoir for many resistant organisms and their resistance genes, and this can lead to infection in the colonized host and transmission of resistance between commensals and pathogens (2–8). Once established in the gut, antimicrobial-resistant bacteria can persist for extended durations even in the absence of additional antimicrobial exposure (3–9). Novel therapeutic approaches are essential to limit or even reverse colonization with resistant organisms and the associated risk of infection and transmission between hosts.

Despite the growing antimicrobial resistance threat, there are no established methods to effectively reverse the effects of antimicrobial exposure on commensal or pathogenic bacteria. Several strategies have been proposed for mitigating the threat of resistance, but each carries risk. For example, using nonabsorbable broad-spectrum antimicrobials such as rifaximin has been proposed as a method of limiting systemic resistance selection (10). Using such orally ingested drugs to treat infections localized to the gastrointestinal tract may indeed limit systemic selection for antimicrobial resistance; however, this practice still leads to resistance selection in the gut and allows opportunistic pathogens to gain access in this critical body habitat (11).

A healthy fecal microbiome defends against pathogen and multidrug-resistant organism (MDRO) invasion through colonization resistance (12). Fecal microbiota transplantation (FMT) has been proposed as a method of restoring the microbiome to a healthy state after treatment with antimicrobials (13). By displacing infectious or resistant microbes, the new community can restore species diversity, antimicrobial susceptibility, and colonization resistance (13). FMT from healthy allogeneic donors (alloFMT) has been remarkably successful in treating *Clostridium difficile* infection (CDI) (13, 14). Some studies indicate that patients who receive alloFMT for CDI may have a reduction in MDROs in feces as well as infections due to intestinal colonizers (15–21). However, this approach has been found to inadvertently allow transmission of resistance genes from donor to recipient (22). Donor feces may also transmit pathogens or pathobionts which are being asymptotically harbored by the allogeneic donor. Additionally, microbiota structure varies significantly between individuals, and a poor donor-recipient match may lead to dysbiosis or FMT failure (23–26). Studies to date of the effects of FMT on the microbiome have primarily relied on 16S ribotype-based analyses of bacterial taxonomic composition and diversity (27–30). While such studies have been transformative in demonstrating how well taxonomic compositions match between FMT donors and recipients over time, they are generally not designed to illuminate functional changes in the microbiome (29, 30). Accordingly, complementary approaches are required to enable a higher-resolution understanding of the impact of FMTs on the composition, dynamics, and transmission of resistance genes and metabolic capacity encoded by the microbiome.

Autologous fecal microbiota transplantation (autoFMT) is a potential method for restoring the gut to a healthy state while avoiding the risks of donor resistance genes

**TABLE 1** Demographics of study population, stratified by study group ( $n = 10$ )

Variable	Saline no. (%)	AutoFMT no. (%)
Age (median [range]), yr	34 (24–56)	26 (25–57)
Female	2 (40)	4 (80)
Nonwhite	0 (0)	1 (20)
BMI		
Underweight	1 (20)	0 (0)
Normal	2 (40)	1 (20)
Overweight	1 (20)	2 (40)
Obese	1 (20)	2 (40)
Preexisting medical condition <sup>a</sup>	2 (40)	3 (60)
Smoker (former or current)	0	0
Alcohol use		
Current	4 (80)	4 (80)
Former	1 (20)	0 (0)
Never	0 (0)	1 (20)
Special diet <sup>b</sup>	1 (20)	0 (0)
Diarrhea in the past yr	2 (40)	3 (60)
Constipation in the past yr	2 (40)	0 (0)

<sup>a</sup>Hysterectomy (two subjects), migraine headaches, mild hearing loss, history of sports injuries, arthritis, history of back surgery, history of ulcer, history of knee surgery, thyroid partial.

<sup>b</sup>Vegan plus fish.

and donor-recipient mismatch. AutoFMT involves storage of a healthy person's fecal material for later use to restore the gut microbiota after perturbation, such as antimicrobial use. Bolstering the commensal microbiome by autoFMT following exposure to antimicrobials may be effective at combating colonization with MDROs (17). AutoFMT theoretically has a more desirable safety profile than alloFMT because the feces originated from the participant and was collected during a healthy state. The purpose of this study was to evaluate the effects of 5 days of amoxicillin-clavulanate (Amox-Clav) on microbiome taxonomic composition, resistance gene content, and predicted metabolic capacity and the effectiveness of autoFMT versus placebo in microbiome restoration.

