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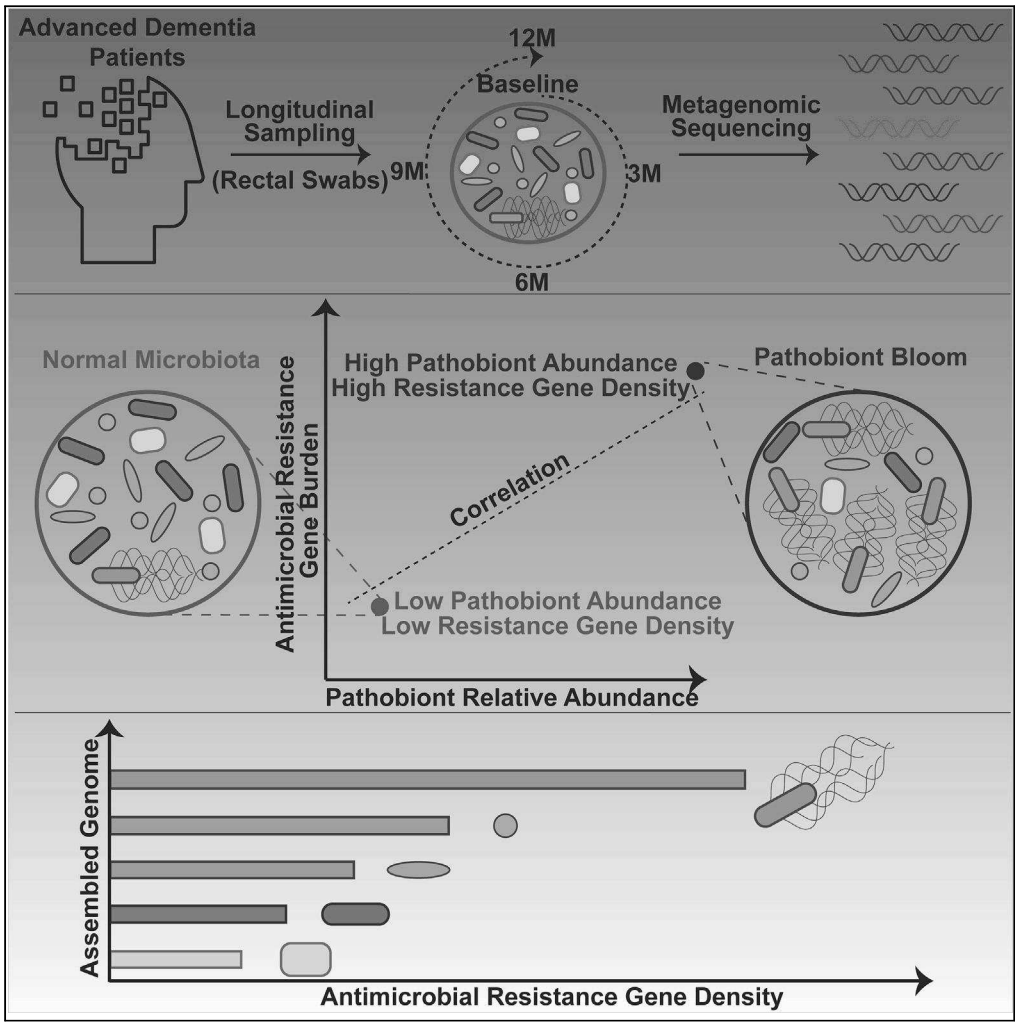

Aislinn D. Rowan-Nash, Rafael Araos, Erika M.C. D'Agata, Peter Belenky

peter_belenky@brown.edu

HIGHLIGHTS

Longitudinal analysis of the rectal microbiota of patients with advanced dementia

The microbiota was temporally unstable and characterized by pathobiont blooms

Antimicrobial resistance gene burden correlated with abundances of pathobionts

Genome assembly revealed that these species carried high levels of resistance genes

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Antimicrobial Resistance Gene Prevalence in a Population of Patients with Advanced Dementia Is Related to Specific Pathobionts

Aislinn D. Rowan-Nash,[1] Rafael Araos,[2,3,4] Erika M.C. D'Agata,[5] and Peter Belenky[1,6,*]

SUMMARY

Long-term care facilities are significant reservoirs of antimicrobial-resistant organisms, and patients with advanced dementia are particularly vulnerable to multidrug-resistant organism (MDRO) acquisition and antimicrobial overuse. In this study, we longitudinally examined a group of patients with advanced dementia using metagenomic sequencing. We found significant inter- and intra-subject heterogeneity in microbiota composition, suggesting temporal instability. We also observed a link between the antimicrobial resistance gene density in a sample and the relative abundances of several pathobionts, particularly *Escherichia coli*, *Proteus mirabilis*, and *Enterococcus faecalis*, and used this relationship to predict resistance gene density in samples from additional subjects. Furthermore, we used metagenomic assembly to demonstrate that these pathobionts had higher resistance gene content than many gut commensals. Given the frequency and abundances at which these pathobionts were found in this population and the underlying vulnerability to MDRO of patients with advanced dementia, attention to microbial blooms of these species may be warranted.

INTRODUCTION

It is well-recognized that there is a growing threat of antimicrobial-resistant (AMR) bacterial strains that threaten the health and lives of millions worldwide. In the United States alone, the Centers for Disease Control and Prevention estimates that at least 2 million people get an AMR infection each year and at least 23,000 die as a result (Centers for Disease Control and Prevention, 2013). A number of factors have driven the rise in AMR bacteria worldwide, including overprescription of antibiotics in the healthcare setting, over-the-counter access to antibiotics in some countries, and widespread use of antibiotics in animal husbandry for non-veterinary purposes (Aslam et al., 2018; Michael et al., 2014; Ventola, 2015). Concerningly, hospitals and other medical institutions are frequent sites of AMR bacteria acquisition, where patients may already be ill or immunocompromised, antimicrobial use is common, and patient-to-patient transmission of AMR isolates can occur via inadequate hygiene or environmental contamination (Paterson, 2006b; Struelens, 1998; Mulvey and Simor, 2009; Cookson, 2005). For example, AMR bacteria are highly prevalent in nursing homes, with estimates that over 35% of nursing home residents are colonized with multidrug resistant organisms (MDROs) (Cassone and Mody, 2015; Aliyu et al., 2017; O'Fallon et al., 2009; O'Fallon et al., 2010; Pop-Vicas et al., 2008; Trick et al., 2001). This is particularly problematic in light of the fact that elderly patients in long-term care facilities may be frequently hospitalized, potentially serving as a mode of bidirectional transport of MDROs between healthcare facilities (van den Dool et al., 2016; Trick et al., 1999; Morrill et al., 2016). They are also prone to infections and are frequently treated with antimicrobials (Bonomo, 2000; Daneman et al., 2011; van Buul et al., 2012), which has long been associated with acquisition of MDROs and may not always be indicated (O'Fallon et al., 2010; McGowan, 1983; Nicolle et al., 2000; Jones et al., 1987; van Buul et al., 2012; Dyar et al., 2015; Peron et al., 2013; Loeb, 2000; Morrill et al., 2016; D'Agata et al., 2013; Mitchell et al., 2014).

The problem of MDRO prevalence and inappropriate antimicrobial use is of particular relevance in elderly subjects with advanced dementia, a population which receives extensive antimicrobial treatment, which becomes more frequent closer to death, calling its benefit and effectiveness into question (D'Agata and Mitchell, 2008; Mitchell et al., 2014). Accordingly, advanced dementia specifically has been shown to be a risk factor for MDRO colonization (Pop-Vicas et al., 2008; Snyder et al., 2011). To examine this issue, the Study of Pathogen Resistance and Exposure to Antimicrobials in Dementia (SPREAD) was undertaken from 2009 to 2013 in order to analyze MDRO acquisition and appropriateness of antimicrobial prescription in elderly adults with advanced dementia residing in nursing homes (Mitchell et al., 2013). Supporting the

[1]Department of Molecular Microbiology and Immunology, Brown University, Providence, RI 02912, USA

[2]Instituto de Ciencias e Innovación en Medicina (ICIM), Facultad de Medicina Clinica Alemana Universidad del Desarrollo, Santiago, Chile

[3]Millenium Nucleus for Collaborative Research on Bacterial Resistance (MICROB-R), Santiago, Chile

[4]Advanced Center for Chronic Diseases (ACCDiS), Facultad de Medicina Clinica Alemana Universidad del Desarrollo, Santiago, Chile

[5]Infectious Diseases Division, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI 02903, USA

[6]Lead Contact

*Correspondence: peter_belenky@brown.edu

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widespread nature of MDRO carriage in this population, analysis of SPREAD subjects revealed that there were significant baseline levels and new acquisitions of MDROs and that there was notable spread of MDRO strains within and even between nursing home facilities (Mitchell et al., 2014; D'Agata et al., 2015).

In addition to potential facilitation of MDRO acquisition or spread, antimicrobial overuse may also have negative impacts on the diversity, composition, or function of the gut microbiota, which may already be vulnerable in elderly populations. Healthy younger adults tend to have a fecal microbiome characterized by relatively high diversity of species and populated primarily by members of the phyla *Bacteroidetes* and *Firmicutes*, largely obligate anaerobes that exist in homeostasis with the intestinal epithelium (Human Microbiome Project Consortium, 2012; Lloyd-Price et al., 2016; O'Toole and Claesson, 2010; Qin et al., 2010; Rowan-Nash et al., 2019). However, it has been found that, during senescence, the gut tends to have higher levels of *Bacteroidetes* and *Proteobacteria* and harbors higher levels of facultative aerobes and potential pathobionts, including *Enterobacterales* such as *E. coli*. These changes become more pronounced as aging progresses, and several studies have indicated that age-related alterations to the gut microbiota are relatively minor in septuagenarians but become more pronounced over time and are clear in centenarians and supercentenarians (Odamaki et al., 2016; Biagi et al., 2016; Biagi et al., 2010; Rampelli et al., 2013; Santoro et al., 2018). This is likely due to a number of factors, including the decline of immune function, onset of age-related diseases (including metabolic disorders), changes to diet and mobility, and the increased likelihood of medication utilization and/or hospitalization (Claesson et al., 2012; Salazar et al., 2017). However, lifestyle of elderly adults has an important impact, as research suggests that community-resident elderly subjects have a distinct and more diverse microbiome compared with those of their hospitalized or institutionalized peers, which was suggested to be at least in part due to nutritional differences (Claesson et al., 2012; Ticinesi et al., 2017). Furthermore, reduced microbiome diversity has been associated with increased frailty of elderly subjects (Claesson et al., 2012; van Tongeren et al., 2005). Accordingly, given that the microbiomes of institutionalized elderly patients are perhaps already at risk, understanding the impacts of antimicrobial use and MDRO acquisition on this population is of importance.

