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Gut microbiome and antibiotic resistance effects during travelers' diarrhea treatment and prevention

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ABSTRACT International travelers are frequently afflicted by acute infectious diarrhea, commonly referred to as travelers' diarrhea (TD). Antibiotics are often prescribed as treatment or prophylaxis for TD; however, little is known about the impacts of these regimens on travelers' gut microbiomes and carriage of antibiotic resistance genes (ARGs). Here, we analyzed two cohorts totaling 153 US and UK servicemembers deployed to Honduras or Kenya. These subjects either experienced TD during deployment and received a single dose of one of three antibiotics [Trial Evaluating Ambulatory Therapy of Travelers' Diarrhea (TrEAT TD) cohort] or took once-daily rifaximin (RIF), twice-daily RIF, or placebo as prophylaxis to prevent TD [Trial Evaluating Chemoprophylaxis Against Travelers' Diarrhea (PREVENT TD) cohort]. We applied metagenomic sequencing on 340 longitudinally collected stool samples and whole-genome sequencing on 54 *Escherichia coli* isolates. We found that gut microbiome taxonomic diversity remained stable across the length of study for most treatment groups, but twice-daily RIF prophylaxis significantly decreased microbiome richness post-travel. Similarly, ARG diversity and abundance were generally stable, with the exception of a significant increase for the twice-daily RIF prophylaxis group. We also did not identify significant differences between the ARG abundance of *E. coli* isolates from the TrEAT TD cohort collected from different treatment groups or timepoints. Overall, we found no significant worsening of gut microbiome diversity or an increase in ARG abundance following single-dose treatment for TD, underscoring that these can be effective with low risk of impact on the microbiome and resistome, and identified the relative microbiome risks and benefits associated with the three regimens for preventing TD.

IMPORTANCE The travelers' gut microbiome is potentially assaulted by acute and chronic perturbations (e.g., diarrhea, antibiotic use, and different environments). Prior studies of the impact of travel and travelers' diarrhea (TD) on the microbiome have not directly compared antibiotic regimens, and studies of different antibiotic regimens have not considered travelers' microbiomes. This gap is important to be addressed as the use of antibiotics to treat or prevent TD—even in moderate to severe cases or in regions with high infectious disease burden—is controversial based on the concerns for unintended consequences to the gut microbiome and antimicrobial resistance (AMR) emergence. Our study addresses this by evaluating the impact of defined antibiotic regimens (single-dose treatment or daily prophylaxis) on the gut microbiome and resistomes of deployed servicemembers, using samples collected during clinical trials. Our findings indicate that the antibiotic treatment regimens that were studied generally do not lead to adverse effects on the gut microbiome and resistome and identify the relative risks associated with prophylaxis. These results can be used to inform therapeutic guidelines for the prevention and treatment of TD and make progress toward using microbiome information in personalized medical care.

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International travelers are frequently afflicted by acute infectious diarrhea, commonly referred to as travelers' diarrhea (TD) (1, 2). Deployed military personnel are also at risk, with as many as three-quarters of American troops in Afghanistan and Iraq experiencing TD and over half having multiple episodes (3, 4). The subsequent operational costs from reduced performance and lost duty days make TD a top infectious disease concern for military medicine (1). Further, disruptions to the gut microbiome caused by TD and international travel can increase the risk of colonization by multidrug-resistant organisms (5–7), which may spread domestically when travelers and military personnel return home (8).

As TD is commonly caused by bacterial etiologies, most notably pathogenic *Escherichia coli* (6), antibiotics are recommended as a therapeutic to treat TD and are often offered as prophylaxis to prevent it (9). Empiric treatment for moderate or severe TD includes fluoroquinolones, azithromycin, or rifaximin as a single dose or for a few days (10). While antibiotic prophylaxis is generally discouraged due to concerns for antimicrobial resistance (AMR), regimens of these drugs are often prescribed to prevent TD (10). When chemoprophylaxis is recommended, it is generally limited to rifaximin due to its demonstrated efficacy against non-invasive enteric pathogens, favorable safety profile, and inability to be absorbed (11, 12). At an individual level, a healthy gut microbiome may reduce infection risk and disease progression by deterring colonization by exogenous organisms (13). However, disruptions to this community—such as the dysbiosis caused by antibiotic use (14)—can interfere with the beneficial functions of the microbiome and could predispose to the invasion and proliferation of diarrheagenic pathogens. Despite advances in understanding the epidemiology, etiology, and comorbidities of TD, most studies into its treatment and prevention have not assessed the impact on the gut microbiome. Clinical outcomes cannot be fully explained without an understanding of unintended consequences potentially occurring in the microbiome, such as gut dysbiosis or acquired AMR, prompting investigations into the impacts of current antibiotic therapeutic and preventative approaches (12).

To address this gap in knowledge, we applied shotgun metagenomic and whole-genome sequencing to longitudinally collected fecal samples and *E. coli* cultured from stool during two US military-led TD clinical trials. The Trial Evaluating Ambulatory Therapy of Travelers' Diarrhea (TrEAT TD) (NCT01618591) (15) trial evaluated the effectiveness of three single-dose antibiotic regimens [azithromycin (AZI), rifaximin (RIF), or levofloxacin (LEV)] with loperamide to treat TD. Single-dose antibiotic therapies have potential importance for use in an operational military environment, where multidose regimens may not be feasible (15). Additionally, single-dose antibiotics may have a reduced effect on colonization with resistant organisms (16). By analyzing the samples from this trial, we sought to determine whether any of these single-dose treatments increased adverse effects on the microbiome and resistome relative to the others. The Trial Evaluating Chemoprophylaxis Against Travelers' Diarrhea (PREVENT TD) (NCT02498301) trial evaluated the effectiveness of the antibiotic rifaximin once or twice daily as prophylaxis vs placebo to prevent TD. By analyzing samples from this trial, we sought to determine whether rifaximin prophylaxis instigated adverse effects on the microbiome or resistome relative to the placebo control. Together, analysis of these interventional cohorts permitted an evaluation of the impacts of international travel, TD, and antibiotic regimens for treatment and prophylaxis on gut microbiome composition and antibiotic resistance burden.

RESULTS

Gut microbiomes are temporally stable over most antibiotic regimen exposures

TrEAT TD subjects were enrolled based on presentation with acute diarrhea. They provided an initial fecal sample at diarrheal onset (day 0) but before antibiotic treatment with a single dose of AZI, RIF, or LEV (15). All study arms also received adjunctive loperamide. Follow-up fecal samples were collected 7 and 21 days after onset (File S1). PREVENT TD subjects were enrolled upon arrival to the destination country. They provided the first (visit 1) fecal sample, then began daily or twice-daily rifaximin prophylaxis or placebo throughout the length of deployment (average of 35.2 days), and then provided the second fecal sample (visit 2) shortly before departing (Table 1; File S2). To characterize the gut microbiomes of servicemembers enrolled in these cohorts, we profiled the collected fecal samples via whole metagenomic shotgun sequencing with microbial taxonomic composition analysis using MetaPhlAn3 (17). In the TrEAT TD cohort, we observed no change in Shannon diversity or species richness as a function of time or treatment group (Fig. 1A and B; File S3). The mean Shannon diversity values for AZI, LEV, and RIF (respectively) were 2.1, 2.0, and 1.9 (day 0); 2.1, 2.3, and 2.3 (day 7); and 2.1, 2.1, and 2.2 (day 21, all ns, ANOVA linear mixed-effect model). The mean species values for AZI, LEV, and RIF (respectively) were 40.8, 41.3, and 40.3 (day 0); 35.5, 44.9, and 39.5 (day 7); and 40, 42.2, and 38.5 (day 21, all ns, ANOVA linear mixed-effect model). The most dominant phylum was *Firmicutes* across all timepoints, ranging from 68% (day 0, SD = 24.1%) to 81% abundance (day 21, SD = 17.3%) (P = 1.0e-2, Wilcoxon). *Proteobacteria* decreased over the course of the trial, starting at 11% (day 0, SD = 19.1%) and dropping below 1% abundance at the end of the trial (day 21, SD = 1.2%) (P = 5.6e-3). *Bacteroidetes* abundance was 6% at TD onset (SD = 15%) and then fell below 1% relative abundance after treatment (SD = 9.9e-2) (P = 4.1e-2) (Fig. 1C).