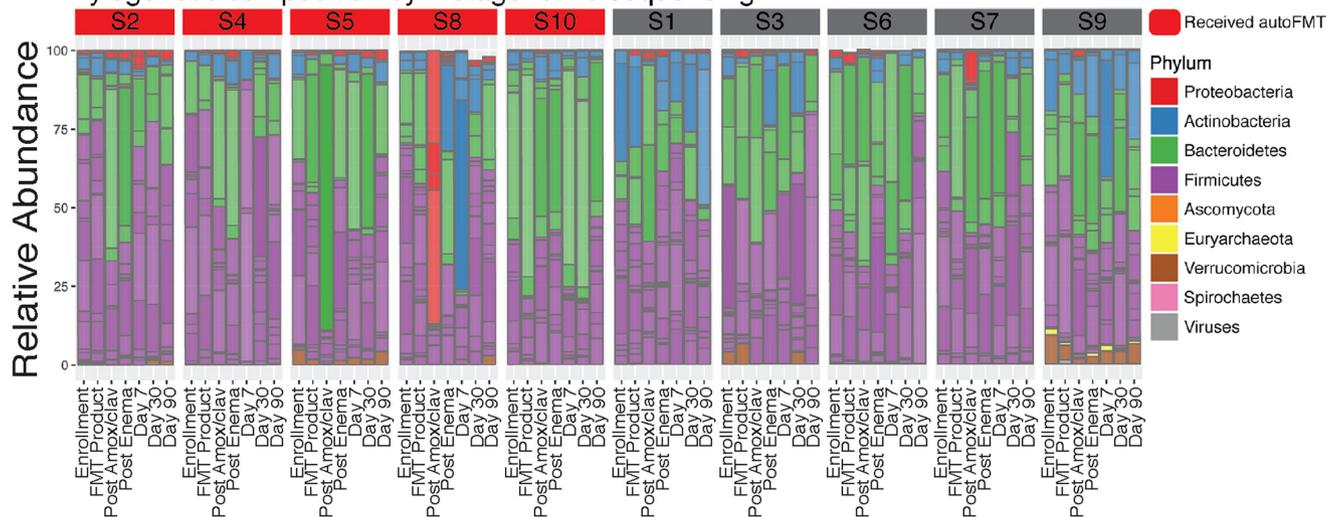
## RESULTS

**Enrollment.** Ten healthy participants were enrolled in the study; participant characteristics are given in Table 1. Two participants experienced adverse events >30 days postenema: one patient was treated with antimicrobials for an ear infection, and the second was diagnosed with *Helicobacter pylori* infection and treated with antimicrobials. Neither infection was determined to be related to Amox-Clav, autoFMT, or saline enema. There was no difference between study groups in the number of bowel movements per day postenema or in bowel movement consistency postenema as measured by Bristol stool type (Mann-Whitney U,  $P > 0.05$  for all).

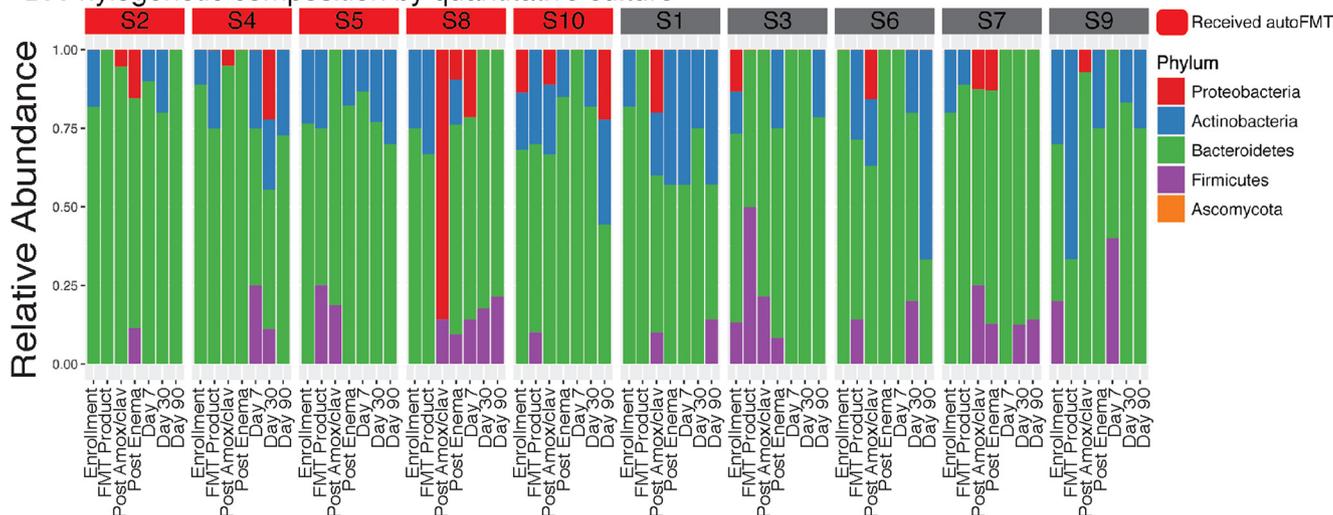
**Taxonomic compositional analysis.** Among healthy volunteers ( $n = 10$ ), 5 days of Amox-Clav resulted in a significant taxonomic shift from the enrollment composition as measured by Bray-Curtis distance ( $P < 0.01$ ). The taxonomic composition across all subjects and time points is visualized in Fig. 1 and 2. Both the autoFMT and saline groups returned to baseline taxonomic composition by 7 days posttreatment (Bray-Curtis distance to enrollment,  $P > 0.05$ ).

Major taxonomic diversity reduction (Shannon index change of at least  $-1$ ) occurred in three of 10 participants. Two of these shifts (in participants 5 and 8) occurred immediately following Amox-Clav. A bloom in *Bacteroidetes* and reduction in *Actinobacteria* and *Firmicutes* contributed to the diversity loss in participant 5. A proteobacterial bloom and loss of *Bacteroidetes* and *Actinobacteria* appeared to drive diversity reduction in participant 8. Both participants were randomized to the autoFMT treatment group. Additionally, participant 4 experienced a reduction in diversity due to loss

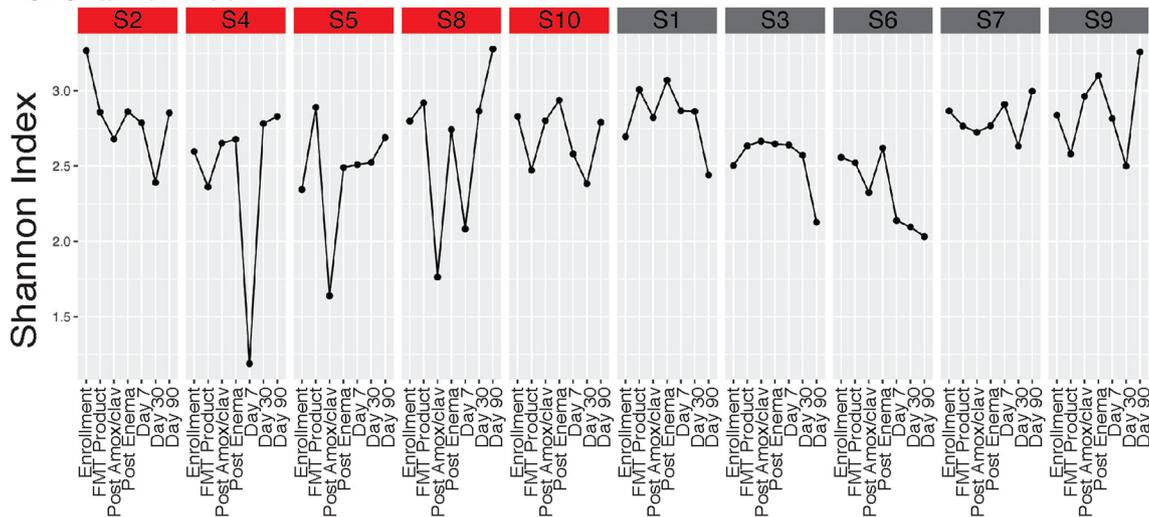
### A. Phylogenetic composition by metagenomic sequencing