We analyzed the gut microbiomes of eleven subjects from SPREAD to examine the impact of antimicrobial use on the gut microbiota composition, function, and antimicrobial resistance gene (ARG) profile of elderly patients with dementia. These subjects were chosen as they were the largest group of subjects in SPREAD who had received a single antimicrobial (levofloxacin) during the collection period, and we anticipated that this intervention could have an impact on the already-vulnerable microbiota of this elderly, institutionalized cohort. Levofloxacin is an antimicrobial of the fluoroquinolone class with high oral bioavailability (Fish and Chow, 1997; Chien et al., 1997; Anderson and Perry, 2008). It has been found to reduce levels of Gram-negative aerobic bacteria, including *Proteobacteria* and particularly *Enterobacterales*, in the fecal microbiota (Inagaki et al., 1992; Edlund and Nord, 1999, 2000; Edlund et al., 1997; Bhalodi et al., 2019; Ziegler et al., 2019; Sullivan et al., 2001), although fluoroquinolone resistance among this taxon has been spreading (De Lastours et al., 2014; Lautenbach et al., 2004; Dalhoff, 2012; Spellberg and Doi, 2015; Acar and Goldstein, 1997; Nordmann and Poirel, 2005; Paterson, 2006a; Ruppe et al., 2015). A maximum of five rectal swab samples, collected every 3 months, were taken from each subject, and both 16S rRNA amplicon and shotgun metagenomics sequencing were performed. We analyzed alpha and beta diversity, taxonomic composition, functional potential, and antimicrobial resistance gene profiles before and after administration of levofloxacin but were unable to detect a specific impact of levofloxacin on any of these measures. However, we did find an association between blooms of particular enteric species and ARG density. Additionally, pathobionts and high ARG density were frequently detected by metagenomics in samples that were MDRO-negative by culture-based methods. Together, these results suggest that certain pathobionts carrying high ARG burdens may frequently colonize this patient group and that metagenomics may allow detection of resistant bacteria not flagged by culture-based methods.

RESULTS

Overview of Subjects

Elderly patients in long-term care facilities, and particularly patients with advanced dementia, are frequently exposed to antimicrobials and are at high risk of acquisition and carriage of MDROs (Cassone and Mody, 2015, Aliyu et al., 2017, O'Fallon et al., 2009, O'Fallon et al., 2010, Snyder et al., 2011, Pop-Vicas et al., 2008, Bonomo, 2000, Daneman et al., 2011, van Buul et al., 2012; D'Agata and Mitchell, 2008, D'Agata et al., 2015, D'Agata et al., 2013, Mitchell et al., 2014). From within the SPREAD cohort, we selected the largest group of subjects who had been administered a single antimicrobial during their participation in

the study. This gave us a group of eleven subjects who had been given the fluoroquinolone levofloxacin, one of the most commonly prescribed antimicrobials. We analyzed up to five rectal swabs, taken every 3 months over the course of a year, from these eleven subjects in the SPREAD cohort (Mitchell et al., 2013), using both 16S rRNA and shotgun metagenomics sequencing (Figure 1A). During their participation in the study, these subjects had received only a single course of levofloxacin (average course of 8 days), which has previously been shown to decrease the proportion of the *Enterobacterales* order of *Proteobacteria* (Sullivan et al., 2001; Inagaki et al., 1992; Edlund and Nord, 1999, 2000; Edlund et al., 1997; Bhalodi et al., 2019; Ziegler et al., 2019). Of the eleven subjects, all but subject I were female and all but subject G were white. They ranged in age from 72 to 101 years, and six members of the cohort did not survive for the full year of the study (Table S1). All but two subjects (C and G) resided in different nursing homes. Overall, there were 38 samples for metagenomics sequencing (Table S1). Culture-based methods indicated that four of the eleven subjects acquired an MDRO during the study: subject A acquired methicillin-resistant *S. aureus* (MRSA) at the 12-month time point, subject B acquired multidrug-resistant *E. coli* at the 3-month time point, and subjects C and D both acquired multidrug-resistant *P. mirabilis* at the 3-month time point (Table S1). Further information on sample collection, sequencing, and data processing can be found in the Transparent Methods section of the Supplemental Information and Table S2.

Alpha and Beta Diversity Metrics

Before focusing on antimicrobial resistance, we first wanted to assess the composition of the community throughout the longitudinal time frame. We initially used the metagenomic sequencing data to compare the alpha diversity, or the diversity within each sample, of samples collected before and after levofloxacin administration. According to Shannon's Diversity Index, which incorporates both richness and evenness of samples, there was no significant difference between the pre- and post-levofloxacin samples (Figure 1B). Furthermore, the alpha diversity was variable over time even within the same subject, and there was no clear trend of recovery in alpha diversity after antibiotic cessation. This suggests a degree of temporal instability, in which the richness and/or evenness of the samples varies over time.

We then examined beta diversity, or the diversity between samples. We utilized the Bray-Curtis Dissimilarity metric, which considers the identity and abundance of taxa shared between samples. Plotting this metric in a principal coordinate analysis (PCoA) revealed no apparent pattern of clustering based on either subject or sample collection point relative to levofloxacin, and in fact, samples from the same subject were often located quite distantly from one another (Figure 1C). We then compared the within-subjects dissimilarity of sequential samples within a subject when both were pre-levofloxacin, both were post-levofloxacin, or one sample was pre- and one was post-levofloxacin; there was no significant difference between any of the groups (Figure 1D), suggesting that levofloxacin may not be associated with community disruption. Furthermore, although within-subject dissimilarity was lower than between-subjects dissimilarity, the effect size was low (0.7013 versus 0.7712, respectively; Figure 1E).

Taxonomic Composition

We utilized Kraken2 in conjunction with the Bayesian Reestimation of Abundance with KrakEN2 (Bracken2) pipeline to assign taxonomy to our metagenomic sequencing samples (Wood and Salzberg, 2014; Lu et al., 2017). Corresponding to the high between-subjects beta-diversity, the taxonomic composition of the gut microbiome varied significantly between subjects. As is typical for the human gut microbiome, most bacteria belonged to the five major phyla of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. However, consistent with the high within-subjects beta diversity, the dominant phylum varied greatly even between samples from the same subject (Figure S1); for example, the most abundant phylum in subject E was *Bacteroidetes* at two time points, *Proteobacteria* at two time points, and *Firmicutes* at one time point (Figure S1F). Overall, the most abundant phylum was *Actinobacteria* in three samples, *Bacteroidetes* in seventeen samples, *Firmicutes* in seven samples, and *Proteobacteria* in eleven samples (Figures S1A–S1L); averaging across all samples, *Bacteroidetes* was the highest at 34.2%, followed by *Proteobacteria* (26.9%), *Firmicutes* (23.3%), and *Actinobacteria* (11.2%) (Figure S1A). Qualitatively, many of the samples from this population represent highly divergent and dysbiotic microbiomes compared with what is typically seen with younger subjects, in which *Proteobacteria* in particular make up a much smaller proportion of the microbiome than in these elderly subjects with dementia (Human Microbiome Project Consortium, 2012).

The genus- and species-level taxonomic composition was also variable. Blooms of potential pathogens (Weiner et al., 2016), including *Campylobacter ureolyticus* (O'Donovan et al., 2014), *Corynebacterium*