In the PREVENT TD cohort, we did not observe differences in Shannon diversity over time nor by prophylaxis group, with visit 1 linear model means of 3.1, 3.0, and 3.0 and visit 2 linear model means of 3.0, 3.0, and 3.0 for placebo, once-daily rifaximin, and twice-daily rifaximin, respectively (Fig. 1D). These subjects had 72.9, 69.7, and 71.3 species on average in their visit 1 gut microbiome sample and 67.9, 64.8, and 64.6 in their post-study sample (Fig. 1E). The subjects on twice-daily rifaximin prophylaxis experienced a significant decrease in microbiome richness at visit 2 relative to visit 1 (71.3 to 64.6 species; 95% CI 2.5–10.8, P = 0.02; Fig. 1E); however, there were no differences between the other groups. When comparing subjects across every treatment

TABLE 1 Demographics of analyzed stool samples[a]

TrEAT TD									
Location	USA to Honduras								
Sex, *n* male (%)	34/37 (92%)								
Age mean (IQR)	31.95 (27–38)								
Study groups	Azithromycin			Levofloxacin			Rifaximin		
Timepoint (in days after TD onset)	0	7	21	0	7	21	0	7	21
Samples	11	11	11	14	12	13	12	12	12
PREVENT TD									
Location	UK to Kenya								
Sex, *n* male (%)	112/116 (97%)								
Age mean (IQR)	27.5 (23–31)								
Study groups	Placebo			Once-daily rifaximin			Twice-daily rifaximin		
Timepoint (relative to travel duration)	Pre-		Post-	Pre-		Post-	Pre-		Post-
Samples	33		33	44		44	39		39
Average time between samples in days (IQR)[b]	34.9 (33–37)			35.8 (33.8–37)			34.8 (33–38)		

[a]Study groups: all single-dose antibiotics with loperamide.
[b]ns, ANOVA.

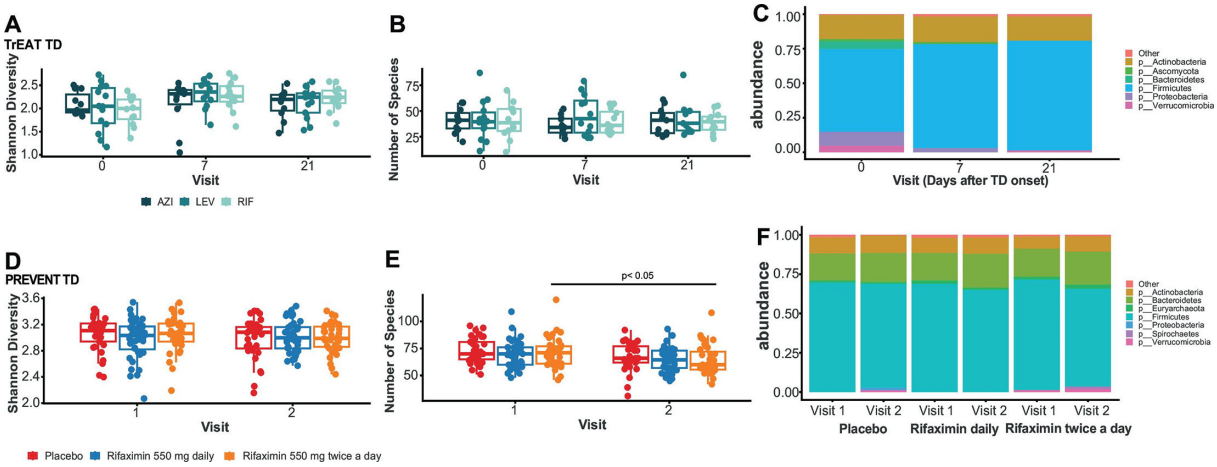

FIG 1 Gut microbiome dynamics based on treatment or prophylactic usage of antibiotics. (A–C) Shannon diversity, richness, and phylum-level relative abundance for the TrEAT TD trial (n = 108). (D–F) Shannon diversity, richness, and phylum-level relative abundance for the PREVENT TD trial (n = 232). Data points are individual fecal samples. The horizontal lines represent the median, and the upper and lower hinges represent the 25th to 75th percentiles (interquartile range). The coloring represents the treatment regimen for Shannon diversity and richness plots. For the relative abundance of phyla, the coloring represents the specified phyla. Subjects who had acute dysentery in TrEAT TD and who did not complete the prophylaxis regimen in PREVENT TD were excluded from the analysis.

group, we did not find any differences in richness or Shannon diversity based on the development of diarrhea (File S3). We also observed no differences in richness as a function of the number of days between samples (File S3). In the PREVENT TD cohort, some of the 232 samples (n = 22) were from subjects who took antibacterial agents (azithromycin, ciprofloxacin, metronidazole, amoxicillin, ciprofloxacin, and flucloxacillin) other than rifaximin, which could impact microbial diversity. Indeed, in linear mixed-effect models, antibacterial use other than rifaximin was found to significantly decrease both richness (P = 6.1e-3) and Shannon diversity (P = 1.4e-3) (File S3). To test whether the observed effects in subjects taking twice-daily rifaximin were driven solely by rifaximin administration, we omitted the subjects who took other antibacterial agents. This retained the same statistical results of the entire cohort, where the only group with significant impacts on microbiome richness were the subjects on twice-daily rifaximin (File S3). Given the stability of microbiomes in both cohorts, we wanted to study the comparative abundances of phyla across studies. Of note, we observed a higher abundance of *Bacteroidetes* in the PREVENT TD cohort relative to the TrEAT TD cohort (P = 2.0e-5) (Fig. 1F). Visit 1 and visit 2 PREVENT TD *Bacteroidetes* average abundances were 17%–18% (SD = 12.8%) and 19%–22% (SD = 13.2%), respectively (ranges are across all treatment groups, P = 0.053). We observed the opposite phenomenon with *Firmicutes*, where visit 1 abundances ranged from 71% to 72% (SD = 12.6%) and visit 2 abundances ranged from 65% to 68% (SD = 11.9%, P = 5.0e-4). We were unable to evaluate the impact of unmeasured covariates including dietary habits prior to and during travel and environmental factors.

Overall, subjects in the TrEAT TD cohort had significantly lower Shannon diversity at TD onset (day 0) and 21 days later relative to PREVENT TD subjects at baseline (Fig. S1A; 2.0 vs 3.1, P < 2e-16, Wilcoxon) and at the end of the trial (Fig. S1B; 2.2 vs 3.0, P < 2e-16, Wilcoxon). Similarly, gut microbiome richness was significantly lower in TrEAT TD subjects at TD onset and at 21 days compared to that in PREVENT TD subjects at baseline (Fig. S1C; 40.0 vs 71.0, P < 8.3e-16, Wilcoxon) and endpoint (Fig. S1D; 39.5 vs 65.0, P < 9.9e-15, Wilcoxon). We next compared taxonomic compositions between the TrEAT TD and PREVENT TD cohorts. We plotted the principal coordinate analysis of Bray-Curtis dissimilarity between the two trials (Fig. S2). Perhaps unsurprising given the differences between the two cohorts (countries of origin, deployment destinations, acute enteric infection status), clustering by trial was statistically significant (P = 3.7e-2).

Escherichia is enriched during diarrhea and associated with the host's immune response

While measures of alpha diversity were largely stable for most subjects, we hypothesized that the relative abundance of certain taxa could vary across the trial period. We identified taxa that were enriched and depleted over time using MaAsLin2 (18) (Fig. 2A and B). For the TrEAT TD cohort, we found that the most depleted genera at each timepoint relative to day 0 were *Bacteroides* (coef = −2.87, qval = 2.3e-2 at day 21), *Escherichia* (coef = −2.07, qval = 0.13 at day 21), and *Parabacteroides* (coef = −1.58, qval = 0.11 at day 21). The most highly enriched genera included *Gemmiger* (coef = 2.78, qval = 4.5e-3 at day 7), *Enterococcus* (coef = 2.60, qval = 0.11 at day 21), and *Adlercreutzia* (coef = 2.59, qval = 2.3e-2 at day 21) relative to day 0 (File S3). For the PREVENT TD subjects, the most depleted genera relative to the placebo group at visit 1 were *Intestinibacter* (coef = −3.17, qval = 0.17 at visit 2 with BID rifaximin), *Streptococcus* (coef = −2.84, qval = 0.08 at timepoint 2 with BID rifaximin), and *Adlercreutzia* (coef = −2.74, qval = 0.09 at timepoint 2 with BID rifaximin) (Fig. 2B). The most highly enriched genera as a function of treatment group and time included *Escherichia* (coef = 4.09, qval = 1.4e-4 at visit 2 with placebo), *Klebsiella* (coef = 2.11, qval = 0.12 at visit 2 with placebo), and *Eggerthella* (coef = 1.32, qval = 0.12 at timepoint 2 with placebo). Thus, we found taxa that were specifically altered over the course of the TrEAT TD and PREVENT TD trials.