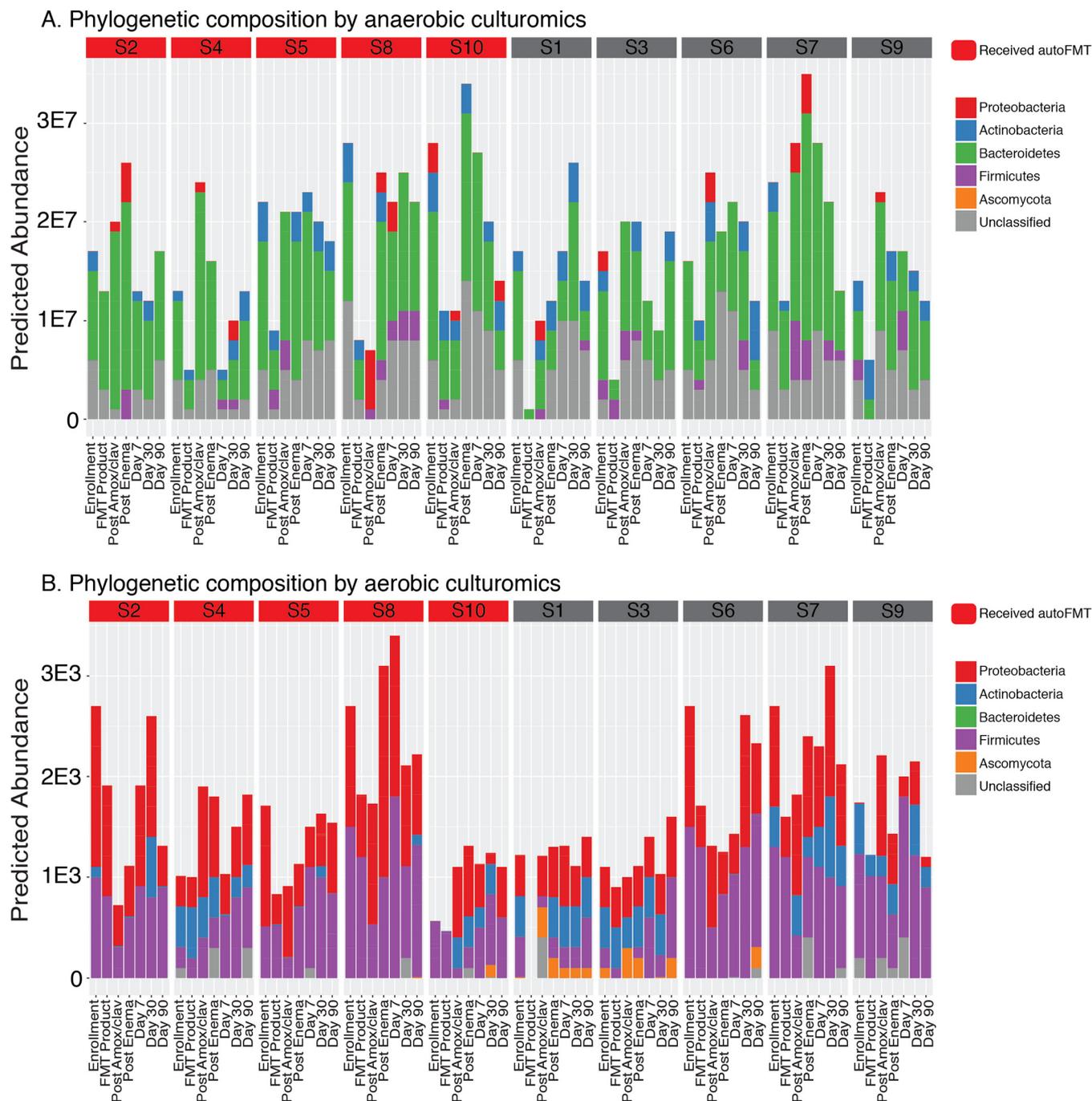
### B. Phylogenetic composition by quantitative culture