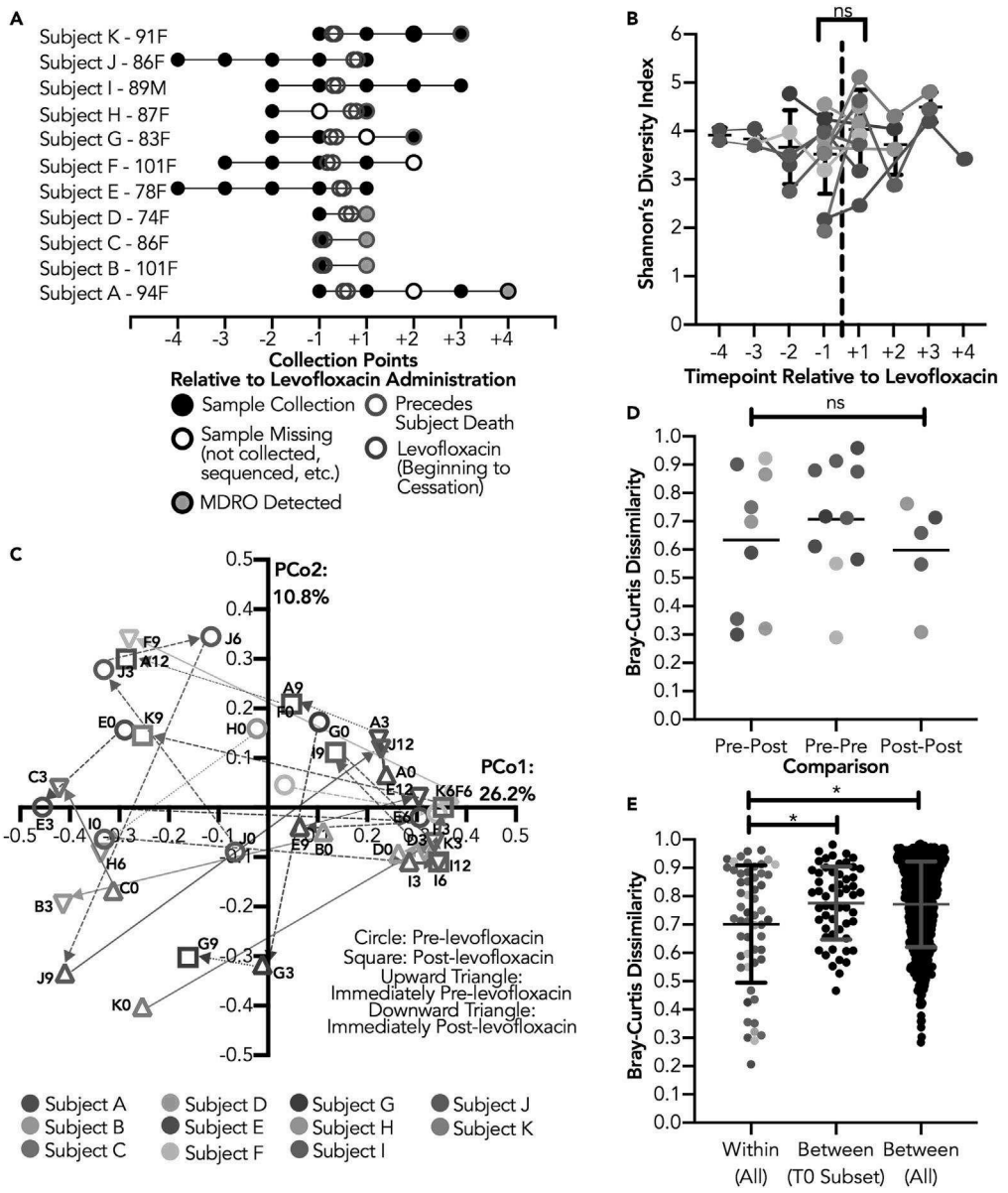

Figure 1. Subject Overview and Diversity Metrics

(A) Metagenomic sequencing was performed on longitudinal samples from eleven subjects from SPREAD who had received a single course of levofloxacin during their participation in the study. Points represent collection of samples, at intervals of approximately 3 months, relative to administration of levofloxacin. See Tables S2, S3, and S4 for metagenomic sequencing results.

(B) Shannon diversity over time of all subjects based on metagenomic sequencing data. The dashed line indicates administration of levofloxacin. ($p = 0.175$ for immediately pre-levofloxacin versus immediately post-levofloxacin samples and $p = 0.1006$ for all pre-levofloxacin versus all post-levofloxacin samples; Mann-Whitney test)

(C) PCoA analysis of Bray-Curtis Dissimilarity based on metagenomic sequencing data. Solid arrows connect immediately pre- with immediately post-levofloxacin samples, dashed arrows connect other sequential samples, and dotted arrows connect samples where an intermediate sample is missing.

(D) Within-subjects Bray-Curtis Dissimilarity of sequential samples based on metagenomic sequencing data. ($p = 0.6248$ between pre-levofloxacin samples, post-levofloxacin samples, or pre-post levofloxacin samples; ANOVA)

(E) Overall within-subjects, T0 between-subjects, and overall between-subjects Bray-Curtis Dissimilarity based on metagenomic sequencing data. ($p = 0.0262$ for overall within-subjects versus T0 between-subjects, and $p = 0.0175$ for overall within-subjects versus overall between-subjects; t test with Welch's correction)

urealyticum (Salem et al., 2015), *Enterococcus faecalis* (Agudelo Higuita and Huycke, 2014; Fiore et al., 2019), *Escherichia coli* (Conway and Cohen, 2015; Woodward et al., 2019), *Oligella urethralis* (Baqi and Mazzulli, 1996; Graham et al., 1990; Pugliese et al., 1993; Wilmer et al., 2013), *Proteus mirabilis* (Schaffer and Pearson, 2015; Chen et al., 2012), *Providencia stuartii* (Wie, 2015; Kurmasheva et al., 2018), *Pseudomonas aeruginosa* (Bassetti et al., 2018; Mittal et al., 2009), *Staphylococcus aureus* (Gordon and Lowy, 2008; Lowy, 1998; Naimi et al., 2003), and *Staphylococcus haemolyticus* (Becker et al., 2014; Czekaj et al., 2015; Froggatt et al., 1989), were fairly common, both before and after levofloxacin administration (Figures 2A and S2). Across subjects, even baseline samples varied in composition, as expected from beta-diversity analysis. Averaging across all samples, the single most-abundant species was *E. coli*, further supporting the qualitatively dysbiotic nature of the gut microbiome of this cohort (Figure 2A). Despite the high proportion of members of *Enterobacterales* in this cohort, the Linear Discriminant Analysis Effect Size (LEfSe) algorithm (Segata et al., 2011) did not reveal biomarkers for pre- or post-levofloxacin samples at the phylum, genus, or species level. Full data on taxonomic composition at the phylum, genus, and species levels can be found in Table S3.

As we had access to full 16S rRNA and shotgun metagenomics data for our samples, we compared their taxonomic identifications at the genus level. The two methods of analysis were generally consistent, and blooms of prominent genera (including *Escherichia, Proteus, Enterococcus, Providencia, Staphylococcus*, and *Bacteroides*) were generally detected by both analysis pipelines (Figure S3A). Metagenomics analysis was unsurprisingly able to detect more distinct genera, and of the genera that were called by both pipelines, LEfSe analysis revealed biases in both methods (Figure S3B). For example, metagenomics analysis by Kraken2 and Bracken2 detected higher levels of *Bacteroides*, whereas 16S rRNA analysis with Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Caporaso et al., 2010) detected higher levels of *Ruminiclostridium* (Figure S3B). Full data on taxonomic abundances at the genus level for both sequencing types can be found in Table S3.

Functional Potential

We used the Human Microbiome Project Unified Metabolic Analysis Network 2 (HUMAnN2) pipeline (Franzosa et al., 2018) to analyze the genetic content of the metagenomic samples. We utilized LEfSe to compare community function at the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog, Gene Ontology (GO) term, and MetaCyc pathway levels. As in the taxonomic analysis, there were no significant biomarkers of either pre- or post-levofloxacin administration samples. However, although the taxonomic profile of the samples varied greatly, the functional capacity was fairly consistent across samples (Figure S4). Full data on functional potential can be found in Table S4.

Antimicrobial Resistance Gene Profile

We used the DeepARG machine-learning program (Arango-Argoty et al., 2018) to detect resistance genes in the metagenomic samples. Across all samples, the most abundant class of ARG was "multidrug," followed by "macrolide-lincosamide-streptogramin" (MLS) and "tetracycline." The most common specific gene detected was the multidrug resistance *rpoB2* variant of the RNA polymerase beta subunit, followed by the MLS resistance gene *macB* and a multidrug ABC transporter (Figure 2B). LEfSe analysis revealed no ARG biomarkers of either pre- or post-levofloxacin samples. Full data on ARG composition can be found in Table S5.

However, we were able to detect changes in specific ARG classes and genes that corresponded with the detection of antimicrobial-resistant organisms in two subjects. Subject A acquired MRSA at the 12-month time point, and a bloom of this species to 25.0% could be detected in the metagenomic taxonomic data (Figures 3A and S2B). Although the overall level of ARGs did not notably increase at this time point, there was a clear expansion in beta-lactam resistance genes (Figures 3B and S5B), including the *mecA/mecR1/mecI* operon, which regulates expression of the low-affinity penicillin-binding protein *mecA* (also known as PBP-2A) (Hiramatsu, 1995; Hiramatsu et al., 2001; Tsubakishita et al., 2010; Enright et al., 2002) (Figure 3C). This operon is characteristic of MRSA strains (Hiramatsu, 1995; Hiramatsu et al., 2001; Tsubakishita et al., 2010; Enright et al., 2002), supporting the culture-based classification of this *S. aureus* isolate as MRSA.

Similarly, subject B acquired multidrug-resistant *E. coli* (resistant to the beta-lactams ampicillin/sulbactam, cefazolin, ceftazidime, and ceftriaxone and to the fluoroquinolone ciprofloxacin) at the 3-month time point, and the proportion of this species expanded to 47.3% of the population in the corresponding sample