Given that enteropathogens that cause TD can cause local inflammation in the gut (19), we evaluated whether specific inflammatory signatures were associated with gut microbiome composition. To investigate the host inflammatory response to TD, we evaluated cytokine concentrations in stool from 56 TrEAT TD subjects at day 0 and at day 21. Nine cytokines were significantly increased at TD onset (day 0) relative to diarrheal resolution 21 days later (Fig. 2C), including IL-6 (P = 7.9e-3) and IL-10 (P = 8.1e-3). No significant differences were found in the study group (-File S3). Interestingly, although fecal inflammation normalizes, we did not observe overall changes in alpha diversity 21 days after diarrheal onset (Fig. 1A and B). We hypothesized that the cytokine levels could be associated with changes in the microbiome composition between TD onset and resolution because we observed taxonomic changes during that interval (Fig. 1C). We compared genus-level microbiome data with cytokine concentrations in stool using DIABLO (20). We found several associations between center log-ratio transformed cytokine and genus-level microbiome abundance (Fig. 2D). We observed a strong inverse relationship between *Asaccharobacter* and the cytokines CCL13, CXCL10, and CXCL11. Of note, we found a positive correlation between *Escherichia* and CXCL11 (Fig. 2D), which also remained at a threshold of r = 0.40 (Fig. S3). *Escherichia* was also associated with other cytokines at a cutoff of r = 0.30, including positive associations between CXCL10 and CCL13 and negative associations with CCL26 (Fig. S3). Notably, CCL13, CXCL10, and CXCL11 have all been reported to have antimicrobial activity against *E. coli* (21). This connection between *Escherichia* abundance and host inflammatory response reinforces previous findings on the role of *E. coli* in diarrheal disease (22). These associations highlight the potential inflammatory effects of *E. coli* abundance and identify *Asaccharobacter* as a potentially anti-inflammatory genus in the gut of military personnel with TD.

Increase in antibiotic resistance gene abundance in the twice-daily rifaximin group

Having characterized the dynamics of changes to the gut microbiome during diarrhea and antibiotic use, we next examined if similar trends exist in the gut resistome. We identified and quantified antibiotic resistance gene (ARG) abundances in the metagenomes of each stool sample using ShortBRED (23). For TrEAT TD subjects, we observed a mean ARG sum per metagenomic sample of 8,731 reads per kilobase per million (RPKM) at day 0, 8,535 at day 7, and 8,723 at day 21 (Fig. 3A, P = 0.99 by LME ANOVA; File S3). The average numbers of unique ARGs per sample were 161, 163, and 157 at days 0, 7, and 21, respectively (Fig. 3B, P = 0.39, File S3). Overall, we observed no change in ARG richness or abundance between timepoints or treatment groups (Fig. 3A and B). Furthermore, when

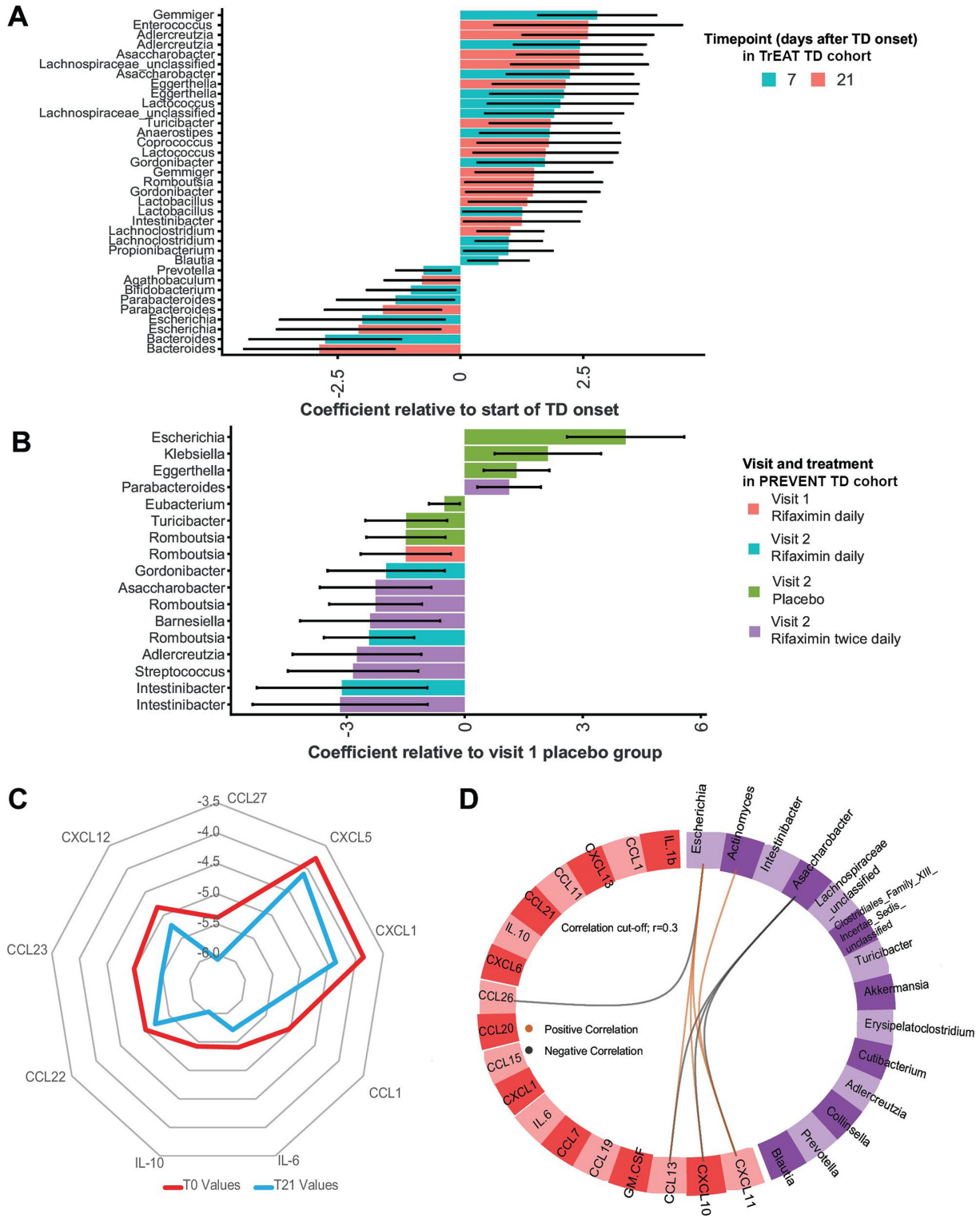

FIG 2 Genus-level dynamics and cytokine differences link to *Escherichia* abundance. (A and B) Microbial genera significantly associated with diarrheal samples detected using MaAsLin2 where subjects were used as random effects and treatment and time (PREVENT TD) or time (TrEAT TD) as fixed effects. The error bars extend to the values within the 95% CI. The colors represent the timepoint for TrEAT TD and treatment and time for PREVENT TD. (C) Radar plot of significantly different cytokines [(pg cytokine/µg protein)/mg feces] between timepoints in TrEAT TD. Using LME models with treatment and time as fixed effects and subject ID as random effects, the cytokines with $P < 0.05$ for time are presented. Values are depicted using a logarithmic scale. The color of the line represents the timepoint. (D) Circos plot using DIABLO demonstrating correlations between gut microbiome genus abundance and fecal cytokine concentration. The color

(Continued on next page)

FIG 2 (Continued)

of the connecting line represents the directionality of correlation. Subjects who had acute dysentery in TrEAT TD and who did not complete the prophylaxis regimen in PREVENT TD were excluded from the analysis.

we subset to ARGs corresponding to resistance for the drugs used in this trial (macrolides, quinolones, and rpoB-targeting drugs), we also observed no differences in resistance over time (Fig. S4). These data demonstrate no discernable impact of three individual antibiotic doses on the gut resistome during the 21-day observation period.