### C. Shannon index



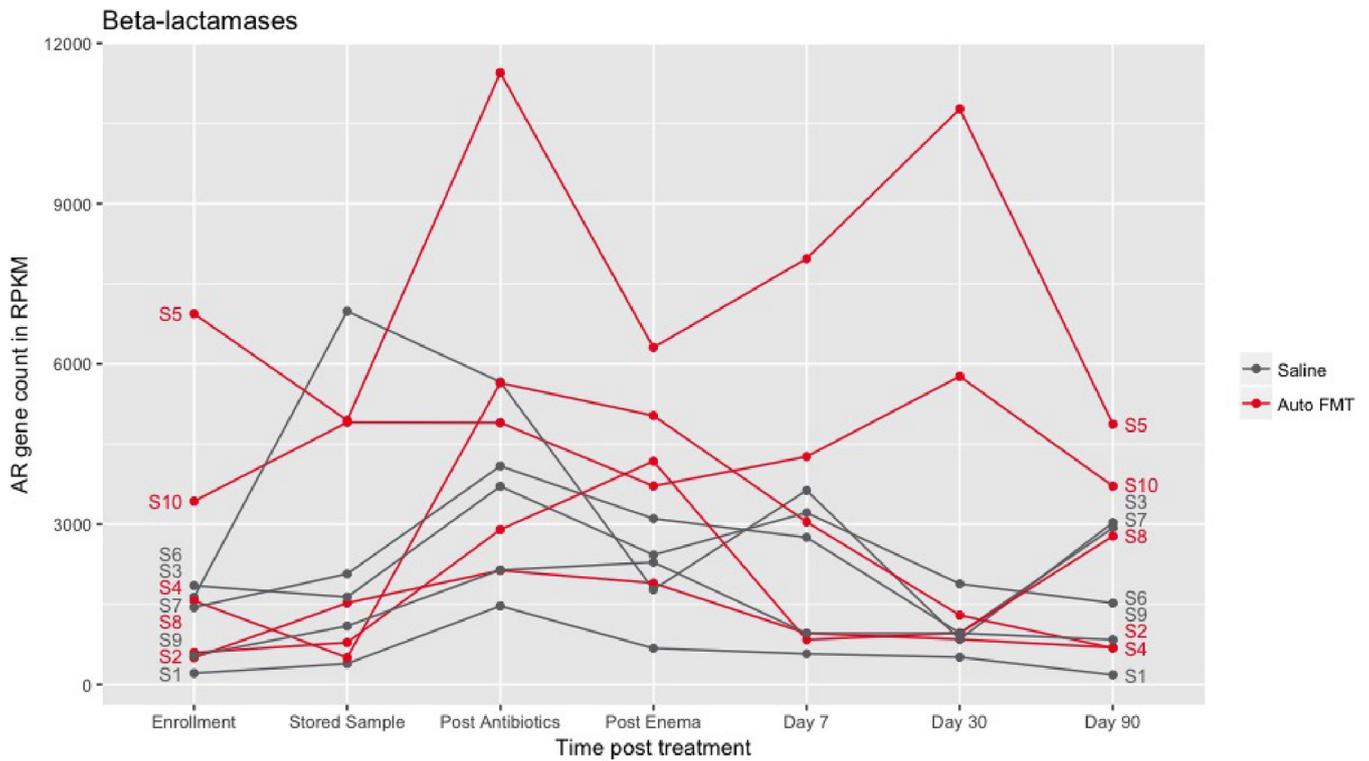
**FIG 1** Taxonomic composition over time was determined by (A) metagenomic sequencing and (B) qualitative culturomics. (C) Shannon index of diversity was calculated using species data from metagenomic sequencing. Species composition was significantly different after Amox-Clav by type II Adonis test of Bray-Curtis distance ( $P < 0.01$ ). The most obvious change post-Amox-Clav was a *Proteobacteria* bloom in subject 8. Numbers preceded by “S” at the top of columns indicate participant identification number.



**FIG 2** Taxonomic composition and predicted absolute abundance over time by quantitative culturomics. (A) Aerobic and (B) anaerobic cultures were quantified. Note that the aerobic cultures were less dilute, allowing greater sensitivity but a lower upper limit of detection. Also note that the relative abundance in Fig. 1B was calculated using growth observed at the greatest dilution for each species while here species may appear in both anaerobic and aerobic cultures.

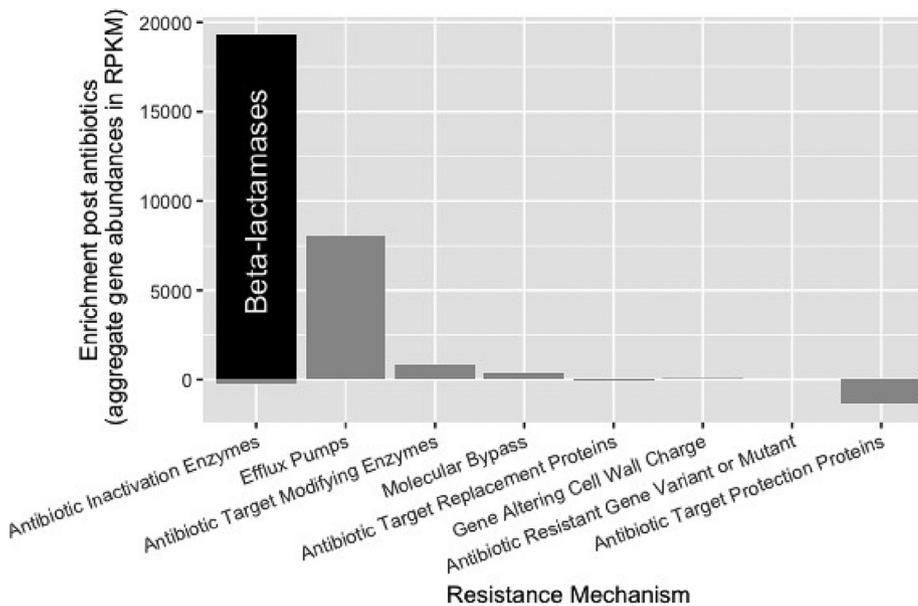
of *Proteobacteria* and *Actinobacteria* 7 days after autoFMT treatment. In each of these three cases, diversity was restored by the next time point (Shannon index within 0.5 of preshift value and restoration of reduced phyla).

**Resistance gene analysis.** The resistance genes detected by metagenomic sequencing included a wide range of common resistance genes. The resistance genes found across the most participants were tetracycline destructases (31, 32) and efflux pumps, but the total number of copies of resistance genes was dominated by beta-lactamases. The beta-lactamases were also the mechanistic category that showed the