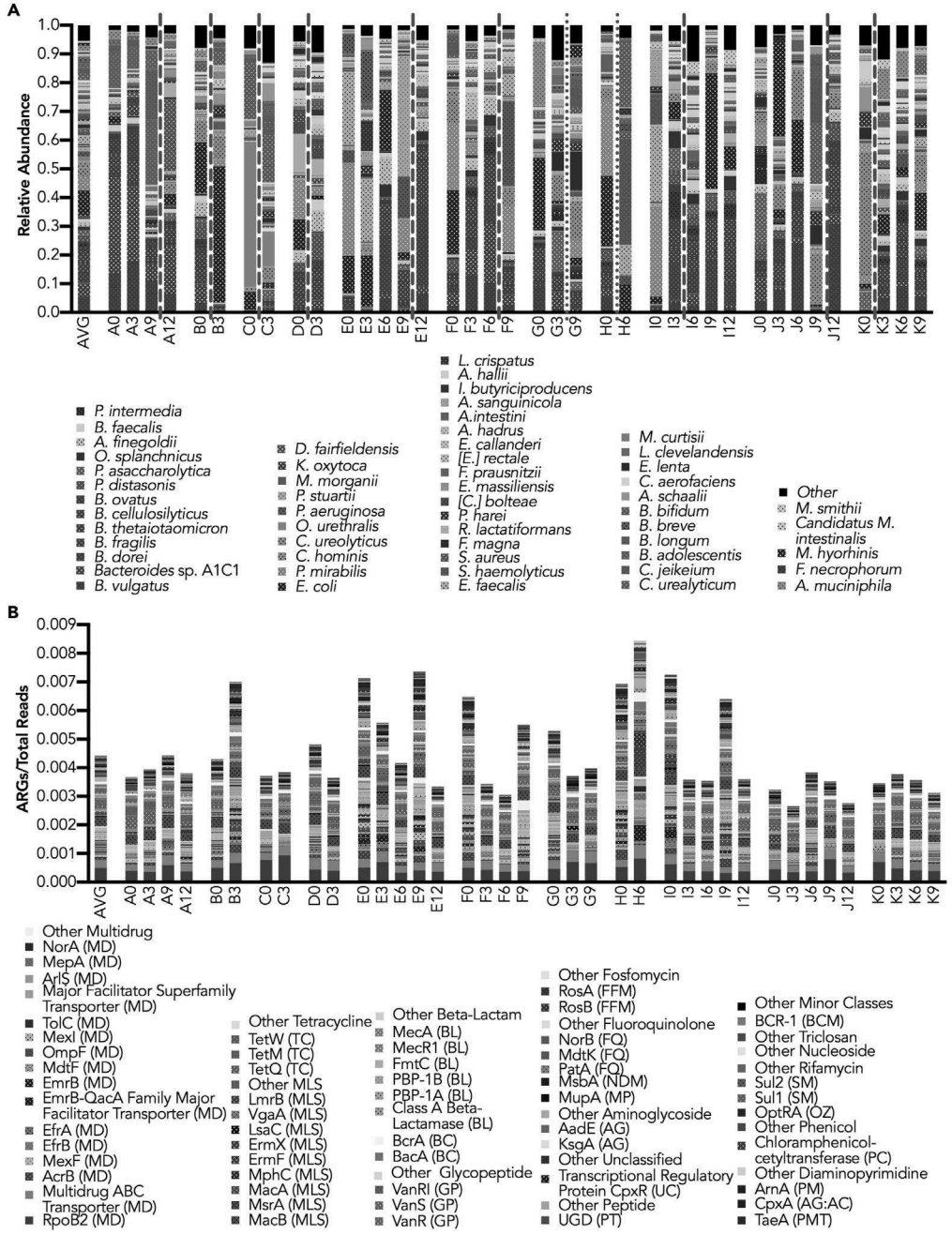

Figure 2. Relative Abundances of Species and Antimicrobial Resistance Genes

(A) Relative abundance of the most abundant species across all samples, with all other species grouped in the "other" category. Species are grouped by genus and phylum and are ranked within those levels by average relative abundance across all samples. Broad color categories distinguish phylum (*Proteobacteria* are red, *Bacteroidetes* are blue, *Firmicutes* are green, and *Actinobacteria* are purple), whereas different species of the same genus are given the same specific background color. Red lines indicate levofloxacin administration; dashed lines indicate usage between consecutive time points, whereas dotted lines indicate usage where the immediately post-levofloxacin sample is missing. See Table S3 for underlying taxonomic abundances.

(B) Relative abundance of the most-abundant antimicrobial resistance genes (ARGs) across all samples. Specific ARGs are grouped by the class of antimicrobials they provide resistance to. Broad color categories distinguish class (Multidrug RGs are blue, MLS RGs are red, etc.), whereas related gene categories (e.g. the *mec* operon or *mex* efflux proteins) are given the same specific background color. All ARGs were normalized to the total number of reads. See Table S5 for underlying ARG abundances.

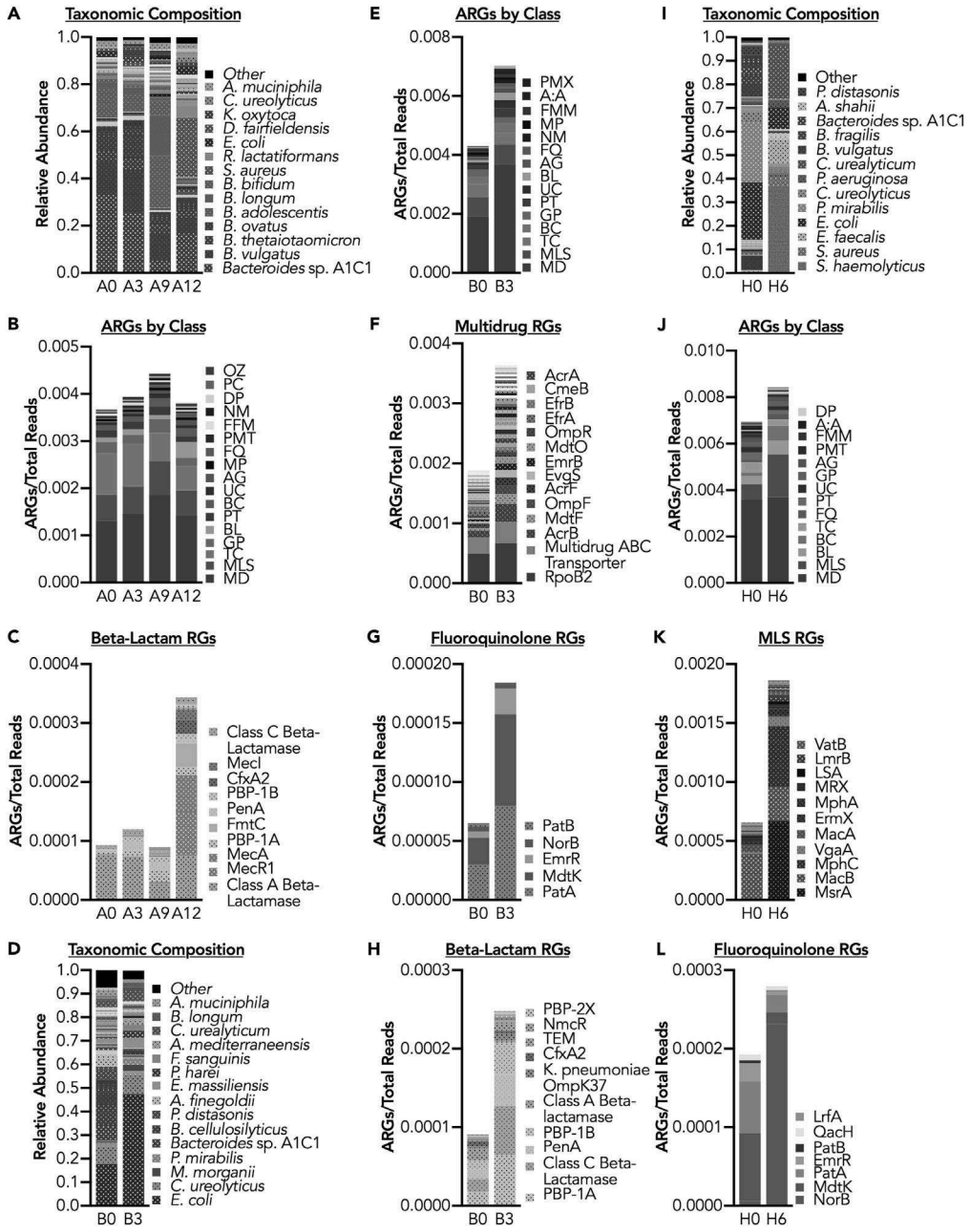

Figure 3. Antimicrobial Resistance Gene Profiles Reflect Taxonomic Observations

(A) Relative abundance of species in subject A, showing a bloom in *S. aureus* at T12.

(B) Relative abundance of ARG classes in subject A, showing an expansion in beta-lactam resistance genes at T12.

(C) Relative abundance of beta-lactam resistance genes in subject A, showing increases in the *mecA/mecI/mecRI* operon at T12.

(D) Relative abundance of species in subject B, showing a bloom in *E. coli* at T3.

(E) Relative abundance of ARG classes in subject B, showing an expansion in multidrug, beta-lactam, and fluoroquinolone resistance genes at T3.

(F) Relative abundance of multidrug resistance genes in subject B, showing increases in various ARGs at T3.

(G) Relative abundance of fluoroquinolone resistance genes in subject B, showing increases in genes including *patA* and *mdtK* at T3.

(H) Relative abundance of beta-lactam resistance genes in subject B, showing increases in genes including penicillin-binding proteins and class C beta-lactamase at T3.

(I) Relative abundance of species in subject H, showing a bloom in *S. haemolyticus* at T6.

Figure 3. *Continued*

(J) Relative abundance of ARG classes in subject H, showing increases in MLS and fluoroquinolone resistance genes.

(K) Relative abundance of MLS resistance genes in subject H, showing an increase in staphylococcal resistance gene *msrA* and others at T6.

(L) Relative abundance of fluoroquinolone resistance genes in subject H, showing an increase in staphylococcal resistance gene *norB* and others at T6.

See Tables S3 and S5 for underlying taxonomic and ARG abundances.

(Figures 3D and S2C). Accordingly, this sample showed a notable increase in the relative abundance of ARGs, which was in large part driven by an increase in a number of multidrug resistance genes (Figure 3E and 3F); there was also a clear increase in several beta-lactam resistance genes, including the low-affinity penicillin-binding protein genes *PBP-1A, PBP-1B,* and *penA* (*PBP2*) as well as class C beta-lactamase genes (Smith and Klugman, 1998; Zapun et al., 2008; Brannigan et al., 1990; Dowson et al., 1989; Thulin et al., 2006; Beceiro and Bou, 2004), and several fluoroquinolone resistance genes, including the transporters *patA* and *mdtK* (Figures 3G and 3H).

Despite the acquisition of multidrug-resistant *P. mirabilis* at the 3-month time point in subjects C and D, there was no corresponding increase in ARGs. ARG levels stayed approximately the same in subject C (0.372% at baseline and 0.384% at 3 months) and decreased in subject D from 0.482% at baseline to 0.364% at the 3-month time point (Figure 2B, S5D, and S5E). However, this corresponds to the taxonomic data; levels of *P. mirabilis* were low and stable in subject C (0.55% at baseline and 0.61% 3 months later), and although *P. mirabilis* made up 13.8% of the population at baseline in subject D, it underwent a reduction to 2.3% at the 3-month time point (Figures 2A, S2D, and S2E). Taken together, these data indicate that our metagenomics pipeline can detect blooms of AMR pathogens and that the corresponding change in ARG levels aligns with culture-based detection of MDROs. At the same time, metagenomic analysis of some samples found blooms of pathogens and ARGs that were not associated with culture-based MDRO detection.