At baseline within the PREVENT TD trial, each gut resistome harbored a mean of 5,134 to 5,390 ARG RPKM (Fig. 3C, ns, ANOVA). After an average of 35 days of twice-daily rifaximin, the ARG burden significantly increased to 5,833 (Fig. 3C; $P = 0.03$, LME ANOVA). This increase was not secondary to specific ARGs as we observed no associations between ARGs and treatment groups with generalized linear mixed-effect models. The ARG burden increased for the other two groups, but this increase was not significant (5,224 to 5,836 RPKM for the placebo group and 5,390 to 5,655 RPKM for the once-daily rifaximin group) (Fig. 3C; $P = 0.14$ and $P = 0.82$, respectively, LME ANOVA). The mean number of metagenomic ARGs ranged from 241 to 268 per sample at baseline at visit 1 (Fig. 3D). After treatment (average duration of 35.2 days), no significant differences were present, and the mean values were 282 for the placebo group, 245 for the daily rifaximin group, and 242 for the BID rifaximin group. We then subset ARGs to those that could provide rifaximin resistance including mutations in *rpoB* and efflux pumps (24). We found no significant changes in *rpoB* ARG count or sum (Fig. S5A and B). We found an increase in the number of ARGs corresponding to efflux pumps in the placebo group at visit 2 from 19.6 to 27.4 (Fig. S5C, $P = 0.02$). This increase likely corresponds to the observed increase in *Escherichia* and *Klebsiella* (Fig. 2B) as these organisms are known to harbor multiple efflux pump ARGs (25). We observed a non-significant increase in efflux pump ARG RPKM in the twice-daily rifaximin group from 332 at baseline to 447 at visit

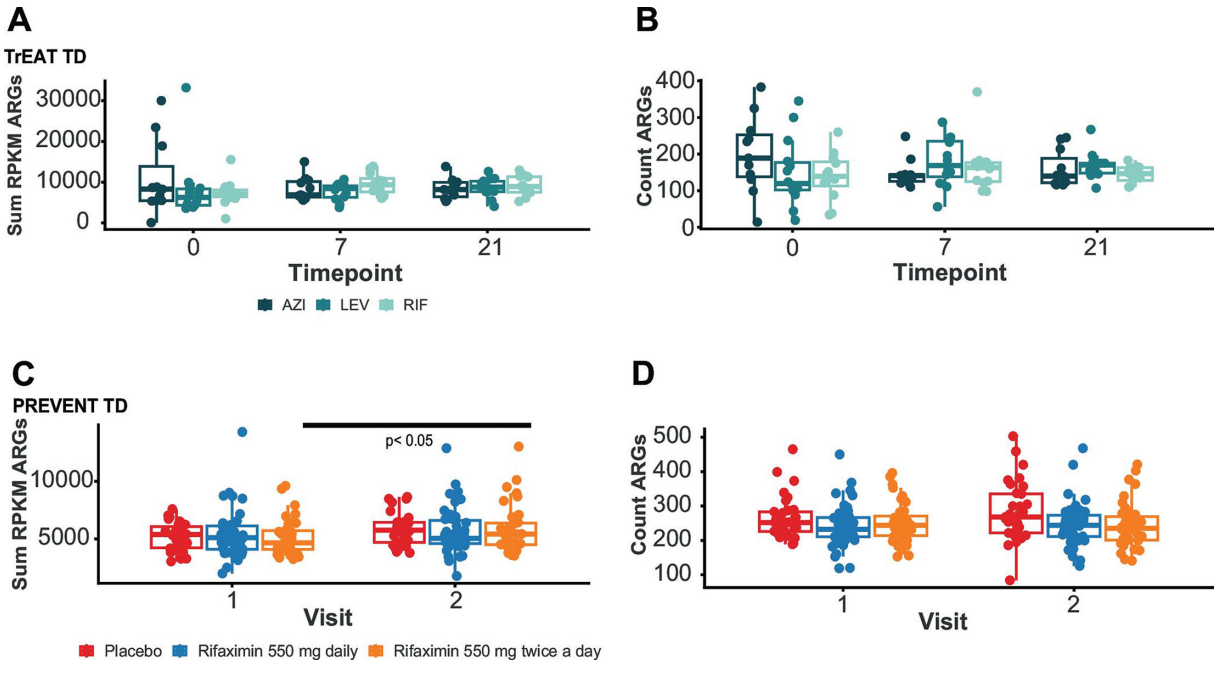

FIG 3 Resistome dynamics based on treatment or prophylactic usage of antibiotics. (A and B) The reads per kilobase per million (RPKM) sum and counts of resistomes for the TrEAT TD trial ($n = 108$). (C and D) The RPKM sum and counts of resistomes for the PREVENT TD trial ($n = 232$). The points are individual fecal samples with a horizontal line at the median, and the upper and lower hinges represent the 25th to 75th percentiles. The coloring represents the treatment regimen for each trial. Subjects who had acute dysentery in TrEAT TD and who did not complete the prophylaxis regimen in PREVENT TD were excluded from the analysis.

2 (Fig. S5D, P = 0.098). Thus, twice-daily rifaximin led to an increase in gene content devoted to ARGs without increasing the absolute number of ARGs. Taken together, these results show that while the host inflammatory response recovers from perturbation by diarrheal disease within 21 days after TD onset in the TrEAT TD trial, the gut microbiome and resistome do not recover during this time as inferred by the baseline samples from the PREVENT TD trial. We also show strong associations between *Escherichia* abundance and diarrhea-induced inflammatory cytokines and that rifaximin prophylaxis reduces the abundance of *E. coli* at the expense of a decrease in microbiome richness and an increase in ARG count for twice-daily prophylaxis.

Phylogeny and ARG content of multidrug-resistant fecal *E. coli*

Given that *E. coli* is a common cause of diarrhea (6), and our findings on the association of the *Escherichia* genus with diarrhea (Fig. 2), we investigated the phylogenetic diversity, ARG content, and virulence potential of TD-associated *E. coli* by performing whole-genome sequencing on 54 *E. coli* isolates collected from 17 US personnel from the TrEAT TD cohort, deployed to Honduras (Fig. S6A). While the microbiome analyses were limited to subjects who experienced watery diarrhea due to the limited sample size, we also included isolates from three subjects who experienced bloody febrile diarrhea and received azithromycin alone or azithromycin plus adjunctive loperamide. Additionally, to get a broader scope of diarrhea-causing *E. coli* overall, we also included 189 *E. coli* genomes from Boolchandani et al. (26), which were isolated from diarrheal and non-diarrheal samples from a cohort of international travelers visiting Cusco, Peru.

We constructed a core genome maximum-likelihood tree of this group of 243 isolates as well as 35 reference *Escherichia* genomes (27) (Fig. 4). The TrEAT TD isolates belonged to *E. coli* phylogroups A, B1, B2, C, D, F, and clade I. The majority of *E. coli* isoaltes overall as well as those specifically from diarrheal samples (day 0) belonged to clade A (overall = 31/54, 57.4%; diarrheal = 18/28, 64.3%) and B1 (12/54, 22.2%; 6/28, 21.4%). No isolates from phylogroups B2, F, or clade I were collected from diarrheal samples. This matches the phylogroup distribution seen in the isolates from Cusco, where the most common phylogroups overall and from diarrheal samples were also A and B1. The TrEAT TD isolates' MLST profiles represented 34 unique sequence types, with ST10 being the most common overall and from diarrheal samples (overall = 9/54, 16.7%; diarrheal = 6/28, 21.4%). ST10 was also the most commonly detected among isolates from the Cusco cohort.

We next screened our isolate genomes for VF and assigned DEC pathotypes based on the presence of specific VF genes (Materials and Methods). DEC pathotypes are used to group *E. coli* strains that possess similar VFs and cause diseases with similar pathology (28). The most prevalent pathotypes in the TrEAT TD cohort overall were EAEC (9/54, 16.7%) and ETEC (8/54, 14.8%). We also observed one hybrid strain (EAEC-DAEC). Nearly half of TrEAT TD isolates were unable to be assigned a pathotype, and so were assigned to the "no pathotype determined" (NPD) group (25/54, 46.3%). Among the TrEAT TD diarrheal samples, EAEC (8/28, 28.6%) and ETEC (5/28, 17.9%) were also the most common, and a smaller relative proportion were NPD (11/28, 20.4%). For the Cusco isolates, EAEC was also the most prevalent pathotype overall and in diarrheal samples (26).

To explore the diversity and distribution of AMR determinants harbored by these isolates, we screened their genomes for known ARGs. In total, we identified 39 unique AMR determinants, comprised of 3 core (present in 100% of isolates) and 36 accessory ARGs. Consistent with Cusco isolates, the core ARGs were the multidrug efflux pumps *emrD* and *acrF* and the beta-lactamase *blaEC*. The most common accessory ARGs were the *mdtM* multidrug efflux pump (50/54; 92.6%), *sul2* sulfonamide resistance gene (31/54, 57.4%), *aph(6)-Id* and *aph(3″)-Ib* aminoglycoside resistance genes (28/54 and 27/54, 51.9% and 50.0%, respectively), and the *blaTEM-1* extended-spectrum beta-lactamase (23/54, 42.6%). These were also the most common accessory ARGs in the TrEAT TD diarrheal samples. We observed no significant difference in the number of ARGs encoded