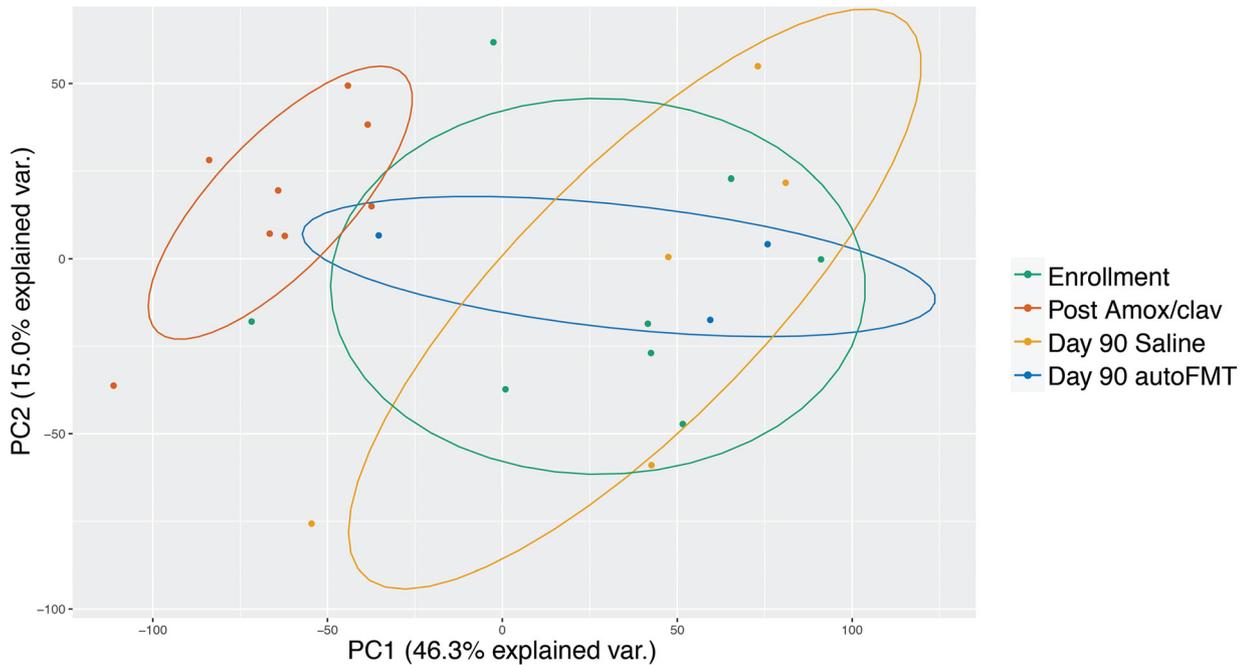


**FIG 3** Beta-lactamase genes were significantly enriched after Amox-Clav (two-tailed *t* test, *P* = 0.0017). Participants randomized to autoFMT (red) and saline (gray) both returned to baseline by day 90. Participant numbers are noted at the beginning and end of each line.

most change correlated with Amox-Clav treatment. As expected, the number of beta-lactamases significantly increased in the study cohort after use of Amox-Clav (*P* = 0.0017), while the count of non-beta-lactamase antimicrobial inactivation resistance genes was not enriched (Fig. 3 and 4). Beta-lactamase gene levels returned to



**FIG 4** Enrichment of resistance genes by mechanism was determined by comparing normalized counts (RPKM) post-Amox-Clav to enrollment. Beta-lactamases (black) were most enriched and formed a majority of the antibiotic inactivation enzymes enriched. Efflux pumps were also enriched, and functional metagenomic selections suggest cooccurrence of beta-lactamases and efflux pumps on a mobile element.



**FIG 5** Principal coordinate analysis (PCA) of metabolic pathway data (IMC) from participants without obvious taxonomic disturbances (all participants excluding 5 and 8). IMC was derived from metagenomic sequencing data. Normally distributed confidence ellipses are shown. Post-Amox-Clav samples have significantly different IMCs than enrollment samples by type II Adonis test of Bray-Curtis distance ( $P < 0.01$ ). This is true with or without participants 5 and 8. IMC returned to baseline state by 90 days with saline or autoFMT at similar rates.

baseline (not significantly different from enrollment level) in both the saline ( $P > 0.05$ ) and autoFMT ( $P > 0.05$ ) groups. The participants who experienced phylogenetic perturbation following Amox-Clav (participants 4, 5, and 8) had enriched beta-lactamase gene content following Amox-Clav treatment. Notably, these participants did not exhibit a common set of resistance genes enriched at enrollment (see Fig. S1 in the supplemental material), indicating that there is not a specific set of genes at enrollment predisposing participants to later taxonomic perturbation. The baseline beta-lactamase composition for participants 4 and 8 ranked in the middle of all enrollment samples. However, participant 5 exhibited the highest baseline beta-lactamase content.

Functional metagenomic selections also provided information about gene cooccurrence. Genes encoding the beta-lactamase CblA and an efflux pump from the AcrB family cooccurred on 22 different functional metagenomic contigs in eight different selections. Twelve such cooccurrences had exactly 114 bp between the genes, indicating a conserved multigene cassette.

**Metabolic capacity analysis.** Metabolic capacity of the microbiome shifted significantly following Amox-Clav, even when subjects with broad phylogenetic differences post-Amox-Clav (participants 5 and 8) were excluded. In order to assess metabolic perturbations, we structured an index of metabolic capacity (IMC) from the metagenomic sequencing data. Genes with metabolic functions were grouped into 37 functional categories. Principal-coordinate analysis of IMC (Fig. 5) demonstrated an effect of Amox-Clav on metabolic capacity. IMC of postantimicrobial samples clusters apart from baseline IMC. The Bray-Curtis distance between baseline and postantimicrobial IMC was significantly different by type II Adonis ( $P < 0.01$ ). This remained true when we excluded participants with obvious taxonomic shifts at these time points (participants 5 and 8) to control for intrinsic metabolic differences between phyla ( $P < 0.01$ ). Additionally, both saline and autoFMT groups returned to baseline composition (not significantly different from enrollment) by day seven posttreatment ( $P = 0.99$  and  $P = 0.84$ , respectively).

Thirteen of the 37 metabolic categories comprising the IMC drove the change in metabolic capacity from enrollment to post-Amox-Clav. Each of these metabolic path-

way categories was enriched post-Amox-Clav. Factors and their relative contribution are found in Fig. S2 and are ranked according to their contribution to this difference in Table S1. AutoFMT did not affect IMC differently than the saline control. IMC returned to baseline state by 30 days with saline or autoFMT. Saline or autoFMT did not result in different rates of return to baseline IMC. We found significant differences between individual participants' IMCs when all time points were compared by type II Adonis ( $P < 0.01$ ).

## DISCUSSION

This pilot study evaluated the impact of Amox-Clav on the taxonomic function, resistome, and metabolic potential of the microbiome, and whether autoFMT could reverse any changes. Analysis of antimicrobial perturbation of the gut microbiome is complicated by the wide variation between baseline taxonomic compositions of healthy guts (33, 34). Additionally, while antimicrobial use has been demonstrated to reduce species diversity and cause diarrhea, the specific taxonomic changes observed vary greatly by individual (33).