Attribution of ARG Density to Specific Species

Although we did not observe that total ARG density within samples varied by levofloxacin administration, there was significant variability between samples. In fact, most samples had similar baseline levels of ARGs of 0.3%–0.4% of the total reads, whereas only a few samples rose above this value to between 0.6% and 0.8%. Close inspection of the taxonomic composition of the samples revealed that samples with higher levels of ARGs tended to have blooms of one or more of the *Proteobacteria* species *E. coli* and *P. mirabilis* and the *Firmicutes* species *E. faecalis*, strains of which are common pathobionts (Archambaud et al., 2019; Butto et al., 2015; Chow et al., 2011; Dzutsev and Trinchieri, 2015; Hamilton et al., 2018; Mirsepasi-Lauridsen et al., 2019; Yang et al., 2019) (Figure 4A). Confirming this association, correlation analysis between ARG levels and the sum of the relative abundances of these three species showed a strong and significant positive correlation ($r = 0.791$, $R^2 = 0.6254$, $p < 0.0001$, Pearson's correlation; Figure 4B). This suggests that, in samples with higher-than-baseline ARG levels, ARG abundance is being driven by high relative abundance of these three species.

However, there were two notable exceptions: samples E9 and H6 had high levels of ARGs without corresponding blooms of these three species. However, *P. stuartii* bloomed to 41.9% relative abundance in sample E9 and *S. haemolyticus* bloomed to 36.9% in sample H6 (Figures 2A, S2F, S2I, and S3H). Both species have long been associated with AMR phenotypes (Overturf et al., 1974; McHale et al., 1981; Hawkey, 1984; Warren, 1986; Oikonomou et al., 2016; Czekaj et al., 2015; Froggatt et al., 1989; Barros et al., 2012; Maleki et al., 2019) and were not found at high levels in other samples, but could explain the higher ARG abundance in these samples (Figure 4A). Supporting this possibility, an examination of the ARGs in sample H6 showed a distinct profile relative to other samples, with high levels of staphylococcal resistance genes including fluoroquinolone resistance gene *norB* and macrolide-streptogramin resistance gene *msrA* (Figures 2B and 3I–3L). Accordingly, addition of *P. stuartii* and *S. haemolyticus* abundances to the analysis resulted in a stronger correlation ($r = 0.933$, $R^2 = 0.8706$, $p < 0.0001$, Pearson's correlation; Figure 4C).

To more rigorously examine the relationship between the species of interest and ARG levels, we performed metagenomic assembly and binning to compare the levels of ARGs in these organisms to levels in other common and abundant species, including likely commensals and potential pathogens (Figure 4D). Specifically, we analyzed bins that passed various quality controls (see Transparent Methods) and corresponded

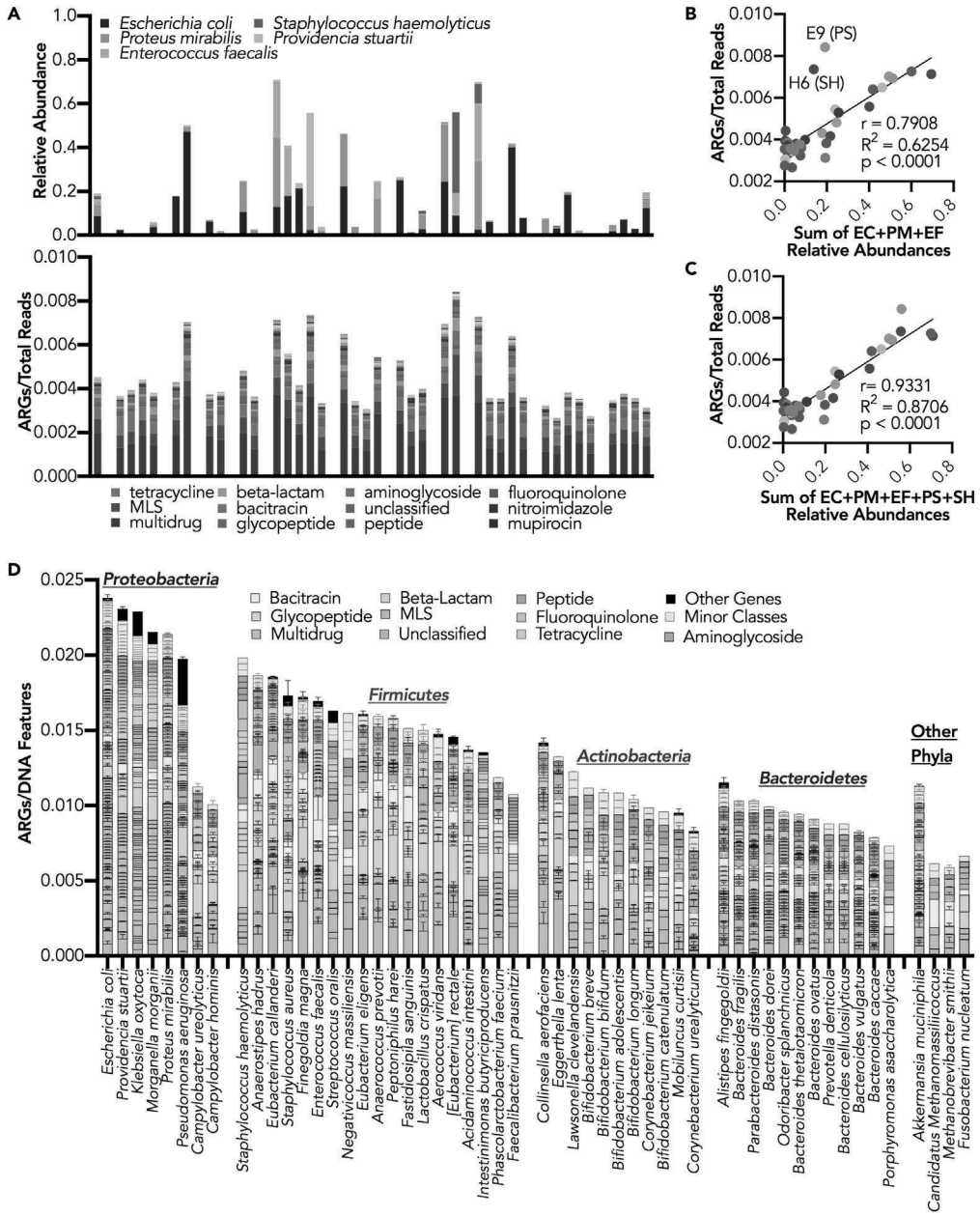

Figure 4. Relationship of ARG Levels to the Relative Abundance of Specific Pathobionts

(A) Correspondence between the relative abundances of key species of interest (*E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus*) and total ARG density in each sample.

(B) Correlation between the sum of the relative abundances of *E. coli* (EC), *P. mirabilis* (PM), and *E. faecalis* (EF) and the total ARG density in each sample ($r = 0.791$, $R^2 = 0.6254$, $p < 0.0001$; Pearson's correlation). Outliers are labeled by sample and with the species that may be driving high ARG levels. PS, *P. stuartii*; SH, *S. haemolyticus*.

(C) Correlation between the sum of the relative abundances of *E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus* and the total ARG density in each sample ($r = 0.933$, $R^2 = 0.8706$, $p < 0.0001$; Pearson's correlation).

(D) Average ARG density in bins of species across all samples in which we were able to construct a bin for that species. Specific genes are grouped and colored by their ARG class, and bins are grouped by phylum and ranked by their total average ARG density within that phylum.

See Tables S3 and S5 for underlying taxonomic and ARG abundances.

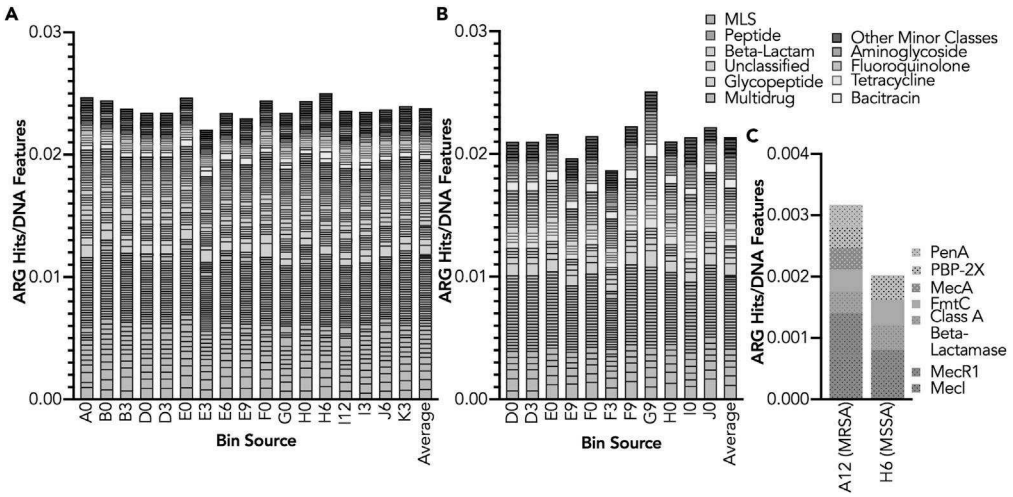

Figure 5. Comparison of MDRO and non-MDRO Bins of the Same Species

(A) ARG density in all *E. coli* bins across samples.

(B) ARG density in all *P. mirabilis* bins across samples.

(C) Beta-lactam ARG density in all *S. aureus* bins across samples.