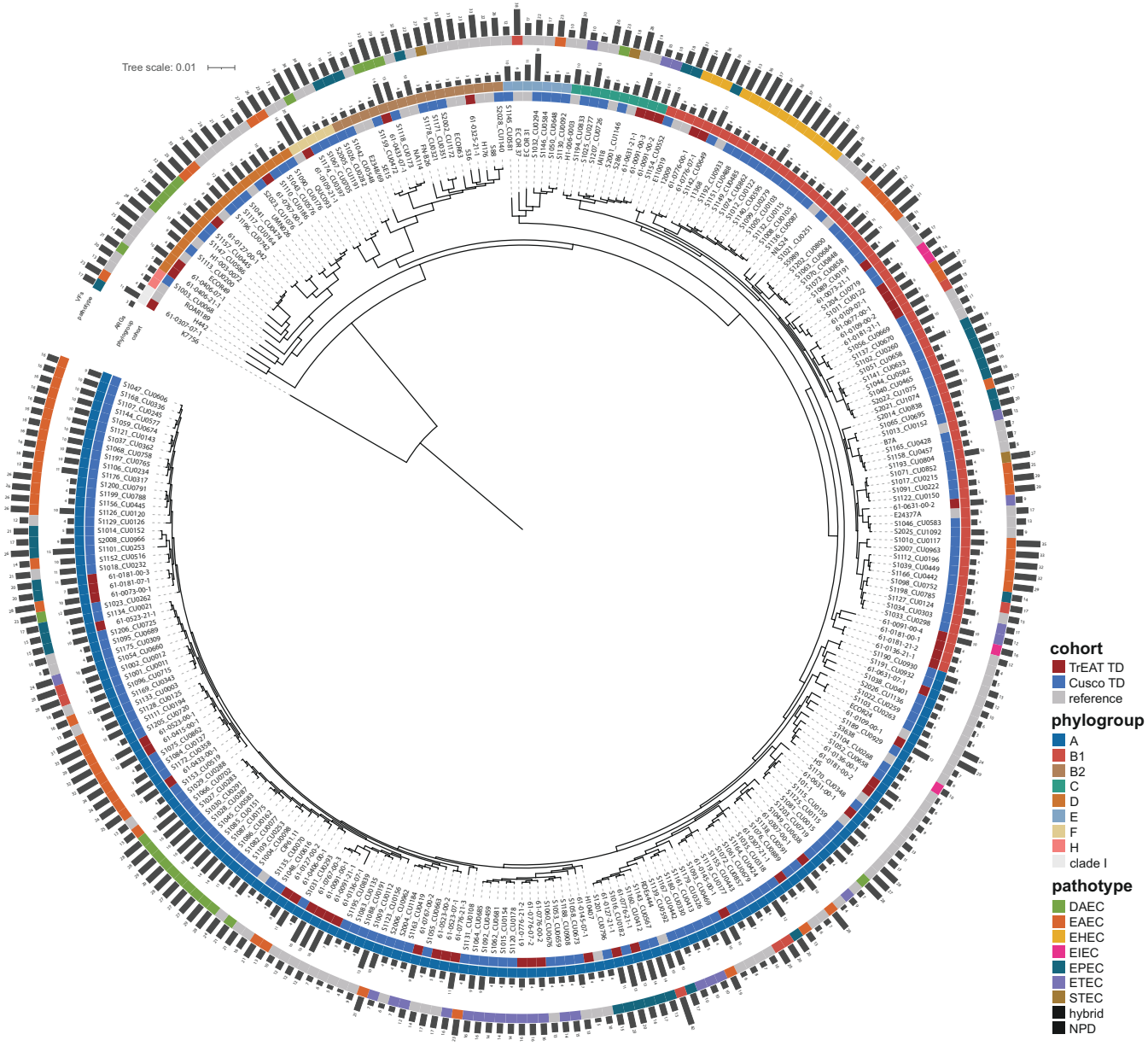

FIG 4 Diarrheagenic *E. coli* isolates are phylogenetically diverse and encode many ARGs and virulence factors (VFs). The phylogenetic tree inferred from the core-genome alignment of 54 *E. coli* isolates from the TrEAT TD cohort (red), 189 published DEC genomes from the Cusco TD study (blue), and 35 *E. coli* reference genomes (gray). Annotations from the inner to the outer ring: cohort, phylogroup, barplot denoting ARG count, *E. coli* pathotype (assigned by the presence/absence of specific VFs), and barplot denoting VF count.

by isolates collected from the different timepoints, pathotypes, nor treatment groups (Fig. 5A and B). This suggests no treatment-driven increase in the risk of multidrug-resistant *E. coli* enrichment and is consistent with findings that ARG counts remain high for weeks after diarrhea (26).

Overall, we observed low rates of ARGs encoding resistance to the drug classes that include our antibiotic treatment regimens (Fig. S6C). Only 2 ARGs against macrolide class antibiotics (AZI, PLA, LOP) were detected—*erm*(*B*) and *mph*(*A*)—which were encoded by just 11/54 isolates. Only 3 ARGs against quinolone class antibiotics (LEV) were detected—*qepA4*, *qnrB19*, and *qnrS1*—which were encoded by just 5/54 isolates. And only 1 ARG against rifamycin class antibiotics (RIF) was detected—*arr*—which was encoded by just 2/54 isolates. Examining the rates of ARG carriage within treatment groups,

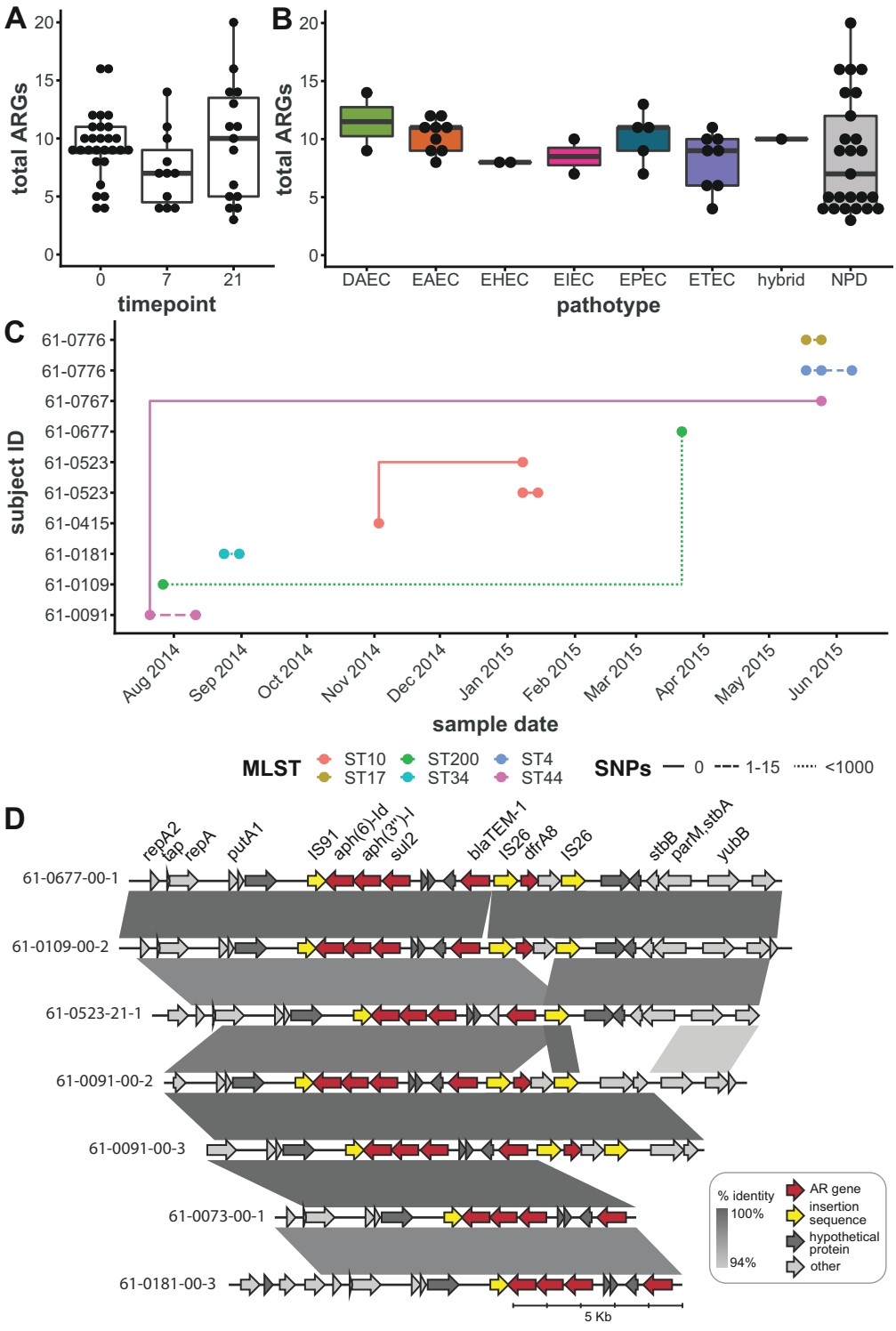

FIG 5 ARG content of *E. coli* isolates. (A) Boxplot of ARG counts of *E. coli* isolates. Each point represents an isolate. Isolates are binned by those collected from diarrheal samples (timepoint 0) and then from stool samples collected 7 and 21 days later. The boxes show median and quartiles; the error bars extend to the values within the 1.5 interquartile range. (B) Boxplot of ARG counts of *E. coli* isolates. Each point represents an isolate. The isolates are binned by *E. coli* pathotype (assigned by the presence/absence of specific VFs) or as no pathotype determined (NPD). The boxes show the median and quartiles; the error bars extend to the values within the 1.5 interquartile range. (C) Linkage of isolates between subjects. High-resolution single nucleotide polymorphism (SNP) comparisons between isolates belonging to the same strain collected from different