Despite the dramatic and rapid perturbations observed acutely following antimicrobial exposure, surprising longer-term robustness and resilience have been previously observed. Most pilot studies and case reports after amoxicillin or ciprofloxacin indicate return to baseline by two months after exposure (33–36). However, while overall diversity and structure may be restored, some species remain missing. These analyses have been limited by resolution of 16S ribotype data and have been unable to detect resistance and metabolic genes. These factors may play critical roles in determining the robustness of the restructured microbiome to antimicrobial perturbation and pathogen invasion. Using shotgun sequencing, we tracked resistance and metabolic genes not detectable in previous 16S analyses of taxonomy.

Among healthy volunteers, 5 days of Amox-Clav exposure (875 mg BID) led to significant taxonomic shifts ( $P < 0.01$ ), beta-lactamase gene enrichment ( $P = 0.0017$ ), and predicted metabolic capacity alteration ( $P < 0.01$ ). Interestingly, only two participants demonstrated obvious phylum-level taxonomic perturbation of the type readily detected by traditional analysis. However, Amox-Clav led to enrichment of 13 metabolic gene categories and significant alteration from enrollment. This significant shift in metabolic capacity was observed even in the absence of phylum-level differences. Considering that interindividual intestinal microbiome metabolic potential is more homogeneous in contrast to the interindividual phylogenetic heterogeneity (37), antimicrobial-induced alterations in functional metabolic potential may be more important than taxonomic alterations when predicting acquisition and/or proliferation of an MDRO.

This study's limitations included small sample size and limited taxonomic perturbation following Amox-Clav. In addition, the most obvious taxonomic shifts occurred in participants randomized to autoFMT. These factors limited assessment of autoFMT's effects on taxonomic restoration. However, this provided an opportunity to assess metabolic perturbation in the absence of broad taxonomic shifts. Small sample size limited our ability to assess the implications of the observed shift in metabolic capacity. Future work is necessary to explore perturbations caused by more disruptive antimicrobial regimens and the ability of autoFMT to restore gut phylogeny.

This study assessed the effects of the dose of Amox-Clav consistent with clinical dosing and previous studies where gut dysbiosis was observed (38). This drug and dose are clinically relevant given UTI and community-acquired pneumonia treatment recommendations. Future work should assess the effects of antimicrobial protocols predicted to result in greater perturbation. In addition, methodological variations for FMT should be tested. The European Society for Clinical Microbiology and Infectious Diseases for FMT treatment of *C. difficile* infection recommends using 30 g of stool in 150 ml of saline (39). Although 100 ml of autoFMT product was administered in this study, 50 g of stool was used. Whether the volume administered and/or amount of stool used to create the FMT product and/or route of administration matters needs to be determined.

Despite these limitations, this study contributes to our understanding of the gut microbiome in several important ways. This study supports the concept of the healthy microbiome's resilience following perturbation. AutoFMT was found to be safe and well tolerated, with no adverse events. By 7 days following Amox-Clav phylogeny, beta-lactamase content and predicted metabolic capacity returned to baseline.

Most strikingly, this study demonstrates the importance of assessing gut metabolic potential, which was significantly altered even in the absence of taxonomic shifts. This shift in metabolic capacity can be detected only using shotgun sequencing or metabolite profiling. Further work is necessary to determine the implications of this shift in metabolic capacity on risk of MDRO colonization and host metabolism.

## MATERIALS AND METHODS

**Overview.** This was a prospective, randomized controlled pilot study (NCT 02046525) to determine the effect of autoFMT on the intestinal microbiome versus placebo (saline enema) after 5 days of Amox-Clav, administered at 875 mg twice per day. The study participants and investigators were blinded to the treatment assignment until after all analyses were completed. This study was approved by the Washington University School of Medicine Human Research Protection Office. Written informed consent was obtained from all study participants.

**Study participants.** Ten healthy volunteers were recruited to participate. Written informed consent was obtained from all study participants. Inclusion criteria included being generally healthy and between 21 and 70 years of age. Exclusion criteria included a history of allergic reaction to beta-lactam antimicrobials or contraindications to amoxicillin-clavulanic acid; any nontopical antimicrobial exposure or tube feeds as a primary source of nutrition in the six months prior to enrollment; pregnancy or risk of becoming pregnant during the study period; gastroenteritis in the last three months; any nonelective hospitalization in the previous 12 months; incontinuity of feces; prior resection or alteration of the stomach, small bowel, or colon; unwillingness to receive an enema/FMT; known colonization with an MDRO; anticipated change in diet, medications, or elective surgery during the study period; or a history of an intestinal disorder.

**Study procedures.** Participants submitted stool samples to study investigators at enrollment, immediately post-Amox-Clav, and at days 1, 7, 30, and 90 post-enema. Once the preantimicrobial specimen was obtained, the participant was instructed to take 5 days of Amox-Clav at 875 mg twice daily (BID). Participants were requested to return the bottles of Amox-Clav to confirm all doses had been taken. The participant was then randomized in a 1:1 fashion to 100 ml of placebo (nonbacteriostatic saline) or autoFMT product. The autoFMT product was thawed overnight at room temperature. The study enema was administered 24 to 48 h after the last dose of Amox-Clav. The participants were instructed to retain the material for as long as possible. All participants retained >90% of the material. Study participants were monitored for 30 min after the enema. Previous studies in FMT for treatment of *C. difficile* infection have demonstrated that FMT effectiveness is not dependent on FMT volume (40). In order to maintain blinding of the study participant, the study enema was delivered to the study clinic in an opaque bag, and the participant was instructed to not turn around when the enema was being prepared and administered. A vial of the participant's autoFMT product was opened in the room, regardless if randomized to placebo or autoFMT, prior to preparing the enema for administration in order to prevent the participant from attempting to guess which study group she or he had been assigned to based on the odor during the procedure. The investigators doing the sequencing work remained blinded to the study group until after the sequencing was completed.

**Fecal processing.** Participants were provided with sealable feces collection devices. After the participant collected a bowel movement, samples were delivered within 2 h of collection. Upon receipt, the feces was immediately processed with 1.5 g reserved for feces culture, 1 g for *Clostridium difficile* culture, and approximately 23 g for feces pulverization and sequence-based analyses, and 50 g of the subject's first sample was used to prepare the FMT product.