See Table S5 for underlying ARG abundances. Related to Figure 4.

to species identified by Kraken2/Bracken2 to make up greater than 0.1% of their source samples; a complete list of analyzed bins, including quality information and source, can be found in Table S6.

As anticipated, we found that the levels of ARGs in bins from *E. coli* and *P. mirabilis* were consistently high compared with other species analyzed. In fact, *E. coli* had the highest average ARG density of any species analyzed, whereas *P. mirabilis* was the fifth highest (Figure 4D). Notably, the ARG composition of the bins of these species from samples in which MDROs were detected (B3, C3, and D3) did not appear to be different from those of other samples (Figures 5A and 5B), although it is possible that some resistance genes were carried on plasmids that were not assembled into genomes. *P. stuartii* had the second-highest average ARG density (Figure 4D), reflecting the expansion of ARGs detected in sample E9, where this species bloomed to 41.9% of the population (Figures 2A, S2A, and S2F). The third and fourth positions were taken by the single bins constructed for *Klebsiella oxytoca* and *Morganella morganii* (Figure 4D), other *Proteobacteria* with pathogenic potential (Singh et al., 2016; Moradigaravand et al., 2017; Liu et al., 2016). *P. aeruginosa* bins rounded out the top six, with similar levels to the other top species (Figure 4D). However, as *K. oxytoca* and *M. morganii* were never present at greater than 3% and *P. aeruginosa* bloomed in only two samples (Figure 2A), they did not significantly contribute to overall ARG density in the samples. Importantly, high ARG density was not a universal feature of *Proteobacteria*, or even of pathogenic *Proteobacteria*; bins constructed for the *Campylobacter* species *C. hominis* and *C. ureolyticus* had universally low ARG levels. Additionally, although we could not construct a high-quality bin for *O. urethralis*, the low ARG densities in the samples in which this species bloomed (C0 and C3) suggests that it also has low genomic ARG content. This suggests that high ARG density among the *Proteobacteria* analyzed was restricted to the *Gammaproteobacteria* class, primarily of the order *Enterobacterales* but also including *Pseudomonadales*.

We were only able to construct two good-quality bins for *E. faecalis*, which varied in their ARG levels, particularly on the basis of bacitracin resistance. On average, although the two bins did not have ARG levels as high as the *Proteobacteria* of interest, they did rank among the highest of the *Firmicutes* bins tested (Figure 4D). We were also able to create a single bin for *S. haemolyticus* from sample H6, in which it made up 36.9% of the population (Figures 2A, S2A, and S2I). This bin had an ARG density higher than the average for any other non-*Proteobacteria* species (Figure 4D), supporting its role in the high ARG levels found in the corresponding sample. As expected from the analysis of the total ARG population of that sample (Figures 3K and 3L), the staphylococcal resistance genes *norB* and *msrA* were found in this bin (Table S5). We were also able to create two bins for *S. aureus*, including from sample A12 where MRSA was detected. The A12 bin contained the characteristic MRSA gene *mecA*, whereas the H6 bin did not, suggesting that the

S. aureus strain found in H6 was not MRSA (Figure 5C and Table S5). In general, bins from the phyla *Actinobacteria* (including *Bifidobacterium* and *Corynebacterium* species) and *Bacteroidetes* (including *Bacteroides* and *Parabacteroides* species) had low ARG levels. Full data on the ARGs and classes found in species-level bins can be found in Table S5.

Prediction of ARG Density from Species Abundances

Our initial analysis only considered the eleven subjects for whom we had longitudinal metagenomics data due to their receiving levofloxacin. We also had access to a larger set of SPREAD samples that underwent shotgun metagenomic sequencing for a related study (Araos et al., 2019), and these sequences can be found at the NCBI Short Read Archive:PRJNA531921. In this case, the data was not longitudinal and encompassed an array of antibiotic treatment conditions across 63 subjects, providing a diverse set of taxonomic and ARG data on which to test whether the relationship between *E. coli*, *P. mirabilis*, and *E. faecalis* and ARG density held true. Taxonomic data, ARG data, and subject metadata for these samples can be found in Tables S3, S5, and S7, respectively. As an initial test, we performed the same correlation analyses between species of interest and ARG levels as on the levofloxacin dataset, finding that both the simple and complex models showed significant correlation ($r = 0.7367$, $R^2 = 0.5427$, $p < 0.0001$ and $r = 0.7811$, $R^2 = 0.6012$, $p < 0.0001$, respectively; Pearson's correlation; Figures 6A and 6B). This provided initial support for the trend being present in the wider dataset.

We then created a multiple linear regression model to predict ARG density using the relative abundances (RA) of the three main species of interest in the initial levofloxacin dataset, with the following equation: *ARG density* = 0.003482 + 0.006221(*E. coli* RA) + 0.006248(*P. mirabilis* RA) + 0.006920(*E. faecalis* RA) (Figure 6C). We then used this equation to predict the ARG density in the larger metagenomics dataset and found that it was able to accurately predict the true ARG level of those samples, with predicted and actual values correlating significantly ($r = 0.7335$, $R^2 = 0.5381$, $p < 0.0001$; Pearson's correlation; Figure 6D). As before, there were a few notable outliers with higher ARG levels than predicted by the model; those three samples contained high levels of *P. stuartii*, *P. aeruginosa*, or *Klebsiella pneumoniae*. This maps well to the fact that we observed high levels of ARGs in bins constructed from *P. stuartii*, *P. aeruginosa*, and the related species *K. oxytoca* (Figure 4D). Importantly, although this model does capture the contribution of pathobionts to ARG density present in the samples, it is likely that other microbial or host-related factors also contribute to ARG levels. Therefore, this model is descriptive of the relationship between ARGs and pathobionts in this dataset rather than predictive in other populations.

We also created a multiple linear regression model that incorporated the relative abundances of *P. stuartii* and *S. haemolyticus*, blooms of which had caused outliers from the original species-ARG correlation: *ARG density* = 0.003253 + 0.006715(*E. coli* RA) + 0.006748(*P. mirabilis* RA) + 0.003461(*E. faecalis* RA) + 0.01123-(*S. haemolyticus* RA) + 0.007569(*P. stuartii* RA)) (Figure 6E). As before, we tested this equation against the larger dataset and found that it slightly increased the accuracy of the predictions; specifically, it eliminated the outlier that had high *P. stuartii* levels and slightly improved the correlation between predicted and actual ARG levels ($r = 0.7901$, $R^2 = 0.6242$, $p < 0.0001$; Pearson's correlation; Figure 6F). However, the simpler model is more broadly applicable, as blooms of *P. stuartii* and *S. haemolyticus* are relatively uncommon. Similarly, although *Klebsiella* spp. and *P. aeruginosa* may also contribute to high ARG density in samples, they do not bloom as commonly in this cohort as the core predictive species of *E. coli*, *P. mirabilis*, and *E. faecalis*.

These results indicate that in this population, levels of only a few key species could predict the majority of ARG abundance beyond background levels. Both the core predictive species (*E. coli*, *P. mirabilis*, *E. faecalis*) and others that are associated with high ARG levels in samples (*P. stuartii*, *S. haemolyticus*, *P. aeruginosa*, *Klebsiella* spp.) are pathogens and/or pathobionts. Monitoring levels of these species may be helpful in elderly, institutionalized populations, as these patients may be vulnerable to developing or transmitting AMR infections from high-level carriage of these species.

DISCUSSION

Overall, we found that the microbial composition of the gut microbiome of elderly patients with advanced dementia was quite variable, both between subjects and over time within the same subject. Even in the absence of antimicrobial treatment, there was notable fluctuation in the abundance of a number of species, including pathobionts such as *E. coli*, *P. mirabilis*, and *E. faecalis*. When comparing the taxonomic composition, functional potential, and resistome of pre- and post-levofloxacin samples, we did not observe

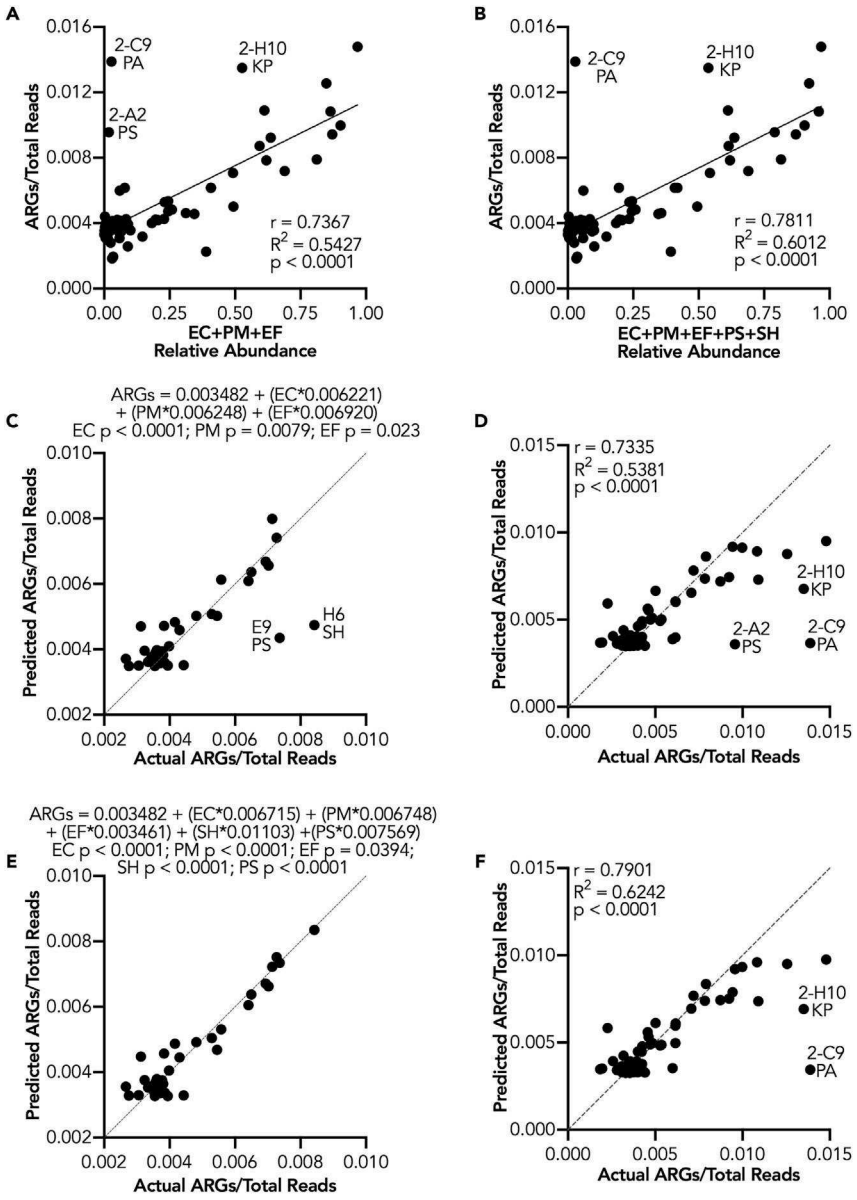

Figure 6. Prediction of ARG Density From Relative Abundance of Specific Pathobionts

(A) Correlation between the sum of the relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis* and the total ARG density in each sample in the test dataset (r = 0.7139, R^2 = 0.5096, p < 0.0001; Pearson's correlation).