(Continued on next page)

FIG 5 (Continued)

subjects and timepoints. The points represent the individual isolates, the type of line indicates the number of SNPs in the comparison, and the coloring of points/lines indicates the isolates' multi-locus sequence type (MLST). Open reading frames (ORF) are colored as follows: ARGs (red), mobile elements (yellow), hypothetical proteins (dark gray), and other genes (light gray). (D) Arrangements of ARGs and mobile elements identified on the same contig and shared between multiple samples. Labeled by the isolate the contig belongs to. The bars connecting the contig regions indicate the regions of similarity with a minimum comparison length of 500 bp, and shading indicates nucleotide identity.

we observed low rates of ARGs encoding quinolone and rifamycin resistance within subjects receiving levofloxacin and rifamycin, respectively. Just 2/25 isolates from 2/8 subjects in the LEV group encoded resistance to quinolones, and 0/7 isolates from 2 subjects in the RIF encoded resistance to rifamycins. However, within the groups that received azithromycin (AZI, PLA, LOP), 8/22 isolates from 5/7 subjects encoded resistance to macrolides. Among these, 2/8 isolates were from day 0 samples (diarrhea onset), 2/8 from day 7, and 4/8 from day 21, suggesting enrichment for macrolide ARGs following treatment with azithromycin. We also performed antibiotic susceptibility tests on a subset of our isolates at the time of collection (15/54), and while we observed high rates of rifamycin resistance across the board (13/15 isolates), we did not observe any associations between resistance phenotype and treatment group over time (Fig. S6D).

As we did not have isolates derived from all TrEAT TD samples or from the PREVENT TD trial, we used our data set of cultured isolate genomes from the TrEAT TD and Cusco trials to identify these strains within these samples using StrainGE (29). We first examined if timepoint had an impact on average strain count per metagenome. We found that there was a significantly higher number of strains detected in day 0 diarrheal samples from the TrEAT TD trial (average strain counts of 1.72 for day 0, 1.17 for day 7, and 1.07 for day 21), but there was no impact on average strain count per sample based on timepoint in the PREVENT TD trial (average strain counts of 1.1 for T1 and 1.33 for T2; Fig. S7A, $P = 1.1e\text{-}2$, Fig. S7B, $P = 0.17$, File S3). Next, we examined if the average number of strains per sample changed based on the antibiotic treatment group. We found no significant impacts on strains per metagenome as a function of treatment group in TrEAT TD (average strain counts of 1.6 for AZI, 1.1 for LEV, and 1.33 for RIF) or PREVENT TD (average strain counts of 1.33 for placebo, 1.35 for rifaximin 550 mg daily, and 1.09 for rifaximin 550 mg twice daily) samples (Fig. S7C, $P = 0.24$, Fig. S7D, $P = 0.30$, File S3). Lastly, we profiled these metagenomes for specific ARGs and VFs encoded by our *E. coli* isolates and found that there was no significant difference in the average number of AMR and VF genes per metagenome between PREVENT TD (29.3 genes) and TrEAT TD (30.0 genes) metagenomes (Fig. S7E, $P = 0.88$, File S3).

Persistence and co-colonization of fecal *E. coli* and ARG clusters

By examining the relatedness of *E. coli* isolates we identified instances of the same strain or clone persisting within a single subject and/or being found in multiple subjects. Within our cohort, we defined a strain as two or more isolates with <200 core genome SNPs (single nucleotide polymorphisms) and identified seven strain groups shared within and between subjects (Fig. S6B). Next, we performed high-resolution whole-genome SNP comparisons between isolates belonging to the same strain group (Fig. 5C). We identified clonal ST44 isolates cultured from different subjects 10 months apart with 0 whole-genome SNPs, as well as ST10 isolates cultured from different subjects 3 months apart with 0 SNPs. Furthermore, while the ST44 0-SNP clones belonged to the NPD pathotype, they encoded among the highest number of ARGs ($n = 16$) of any collected isolates (Fig. 5B). Taken together, this shows that phylogenetically diverse DEC, both pathotyped and non-pathotyped, can encode high numbers of ARGs and persist and infect multiple travelers. Further studies with more dense sampling while traveling and surveillance for several months post-travel could uncover the precise dynamics of acquisition and persistence and determine the risk posed by colonization with these multidrug-resistant strains after travel has concluded.

Lastly, we observed highly similar ARG gene clusters shared among phylogenetically diverse *E. coli*. One cluster was comprised of five ARGs against four antibiotic classes —*blaTEM-1* (beta-lactam), *dfrA8* (trimethoprim), *sul2* (sulfonamide), and *aph(3")-Ib* and *aph(6)-Id* (aminoglycoside)—and was shared among isolates from six subjects and three phylogroups (Fig. 5D). The isolates from subjects 61-0073, 61-0091, 61-0109, and 61-0181 were all from diarrheal samples collected within a 1.5-month span (July to August 2014) (Fig. S5A). The remaining isolates from subjects 61-0523 and 61-0677 were collected >5 months later, with the latter also from a diarrheal sample but the former from a sample collected from a non-diarrheal sample 21 days post-diarrhea. Furthermore, these ARGs were flanked by IS26 and IS91 transposases, and 6/7 were found in isolates collected from diarrheal stool samples, suggesting the potential for mobilizability and the presence of these ARGs in diarrhea-causing pathogens.

DISCUSSION

In this study, we evaluated the impact of different antibiotic regimens for the treatment and prophylaxis of TD on the gut microbiome, resistome, inflammatory status, and strain dynamics using well-characterized samples from two large clinical trials. We found that microbiome diversity remained stable for the duration of the study for most treatment groups. However, twice-daily rifaximin significantly decreased microbiome richness over time. Similarly, antibiotic resistance gene diversity and abundance were mostly stable but were significantly increased for the twice-daily rifaximin prophylaxis group in the PREVENT TD trial.

The TrEAT TD cohort was limited by the lack of placebo controls and an inability to collect samples before the onset of diarrhea. In the absence of these, we speculate that the observed taxonomic stability actually represents the persistence of a decreased diversity state instigated by diarrhea, which does not recover for at least 21 days post-diarrhea—well past the symptomatic period. Indeed, these diarrheal TrEAT TD samples had significantly lower richness and Shannon diversity when compared to those of the non-diarrheal PREVENT TD samples, including the lower diversity twice-daily rifaximin post-travel samples. This perturbation to the gut microbiome observed during our 21-day sampling interval is consistent with a previously reported TD cohort of travelers visiting Cusco, Peru, which observed significantly decreased richness for up to 2 weeks after diarrhea (22). Together, this suggests that while prophylaxis perturbs the microbiome, with the twice-daily rifaximin incurring a significantly decreased taxonomic diversity, this perturbation was less than that caused by experiencing diarrhea and antibiotic treatment. Additionally, while we did not observe microbiome recovery 21 days post-diarrhea, inflammatory cytokines significantly decreased over the same period indicating recovery of diarrhea-associated inflammation. This builds on evidence that the host response and recovery from diarrhea is complex, and while clinical symptoms may cease and local inflammatory markers may resolve, the microbiome community structure remains altered (30).

Our results are consistent with a previous study on the TrEAT TD cohort, which quantified differences in functional composition using the HuMiChip array and the TaqMan Array Card (31). The HuMiChip characterizes the functional capabilities of human microbiomes using ~134,000 probes that target key microbial gene families (32). The TaqMan Array Card also characterizes fecal microbiome compositions, using 384 probes to detect 19 enteropathogens (33). Using these tools, no differences in microbiome compositions or ARG abundances were detected between timepoints and antibiotic regimens.

Here, we observed that rifaximin prophylaxis depletes *E. coli* abundance compared to placebo controls, suggesting efficacy in targeting this potential diarrhea-causing pathogen. This is consistent with previous studies that have evaluated the success of rifaximin in the prevention of TD (34, 35) and other studies that have found an acquisition or enrichment for *Enterobacteriaceae* and *Escherichia* post-travel (36–38). However, we add the caveat that not necessarily all *E. coli* depletion is diarrheagenic

E. coli and likely includes non-pathogenic commensal strains. Future trials that simultaneously capture a pre-, during, and post-diarrheal state would be useful to evaluate the fluctuation in the prevalence of *E. coli* in the microbiomes, to show that a lack of prophylactic treatment leads to a flourishing of *E. coli* that culminates in symptomatic diarrhea.