**FMT product preparation.** To prepare the FMT product, approximately 50 g of feces was weighed and transferred to a sterile container. Nonbacteriostatic saline was added to the feces in a volume of twice the weight of the feces. A sterile spatula was used to emulsify the mixture for 3 to 5 min. Then the mixture was allowed to rest for 5 min. The feces-saline mixture was then poured through a stainless steel strainer to remove large particulate matter. Four 2-ml aliquots were frozen for genomic analysis of the stored FMT product. The remaining filtrate was drawn into 60-ml syringes (50 ml filtrate each). If the patient was randomized to saline, two 60-ml syringes were filled with nonbacteriostatic saline. The filled syringes and aliquots were stored at  $-80^{\circ}\text{C}$ .

**Quantitative culturomics.** Fresh feces (1.5 g) was added to an equal amount of  $1\times$  PBS and mixed thoroughly. Immediately, six 10-fold serial dilutions were made from the homogenized specimen. Ten microliters and 100  $\mu\text{l}$  of the 1/10 and 1/100 dilutions were plated to two each of the following media: TSA II with 5% sheep blood (BAP; BBL BD, Franklin Lakes, NJ), Columbia colistin nalidix agar with 5% sheep blood (CNA; BBL BD), MacConkey (MAC; BBL BD), and chocolate agar (CHOC; BBL BD). The BAP and CHOC were incubated at  $35^{\circ}\text{C}$  in  $\text{CO}_2$ . The CNA and MAC were incubated at  $35^{\circ}\text{C}$  in air. The plates were read at 24 h and 72 h. Ten microliters and 100  $\mu\text{l}$  of the 1/10<sup>3</sup> and 1/10<sup>6</sup> dilutions were plated to two each of the following media: brucella blood agar (BBA; Anaerobe Systems, Morgan Hill, CA), *Bacteroides* bile esculin agar (BBE; Anaerobe Systems), laked blood with kanamycin and vancomycin (LKV; Anaerobe Systems), cycloserine-cefoxitin fructose agar with horse blood and taurocholate (CCFA\_HT; Anaerobe

Systems), and phenylethyl alcohol blood agar (PEA; Anaerobe Systems). These plates were incubated at 35°C anaerobically for 7 days. The plates were read at 48 h, 4 to 5 days, and 7 days. One gram of fresh feces was processed for culture of *C. difficile*, as previously described (41).

All growth was observed and recorded semiquantitatively. All distinct colonies were identified using Vitek MS IVD v2.0 MALDI-TOF MS (bioMérieux). For any isolate that was not identified, a Gram stain was performed. All isolates were stored in TSB with glycerol at –80°C.

**Metagenomic DNA extraction and sequencing.** Metagenomic DNA was extracted from 0.5 g of feces via phenol-chloroform for each sample as previously described (18). DNA from each sample was sheared to 500 to 600 bp using the Covaris E220 sonicator (intensity, 4; duty cycle, 10%; cycles per burst, 200; treatment time, 75 s; temperature, 4°C; sample volume, 130  $\mu$ l). The sonication product was purified with the Qiagen PCR purification kit and eluted in 63  $\mu$ l nuclease-free water (prewarmed at 50°C).

End repair and barcode ligation reactions were performed in triplicate for each sample. End repair was performed in a Bio-Rad thermocycler using the following reagents: 2.5  $\mu$ l T4 DNA ligase buffer with 10 mM ATP (10 $\times$ ) (NEB; B0202S), 1  $\mu$ l dNTP (1 mM), 0.5  $\mu$ l T4 polymerase (NEB; M0203S), 0.5  $\mu$ l T4 PNK (NEB; M0201S), and 0.5  $\mu$ l *Taq* polymerase (NEB; M02675).

The barcode ligation reaction was performed by adding 2.5  $\mu$ l of unique sequencing barcode at 1  $\mu$ M to 500 ng of end-repaired DNA. An 0.8- $\mu$ l amount of T4 DNA ligase (NEB; M0202M) was incubated with the barcode and sample DNA mixture in a Bio-Rad thermocycler. Following barcode ligation, samples were pooled into groups containing 6 barcodes. Pools were purified using the Qiagen PCR purification kit and MinElute columns and eluted in 15  $\mu$ l of EB.

Fragment size selection was performed using a Use 0.5 TBE 1.5% agarose gel and visualized with SYBR Safe DNA stain. Barcoded DNA fragments sized 400 to 900 bp were cut from the gel, purified with the Qiagen Gel Extraction kit and MinElute columns, and eluted in 12  $\mu$ l buffer EB. Two microliters of the eluted mixture was used for PCR enrichment of size-selected products. The enrichment reaction was prepared as follows: 12.5  $\mu$ l 2 $\times$  Phusion HF Master Mix, 9.5  $\mu$ l nuclease-free water, 1  $\mu$ l Illumina PCR primer mix (F+R) (10  $\mu$ M), and 2  $\mu$ l gel-purified DNA. The reaction was performed in a Bio-Rad thermocycler using the following program: 17 cycles of 98°C for 30 s (0:30), 65°C for 30 s (0:30), and 72°C for 30 s (0:30); 72°C for 5 min; and 4°C forever.

Size selection of the enriched products was performed using a Use 0.5 TBE 1.5% agarose gel, and products were visualized with SYBR Safe DNA stain. Fragments sized 400 to 900 bp were cut from the gel, purified with the Qiagen Gel Extraction kit and MinElute columns, and eluted in 15  $\mu$ l of EB. DNA concentration was quantified using a Qbit fluorometer, and all sample pools were combined at equal concentrations for sequencing. Prior to sequencing, pooled fragment size was assessed with a Bioanalyzer trace and barcode read distribution was assessed using a spike-in run on the Illumina sequencing platform.

To generate metagenomic sequencing reads, the Illumina NextSeq platform was used with the high output kit and settings to generate a minimum of 400 million paired-end reads per run. In total, 70 samples were sequenced at least 1 million reads per sample to allow microbiome and resistome analyses. The depth of 1 million reads was established using previous studies of the gut microbiome (42–44).