(B) Correlation between the sum of the relative abundances of *E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus* and the total ARG density in each sample in the test dataset (r = 0.7753, R^2 = 0.6012, p < 0.0001; Pearson's correlation).

(C) Multiple linear regression of relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis* to ARG density in samples in the levofloxacin dataset. (p < 0.0001 for *E. coli*, p = 0.0079 for *P. mirabilis*, p = 0.023 for *E. faecalis*; multiple linear regression).

(D) Correlation between the predicted ARG density and actual ARG density in the test dataset based on the relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis*. (r = 7.335; R^2 = 0.5381, p < 0.0001; Pearson's correlation).

(E) Multiple linear regression of relative abundances of *E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus* to ARG density in samples in the levofloxacin dataset. (p < 0.0001 for *E. coli*, p < 0.0001 for *P. mirabilis*, p = 0.0394 for *E. faecalis*, p < 0.0001 for *S. haemolyticus*, p < 0.0001 for *P. stuartii*; multiple linear regression).

(F) Correlation between the predicted ARG density and actual ARG density in the test dataset based on the relative abundances of *E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus*. (r = 0.7901, R^2 = 0.6242, p < 0.0001; Pearson's correlation).

See Tables S3 and S5 for underlying taxonomic and ARG abundances. Outliers are labeled by sample and with the species that may be driving high ARG levels. KP, *K. pneumoniae*, PA, *P. aeruginosa*; PS, *P. stuartii*; SH, *S. haemolyticus*.

any significant differences. One potential reason for this finding is that oral levofloxacin is well absorbed by the host, with greater than 99% bioavailability (Noel, 2009; Fish and Chow, 1997; Bush et al., 2011; Chien et al., 1997; Croom and Goa, 2003), and therefore may not be directly available to the luminal microbiota of the lower gastrointestinal tract at high levels. Furthermore, other studies have suggested that levofloxacin has a relatively minor impact on the gut microbiome, primarily reducing levels of *Enterobacterales* (Inagaki et al., 1992; Bhalodi et al., 2019; Ziegler et al., 2019; Edlund and Nord, 1999, 2000; Edlund et al., 1997; Sullivan et al., 2001), and it may be less associated with *Clostridiodes difficile*-associated diarrhea outbreaks than other antimicrobials, including other fluoroquinolones (Deshpande et al., 2008).

Additionally, in this cohort, levofloxacin was typically administered at least 2 weeks prior to collected time points, potentially allowing sufficient time for the microbiome to recover from or shift away from its immediately post-antibiotic state. Furthermore, the impacts of levofloxacin on the gut microbiome may be dependent upon the initial state during administration. If the microbiome is initially relatively diverse and healthy, antimicrobial administration may be disruptive and allow blooms of atypical dominant species such as members of *Proteobacteria*; such an occurrence might be observed in subject F, where a diverse *Bacteroides*-dominated microbiome was overtaken by several *Enterobacterales* after levofloxacin treatment (Figure S2G). Alternatively, if the microbiome is initially dominated by one or more pathogens, antimicrobial administration may correct such blooms and allow for the restoration of a diverse community, as might have occurred in subject E where a *P. stuartii* bloom was eliminated (Figure S2F).

Finally, since the pre-existing temporal instability of this community was high, levofloxacin-related changes may not be detectable through the noise of this cohort's general microbiome instability, especially in the context of the relatively low sample size. In contrast to our observations, studies in healthy adults have generally found that the within-subjects dissimilarity is much lower than between-subjects dissimilarity, in line with the fact that the gut microbiome tends to be relatively stable within the same subject over time, including in an elderly cohort (Claesson et al., 2011, Human Microbiome Project Consortium, 2012; Liskiewicz et al., 2019; Lloyd-Price et al., 2016; Mehta et al., 2018). This suggests that the gut microbiomes of the subjects in this study were potentially less stable than that of other cohorts; this may indicate that this institutionalized population with advanced functional impairment is more prone to infections or pathobiont blooms, possibly due to weaker immune systems less able to control them, than young healthy adults or even community-resident elderly adults. One consideration is that this study utilized rectal swabs rather than fecal samples. Although swabs are often used as a proxy for the colonic microbiota, they may also be more sensitive to the timing of sample collection and harbor higher levels of skin commensal or aerotolerant organisms; these factors could contribute to the taxonomic instability and high levels of facultative anaerobes and organisms not specialized for gut residence found in this study (Araujo-Perez et al., 2012; Bassis et al., 2017; Biehl et al., 2019; Budding et al., 2014; Fair et al., 2019; Jones et al., 2018). This high noise level may be an important consideration for future studies of patients with advanced dementia, as more subjects than expected may be required to detect relevant patterns if there are similar levels of noise. Interestingly, despite the taxonomic variability, the functional composition of the cohort was relatively similar across samples and subjects. This is in line with previous studies of the human gut microbiome, which suggest that variable taxa can fill the same functional niches, resulting in a more similar functional composition across individuals despite inter-individual differences in the taxonomic composition (Human Microbiome Project Consortium, 2012; Qin et al., 2010; Turnbaugh et al., 2009).

As all of the subjects had been given an antimicrobial, we were particularly interested in the antimicrobial resistance gene profile of the subjects before and after levofloxacin administration. However, as observed in the taxonomic and functional data, there was no apparent association of any resistance genes or classes with either pre- or post-levofloxacin status. This may be because levofloxacin did not have any specific impacts on the resistome of this group or due to the factors that may have concealed any impacts of levofloxacin, as discussed above. However, we were particularly intrigued by the finding that ARG density in a particular sample could be linked to the abundance of a few key species. *E. coli*, *P. mirabilis*, and *E. faecalis* are all pathobionts that are often found at low levels in a healthy microbiome but bloomed frequently at various time points across a majority of our subjects. All three species can cause severe illness, have been previously observed to colonize nursing home residents, and include well-known multidrug-resistant strains (Agudelo Higuita and Huycke, 2014; Archambaud et al., 2019; Butto et al., 2015; Chow et al., 2011; D'Agata et al., 2015; Davies et al., 2016; Dzutsev and Trinchieri, 2015; Hamilton et al., 2018; Mirsepasi-Lauridsen et al., 2019; Mitchell et al., 2014; O'Fallon et al., 2009, 2010; Paterson, 2006; Pop-Vicas et

al., 2008; Snyder et al., 2011; Yang et al., 2019). In fact, three of the subjects (B, C, and D) are known to have acquired multidrug-resistant strains of *E. coli* and *P. mirabilis* during the study. However, we observed an association between these three species and ARG levels across the entire sample set (Figure 4B), and the ARG composition of the bins of *E. coli* and *P. mirabilis* from samples where MDROs were detected were not distinct from their other bins (Figures 5A and 5B). This suggests that metagenomic sequencing may allow the identification of antimicrobial-resistant organisms that escape detection via culture-based techniques, although it is also possible that the multidrug-resistant isolates contained ARG-carrying plasmids that were not captured by our assembly and binning strategy.

A major implication of this finding is that metagenomic analysis could be a particularly useful tool to track antimicrobial resistance in institutions like nursing homes and hospitals, particularly with the capability to construct contigs and bins that allow examination of specific genomes. In this case, it has allowed us to connect the high levels of ARGs in certain samples with correspondingly high levels of specific pathobionts, which had high proportions of ARGs within their genomes even in samples where MDROs were not detected. In a vulnerable population already prone to infections and carriage of MDROs, metagenomics could be a useful surveillance tool to assess the prevalence or transmission of ARGs in long-term care facilities.

Importantly, all of the subjects in our study were institutionalized in nursing homes, and there exists significant potential for transfer of bacteria between patients. As all but two of our subjects (C and G) lived in different homes, we could not directly examine this possibility ourselves, but it is possible that the high abundance of pathobionts and/or ARGs in our cohort is related to the spread of isolates within nursing homes. In fact, transmission of isolates from other nursing home residents who were treated with antimicrobials could potentially contribute to the high levels of AMR bacteria observed in our samples, even in the absence of direct antimicrobial selection in our subjects. This also raises the possibility that we would not find a similar association between pathobionts and ARG levels in a healthy or community-based elderly cohort, who might be less likely to harbor or transmit such high levels of these bacteria. However, if an association between particular "sentinel" species and ARGs holds true in other elderly institutionalized populations, qPCR detection of the loads of these pathobionts may allow for prediction of resistant bacterial outbreaks before they occur.