Our study builds on previous studies of the impact of international travel and TD on gut microbiome changes and ARG acquisition (7, 8, 22, 36, 37, 39–42). These also observed increases in the abundance of *Enterobacteriaceae*, particularly *Escherichia* (7, 22, 36, 40). Our observations that *Proteobacteria* and *Bacteroidetes* decreased following diarrhea while *Firmicutes* increased (TrEAT TD trial) is consistent with previous reports characterizing diarrheal samples as being enriched for *Proteobacteria* and *Bacteroidetes* and depleted in *Firmicutes* (22, 39). Interestingly, while previous reports did not observe an increase in the overall abundance of ARGs post-travel—although the abundance of genes to specific drug classes could increase (22, 40)—we did observe a significant increase in ARG abundance in the PREVENT TD twice-daily rifaximin group. Lastly, our findings build on those from the TrEAT TD clinical trial study, which found all single-dose treatment groups were also comparable in terms of time to clinical cure, rate of treatment failure, and adverse effects (15).

In addition to these microbiome analyses, we analyzed *E. coli* isolates cultured from TrEAT TD Honduras diarrheal stool samples and from samples collected following symptom resolution. Prior studies also observed high rates of acquisition and persistence of multidrug-resistant extended-spectrum beta-lactamase (ESBL)-encoding *Enterobacteriaceae*, such as *blaTEM*, *blaCTX-M*, and *blaOXA* (7, 8, 40). We also observed similar proportions of *E. coli* pathotypes as the Cusco TD Peru study (22). While we did not have pre-diarrheal isolates to compare to, we note that the ARG count of isolates from diarrheal samples was significantly higher than those collected from the non-diarrheal samples of Cusco subjects who did not experience diarrhea (P = 1.96e-3) and not significantly different from subjects who did experience diarrhea.

Further studies directly evaluating the long-term recovery of the microbiome and resistome along with clinical outcomes following TD treatment and prophylaxis are needed to assess for potential delayed consequences. Our study represents the next phase in the evaluation of antibiotic effects on the gut microbiome and ARG emergence needed to more fully understand and wisely apply antibiotic stewardship while supporting the development of effective clinical practice guidance.

MATERIALS AND METHODS

Study designs and samples analyzed

The overall TrEAT TD study was a randomized, double-blind trial conducted in four countries (Afghanistan, Djibouti, Kenya, and Honduras) between September 2012 and July 2015 (NCT01618591). The US and UK servicemembers with acute watery diarrhea were randomized and received either single-dose azithromycin (500 mg; 106 persons), levofloxacin (500 mg; 111 persons), or rifaximin (1,650 mg; 107 persons), in combination with loperamide. The overall PREVENT TD study was a randomized, placebo-controlled, double-blind, clinical trial conducted in US and UK military personnel deploying overseas between 2016 and 2019 (NCT02498301). Subjects were recruited <72 hours after arrival and randomized to receive either once-daily rifaximin (550 mg), twice-daily rifaximin (550 mg ×2), or placebo, to be taken while deployed. Chemoprophylaxis was maintained for the duration of travel or a predetermined period of up to 6 weeks and at least 2 weeks.

Due to sample availability and transport considerations, we analyzed stool samples from a subset of the subjects and timepoints reported in these studies. For the TrEAT TD cohort, we analyzed 108 stool samples from 37 US servicemembers deployed to Honduras who experienced acute watery diarrhea (Table 1). Samples were collected at the presentation of diarrhea but prior to antibiotic treatment (day 0) and then 7 and 21

days after. Additionally, *E. coli* isolates were collected from the stool samples of 17 TrEAT TD subjects. For the PREVENT TD cohort, we analyzed 232 stool samples from 116 UK servicemembers deployed to Kenya (average travel time = 35.2 days) (Table 1). The first stool samples were collected after arrival in Kenya and before the first dose of rifaximin, and the second stool samples were taken shortly before leaving Kenya and <96 hours from the last dose of rifaximin (the majority taken almost immediately after).

Stool samples were self-collected and stored in a cool box (2–8°C) and then taken to the on-site lab within 2 hours. Macroscopic observations—consistency (watery, loose, semisolid, or formed), atypical components (mucoid, blood, and others, e.g., color)—were graded. Within 24 hours of collection, the stool was frozen at −80°C. Stool samples were cultured for bacterial enteropathogens, including *E. coli*, using conventional microbiologic techniques (43, 44). Enteropathogens were identified according to their growth on differential and selective agar plates (45). Stool specimens were streaked onto MacConkey agar, *Salmonella-Shigella* agar (SS), Hektoen Enteric agar (HE), thiosulfate citrate bile salt sucrose agar (TCBS), and *Campylobacter* blood-free agar base (CBF). Five lactose fermenting colonies with morphology compatible with *E. coli* were selected from each MacConkey agar plate. After incubation, enteropathogen differentiation was performed according to their growth characteristics in the different agars and differential biochemical reactions (45). Antibiotic susceptibility tests were performed using the Kirby-Bauer disk diffusion method, with each antibiotic reported as sensitive (S), intermediate (I), or resistant I according to the CLSI guidelines (46). For transportation, colonies were incubated in MacConkey agar at 37°C for 24 hours, and isolated colonies were resuspended in cryovials with trypticase soy broth with 15% glycerol, frozen at −70°C, and shipped overnight on dry ice. Samples were shipped to Washington University School of Medicine in St. Louis (WUSM), MO, USA, where they were stored at −80°C until DNA extraction.

A freezer failure affected a subset of samples from the TrEAT TD cohort. We first compared 43 paired aliquots collected from the same sample, where one aliquot was in the freezer failure and the other maintained temperature at −80°C. When comparing microbiome richness, Shannon diversity, and Bray-Curtis dissimilarity, we found no statistically significant differences between any metric in paired samples (Supplementary File 3). As this indicated that the freezer failure did not significantly alter sample compositions, we therefore also included 30 samples affected by the freezer failure, which did not have an unaffected paired sample. For the paired samples, we only included the non-freezer failure sample in downstream analyses.

Metagenomic analysis

Approximately 250 mg of frozen stool was chipped from an aliquot shipped to the Dantas laboratory at WUSM and processed with the DNeasy PowerSoil Pro Kit (Qiagen) per manufacturer instructions with two rounds of bead beating for 2 min at 2,500 oscillations/min on a Mini-Beadbeater-24 (BioSpec Products) with 5 min on ice. The Quant-iT PicoGreen fluorescence assay (Invitrogen) and Qubit fluorometer dsDNA HS assay (Invitrogen) were used to quantify DNA concentrations. In addition, 0.5-ng input DNA was then used to generate sequencing libraries with the Nextera Flex reagents (Illumina) and purified using the Agencourt AMPure XP system (Beckman Coulter). The Illumina NextSeq 550 platform was used to generate 2 × 150 paired-end sequencing reads at a target read depth of 5 million reads per sample. Read counts for each sample are provided in Files S1 and S2.

Bioinformatic tools used the default parameters except where indicated. Sequencing reads were demultiplexed by barcode and had adapters removed with Trimmomatic v0.38 (24695404) with the following parameters: *ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:1:true SLIDINGWINDOW:4:20 LEADING:10 TRAILING:10 MINLEN:60*. Read quality was assessed using FastQC v0.11.7 (47) and MultiQC v1.2 (48). Reads that are mapping to the human chromosome were removed with DeconSeq v0.4.3 (49), and disordered reads were repaired using BBMap v38.90 (50). Taxonomic profiling

was performed with MetaPhlAn3 v3.0.7. Richness, Shannon diversity, and Bray-Curtis similarity were calculated on these outputs directly. The MaAsLin2 package was used for linear mixed modeling to identify microbial taxa, ARGs, and pathways associated with the timepoint or treatment status of default parameters (18). Fixed and random effects are denoted in individual analyses in File S3 and our publicly available code on GitHub.

ShortBRED protein markers were built from the Comprehensive Antibiotic Resistance Database (CARD) 3.0 2020 database as well as previously validated hits from prior functional metagenomic screens (51–53) using *shortbred-identify.py* with a cluster identity of 90% and screened against UniRef9 v0.9.4. The number of hits for each gene was determined with ShortBRED-quantify, which normalizes the reads based on marker length and read depth. A Gaussian linear mixed-effect model created using the lmer function of the lmer4 package in R was used to predict ARG totals based on the time of treatment. The formula for the full model was ARGs ~ Treatment Time + (1 | SubjectID). ARG subsetting was based on annotations from the CARD 3.0 2020 database (51).