Multiplexed Illumina paired-end shotgun metagenomic sequence reads were demultiplexed by barcode. Reads without exact match to barcode were discarded. The remaining reads were quality filtered using Trimmomatic v0.35 and parameters optimized by the Dantas lab (seed mismatches, 2; palindrome clip threshold, 30; simple clip threshold, 10; minimum adapter length, 1; keep both reads, TRUE; window size, 4; required quality, 20; leading, 10; trailing, 10; minimum length, 60).

**Microbiome taxonomic composition prediction.** Taxonomic composition was determined by comparing unique indicator sequences from sequencing reads to clade-specific marker genes from approximately 17,000 reference genomes using MetaPhlan 2.6.0 (18). Analysis was performed using metaphlan2/2.6.0 with the following parameters: –blastdb./metaphlan2/blastdb–input\_type multifasta. The difference in species diversity was calculated using the Shannon index function from the R vegan package. The difference in species composition was calculated using Bray-Curtis distance between samples, and significance was tested with type II Adonis.

**Resistance gene prediction.** The metagenomic DNA sequence was analyzed for both known and sequence-novel antimicrobial resistance genes using functional metagenomic selections and curated resistance gene databases for resistance gene identification and ShortBRED for resistance gene abundance estimation (45). To supplement known resistance gene markers available via the well-curated CARD database, markers from novel, cryptic resistance gene unique to the studied samples were obtained by performing functional metagenomic selections on bacterial metagenomic DNA pooled by participant (46). Functional metagenomic identification of genes that conferred resistance to Amox-Clav was performed as previously described (18, 47, 48) by randomly shearing metagenomic DNA from each pool of feces into fragment libraries. These libraries were cloned into the natively pan-susceptible host *Escherichia coli* DH10B using vector PZE21. For each sample, the host cells containing library fragments were selected against amoxicillin and ampicillin at concentrations lethal to the untransformed host. Surviving colonies were pooled, and the inserted fragments were sequenced via the Illumina MiSeq platform (2  $\times$  150 bp). Sequencing reads were assembled into contigs with the PARFUMS pipeline (49). The contigs were searched for open reading frames with MetaGeneMark (50) and annotated by hmmscan function of HMMER3 (51) against the Resfams core database (52), Pfams (46), and TIGRFAMS (46). Resulting annotations were then hand curated (46) in the following manner. Selections were excluded if >100 contigs were assembled, because this suggests a failure in the assembly or selection since <100 unique resistance contigs are expected per selection. Within each contig, annotations were ranked by specificity to the selective agents and lowest E value with preference given to Resfams annotations over Pfams or TIGRFAMS. In the absence of a clear specific causative gene annotation (i.e.,

a beta-lactamase), the two best annotations with <90% overlap were accepted. The sequences corresponding to accepted annotations were then pooled with known antimicrobial resistance gene sequences from CARD 2017 (46). The genes were then quantified in unassembled metagenomic sequence using the ShortBRED pipeline with a clustering identity of 1.

**Metabolic pathway prediction.** The metabolic potential of the fecal microbial communities was inferred through functional potential profiling. The presence and abundance of metabolic pathways in the microbial communities were assessed using HUMAnN2, which maps unassembled shotgun sequencing reads to functionally annotated species pangenomes in order to predict function (53). Default parameters were used, and details can be found at <http://huttenhower.sph.harvard.edu/humann2>.

In order to assess metabolic perturbations, we implemented an index of metabolic capacity (IMC) from the metagenomic sequencing data. To form this index, we grouped genes with metabolic functions into 37 metabolic pathway categories using Gene Ontology (GO) terms. GO term grouping was performed using ASaiM (54) and custom scripts. The ability of the predicted metabolic capacity to discriminate between sample groups was visualized using principal component analysis (PCA), and significance was tested using type II Adonis of Bray-Curtis distance between samples. This test of significance was conducted with and without participants 5 and 8 to determine whether any difference observed was driven by the large taxonomic perturbations seen in those participants. Each metabolic category comprising the IMC was tested for contribution to the difference between enrollment and post-Amox-Clav IMC using a Random Forests model. This was implemented using the Boruta package in R (55). Factors were ranked according to their contribution to this difference.

**Statistical analysis.** Significance of differences between taxonomic composition at baseline and post-Amox-Clav was calculated by determining Bray-Curtis distance between the communities and using a type II Adonis with a significance level of  $P < 0.05$  with  $n = 10$ . Significance of differences between IMC at enrollment and post-Amox-Clav was calculated similarly. Bray-Curtis distance between IMCs was calculated, and a type II Adonis with a significance level of  $P < 0.05$  with  $n = 10$  was used. Because these are permutation tests, iteration cutoffs were set to detect significance up to  $P < 0.01$ . Saline and autoFMT treatment group taxonomy and IMC were compared to enrollment for return to baseline comparison. Enrichment of beta-lactamase genes in postantimicrobial samples relative to enrollment was calculated using a two-tailed *t* test and a significance level of  $P < 0.05$ . This test was also applied to the comparison between saline and autoFMT to enrollment baseline.

**Index of metabolic capacity validation.** To determine the likelihood of obtaining a significant difference in IMC between groups of microbiome samples by chance, we performed bootstrap analysis using the Human Microbiome Project 2 (HMP2) Inflammatory Bowel Disease (IBD) cohort. This publicly available data set contains 364 samples from 27 healthy participants and 375 samples from 38 ulcerative colitis patients (UC) (56). We randomly selected 2 groups of 10 healthy samples (without replacement) and saw a difference in IMC ( $P < 0.05$ ) in 39 of 1,000 such iterations. This is consistent with the expectation that we are not likely to see a significant difference in IMC by chance alone. Note that we selected 10 samples for each group to mirror the autoFMT study design, where a significant difference in IMC ( $P < 0.01$ ) was noted between the 10 enrollment and 10 post-Amox-Clav samples.

**Accession number(s).** All nucleotide sequences generated during this study have been uploaded to NCBI under BioProject accession no. [PRJNA446061](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA446061).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphereDirect.00588-18>.

**FIG S1**, TIF file, 0.7 MB.

**FIG S2**, TIF file, 1.5 MB.

**TABLE S1**, DOCX file, 0.01 MB.

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The authors declare no conflicts of interest.

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