Cytokine measurements from stool

Concentrations of 40 cytokines were measured by Bio-Plex Pro Human Chemokine 40-plex Panel (Bio-Rad) in stool. Fecal samples from timepoints 0 and 21 from subjects in the TrEAT TD trial were processed by adding 500-ul sample diluent HB with a dissolved protease inhibitor tablet (one tablet per 10 mL buffer) (Thermo A32965) as previously published (54). Then, samples were vortexed for 30 sec twice and then spun down at 10,000 RPM for 10 min. Samples were then filtered through a 0.45-um filter (VWR cellulose acetate) into a 96-well plate. Bradford assays for protein quantification were performed on the samples to normalize cytokine concentration to total protein. Samples were then run per the manufacturer's instructions at half concentration. Samples were only used if they were not included in the freezer failure.

Isolate sequencing, assembly, and annotation

The *E. coli* isolates stored at −80°C in 15% glycerol were inoculated in 1.5-mL tryptic soy broth and were grown overnight at 37°C with shaking. Genomic DNA was extracted using the BiOstic Bacteremia DNA Isolation Kit as per the manufacturer's protocol. DNA concentration was quantified using the Qubit fluorometer and stored at −20°C. Isolate sequencing libraries were prepared similarly to stool samples. Processed reads were assembled into draft genomes using SPAdes v3.14.0 (55) with the following parameters: *spades.py -k 21,33,55,– --careful*. Assembly quality was assessed by BBMap v38.82 (56), QUAST v4.5 (57), and CheckM v1.0.7 (58), with the following inclusion criteria: >90% completeness, <5% contamination, and <500 contigs > 500 bp. Species assignment was verified *in silico* using Mash v2.2 and (59) and FastANI v1.3 (60), with an inclusion criterion of >95% average nucleotide identity (ANI) to the *E. coli*-type strain GCF_003697165.2.

Phylogroup, ARG, VF, and pathotype prediction

Draft genomes were annotated using Bakta v1.5.1 (61) wi– *--mincontiglen 200*. A core genome alignment of the 54 isolates from this study, plus 189 isolates from the Cusco TD study (22), and 30 reference *Escherichia* genomes (27) was generated using Panaroo v1.2.10 (62) wi– *--clean-mode strict* a– *--aligner mafft*. The output *core_gene_alignment.aln* file was used to build a maximum-likelihood phylogenetic tree using FastTree v2.1.10 (63) and visualized in iTOL (64) rooted at the outgroup *E. albertii* K7756. The TrEAT TD genomes' phylogroups were assigned with ClermonTyping (65) using the *mash_group* output and verified by clustering with known reference genomes. MLST was assigned using MLST v2.4 (https://github.com/tseemann/mlst) using the *ecoli_achtman_4* scheme. ARGs were identified using AMRFinder v3.10.42 (66), and ARG counts were compared using the ANOVA *post hoc* Tukey test. VFs were identified in *E. coli* genomes using AMRFinder and VirulenceFinder (67). Isolates were assigned a DEC pathotype *in silico* using PathotypeR (https://github.com/kevinsblake/PathotypeR) according to the presence/absence of specific VF genes, namely:

- Shiga toxin-producing *E. coli* (STEC): *stx1* and/or *stx2* (without *eae*)
- Enteropathogenic *E. coli* (EPEC): *eae* and/or *bfpA* (without *stx1* and/or *stx2*)
- Enterohemorrhagic *E. coli* (EHEC): *stx1* and/or *stx2*, and *eae*
- Enteroinvasive *E. coli* (EIEC): *ipaH*
- Enterotoxigenic *E. coli* (ETEC): *ltcA* and/or *sta1*
- Enteroaggregative *E. coli* (EAEC): *aatA* and/or *aaiC* and/or *aggR*
- Diffusely adherent *E. coli* (DAEC): *afaC* and/or *afaE*
- NPD: does not encode any of the above VFs
- Hybrid pathotypes: encode the requisite VFs for >1 pathotype.

For genomic context analyses, isolates containing an ARG of interest (e.g., *blaTEM*) were identified, underwent pairwise blastn, and then filtered for pairs with high identity (>99%) and coverage (>9,000 bp). These were visualized in Easyfig v2.2.2 (68) using the Bakta-outputted .gbff files, with a minimum BLAST length of 500 bp.

Strain and clonality analyses

Pairwise SNP distances were calculated between our 54 TrEAT TD isolates using Prokka v1.14.5 (69) wi– *--mincontiglen 200*, Roary v3.12.0 (70), and SNP sites v2.4.0 (71). Based on a histogram of core SNP counts, we assigned isolates to the same strain if they had <200 core SNPs. Next, we quantified whole-genome SNP counts between isolates from the same strain by aligning an isolate's processed reads to the assembly of the earliest isolated isolate of that strain using the *snippy-multi* function of Snippy v4.4.3 (https://github.com/tseemann/snippy). As a control, we also aligned these earliest isolates' assemblies and their own reads and masked any spurious SNPs found here from the final counts in the other comparisons. Strains were identified in metagenomes with our TrEAT TD and Cusco TD isolates as references using StrainGE v1.3.3 (29) against PREVENT TD and TrEAT TD metagenomes. AMR and virulence factors specific to *E. coli* within metagenomes were done by cross-referencing isolate AMRFinder outputs with the resultant StrainGE analysis.

DIABLO/mixOmics

The mixOmics (20) package in R was used for cross-correlation analysis of both metagenomic and cytokine abundance data. To avoid over-fitting on the large number of variables in our data sets, we utilized sPLS-DA. To find the number of variables from each data set to keep in the final model, we estimated model error rates for all combinations of seq(10,20,2) variables for both metagenomic and metabolomic data sets, using the function tune.block.splsda (10-fold cross-validation, repeated 10 times, "centroids.dist" distance metric). Circos plots were generated using the indicated correlation cutoff between variables. We used correlations of $r = 0.3$ and $r = 0.4$ to maximize potential correlations and minimize false positives (20).

Statistical analyses and data visualization

Statistical comparison between groups was done using vegan v2.6–4 (72). Figures were generated in RStudio, using ggplot2. A Gaussian linear mixed-effect model created using the lmer function of the lmer4 package in R was used to predict ARG totals based on the time of treatment. The formula for the full model was *ARGs ~ Treatment Time + (1 | ParticipantID)*. For inflammation analysis, each cytokine had a linear mixed-effect model constructed with antibiotic type and timepoint as fixed effects and participant ID as random effects.

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DIRECT CONTRIBUTION

This article is a direct contribution from Gautam Dantas, a Fellow of the American Academy of Microbiology, who arranged forand secured reviews by Ilana Brito, Cornell University, and David Haslam, Cincinnati Children's hospital, University of Cincinnati.

DATA AVAILABILITY

The datasetsdata sets generated from shotgun metagenomics and isolate sequencing are available from the NCBI SRA under BioProject ID PRJNA945408. The analysis scripts are available at: https://github.com/dantaslab/2023_Blake_TrEAT-PREVENT-TD

ETHICS APPROVAL

The clinical trials from which data and samples were obtained were reviewed and approved by the Uniformed Services University of the Health Sciences, Naval Medical Research Command, Walter Reed Army Institute of Research, and Kenya Medical Research Institute Institutional Review Boards (IRBs), as well as by the UK Ministry of Defence Research Ethics Committee in compliance with all applicable local, federal, and Department of Defense regulations governing the protection of human subjects. The protocol under which this work was performed was reviewed and approved by the Naval Medical Research Command (NMRC.2021.0005), Uniformed Services University of the Health Sciences, Walter Reed Army Institute of Research, Kenya Medical Research Institute, and Washington University in St. Louis.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

File S1 (mBio02790-23-s0001.xlsx). TrEAT TD metadata.
File S2 (mBio02790-23-s0002.xlsx). Prevent TD metadata.
File S3 (mBio02790-23-s0003.xlsx). Statistics.
Figure S1 (mBio02790-23-s0004.pdf). Higher Shannon diversity at baseline and endpoint and richness for PREVENT TD trial relative to TrEAT TD trial.
Figure S2 (mBio02790-23-s0005.pdf). Bray Curtis dissimilarity index plot between trials using MetaPhlAn3 species outputs.
Figure S3 (mBio02790-23-s0006.pdf). Circosplot with DIABLO.
Figure S4 (mBio02790-23-s0007.pdf). The sums of resistomes for the TrEAT TD trial as subsetted by AMR class.
Figure S5 (mBio02790-23-s0008.pdf). ARG count and RPKM for specific RpoB resistance genes and efflux pump ARG count and ARG sum within the Prevent TD cohort.
Figure S6 (mBio02790-23-s0009.pdf). Timeline, histogram, heatmap, and grouping of AST results.
Figure S7 (mBio02790-23-s0010.pdf). Strain counts.

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