In addition to the increased potential for spread of resistant strains through institutions, there are some other potential explanations for the association between ARGs and these species. In particular, all of the species that we found to be associated with ARG density are potential human pathogens, can be grown *in vitro*, and have been previously associated with AMR phenotypes. ARGs, as well as mobile genetic elements carrying them, from these species may be better-studied than those from organisms less likely to pose a threat to human health, including gut commensals. If ARGs from these organisms are well-represented in databases, it could potentially bias analyses based on these databases toward detecting pathogen-associated over commensal-associated ARGs. However, given the high frequency of AMR isolate carriage in the population under study, a potential bias toward clinically-relevant ARGs and pathobionts may not be as significant an issue in this context. Regardless, it should be noted that there has been significant work done on the resistome of the human commensal microbiome, including functional metagenomics, to detect new ARGs. These have found that commensal anaerobes may serve as significant reservoirs of ARGs and may in some cases contribute to the transfer of resistance to pathobionts (van Schaik, 2015; Francino, 2015; Penders et al., 2013; Hu et al., 2013; Jackson et al., 2011; Scott, 2002; Salyers et al., 2004; Kazimierczak and Scott, 2007). Commensal carriage of antimicrobial resistance genes may correspond to the baseline level of 0.3%–0.4% ARGs observed in samples without pathobiont dominance.

In general, the gut microbiome was highly variable both between and within subjects, with frequent blooms and reductions of bacterial species both before and after levofloxacin treatment. We did not observe a consistent impact of levofloxacin on specific taxa or functions, levels of antimicrobial resistance genes, or overall microbiome diversity in these subjects. However, although we could not link levofloxacin to antimicrobial resistance gene levels, there were a number of samples that had higher relative abundances of these genes. In our original metagenomics dataset, we were able to identify that levels of these genes could be linked to blooms of specific bacterial species, including *E. coli*, *P. mirabilis*, and *E. faecalis*. We were able to build a model to describe the relationship between total ARG levels and the relative abundances of these species in a sample and confirm the validity of this model in a larger metagenomics dataset

from the rest of the SPREAD study, including subjects taking a range of antimicrobials. Furthermore, use of metagenomic assembly and binning allowed us to confirm that our species of interest carry greater ARG densities than other abundant members of the microbiome, even in subjects where MDROs were not detected by culturing.

This work demonstrates that there is a significant amount of information that can be obtained from metagenomic assembly and binning. With sufficient depth, powerful computational tools allow whole genomes to be assembled from short-read metagenomic sequencing to interrogate the functional potential of specific species in complex microbial communities. In our case, we were able to confirm the association between pathobiont blooms and ARG levels in the gut, showing that the genomes of pathobionts contained a greater proportion of ARGs than gut commensals such as *Bacteroides* and *Bifidobacterium* species. This suggests that, although the commensal microbiota are known to serve as reservoirs of antimicrobial resistance, in this population blooms of pathobionts may serve as the driver of ARG levels in the gut microbiome. Given how frequently these blooms occurred, special attention should be paid to these species in patients with dementia in long-term care facilities, a vulnerable group that is often immunocompromised, frequently administered medication including antimicrobials, and may carry MDROs at relatively high levels.

Limitations of the Study

Some limitations to the findings of this study must be acknowledged. First, as for all database-based methodologies, we are limited by accuracy and completeness of those databases. Although the human gut microbiome is fairly well characterized, there may be so-called microbial dark matter that is not well-represented in the taxonomic database used for species identification. We also used a database composed of bacterial and archaeal genomes, excluding consideration of bacteriophage and microbial eukaryotes from our analyses. As mentioned, database representation is particularly relevant for our ARG analysis, as the genes in this database may be skewed toward easily culturable and pathogenic source species, and our analysis may have missed ARGs found in commensal or unculturable gut species. Additionally, critics have noted that some genes found in ARG databases used have unclear links to resistance phenotypes and may perform regulatory, efflux, or other functions not always related to antimicrobial resistance (van Schaik, 2015; Martinez et al., 2015).

Second, we were limited by the original SPREAD population, in which few subjects received only a single antimicrobial during the course of the study, thereby limiting the sample size of our investigation; this makes it difficult to say whether the temporal variability we observed was widespread in the cohort, although the fact that there were frequently high pathobiont levels observed in the larger, cross-sectional metagenomics dataset we used to test our regression suggests that this may be the case. Third, in this study we worked with rectal swabs, which are similar but not identical to fecal samples and may be susceptible to cross-contamination from non-gut-resident bacteria including urinary pathogens or skin flora, particularly in patients with advanced dementia who may suffer from incontinence; furthermore, rectal swabs may be more sensitive to the timing of sample collection and may harbor more oxygen-tolerant taxa than fecal samples, potentially contributing to some of the observed blooms and instability (Araujo-Perez et al., 2012; Bassis et al., 2017; Biehl et al., 2019; Budding et al., 2014; Fair et al., 2019; Jones et al., 2018). Relatedly, as blank controls were not included at the time of DNA extraction and sequencing, potential contaminants cannot be ruled out. Fourth, metagenomic assembly has limitations. It cannot create bins of all species found in a given sample, genome reconstruction is based on the isolates present in the database used, and analysis of assembled genomes may exclude consideration of plasmids, which are often sources of ARGs. Finally, as we analyzed metagenomic data, we cannot comment on the actual antimicrobial resistance phenotypes of the communities or individual bacteria that we studied.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

The accession number for the shotgun metagenomics and 16S rRNA sequencing reads for the longitudinal, levofloxacin-treated dataset reported in this paper is NCBI Short Read Archive:PRJNA573963. The accession number for the shotgun metagenomics sequencing reads for the cross-sectional test dataset reported

in this paper is NCBI Short Read Archive:PRJNA531921. All code implemented for analysis can be found in Data S1.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100905.

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AUTHOR CONTRIBUTIONS

A.D.R.-N. conceptualized the project, performed analysis, generated figures, and wrote the manuscript. R.A. collected the data and contributed to manuscript preparation. E.M.C.D. collected the data and contributed to manuscript preparation. P.B. conceptualized the project and wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

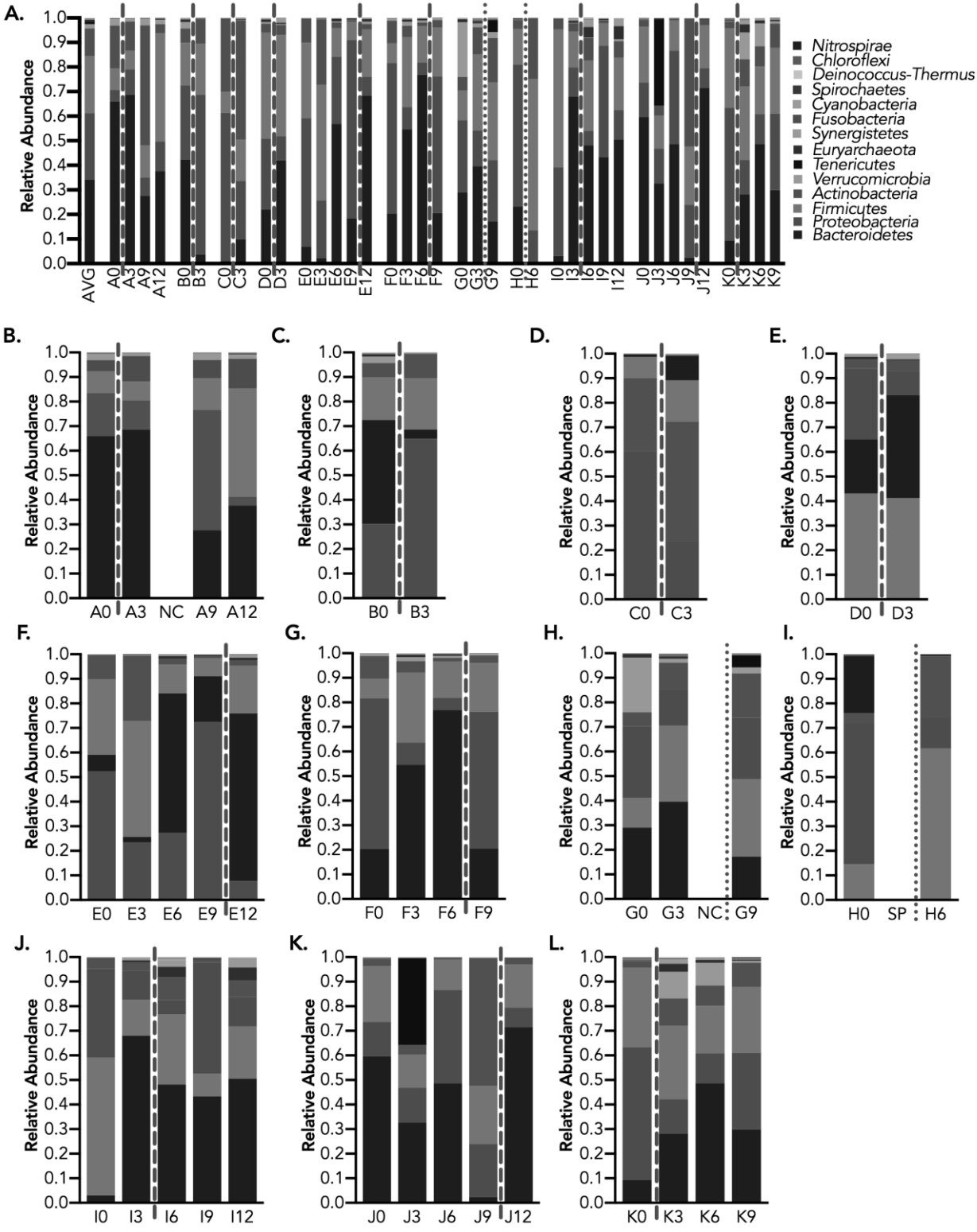
Antimicrobial Resistance Gene Prevalence in a Population of Patients with Advanced Dementia Is Related to Specific Pathobionts

Aislinn D. Rowan-Nash, Rafael Araos, Erika M.C. D'Agata, and Peter Belenky

SUPPLEMENTAL FIGURES

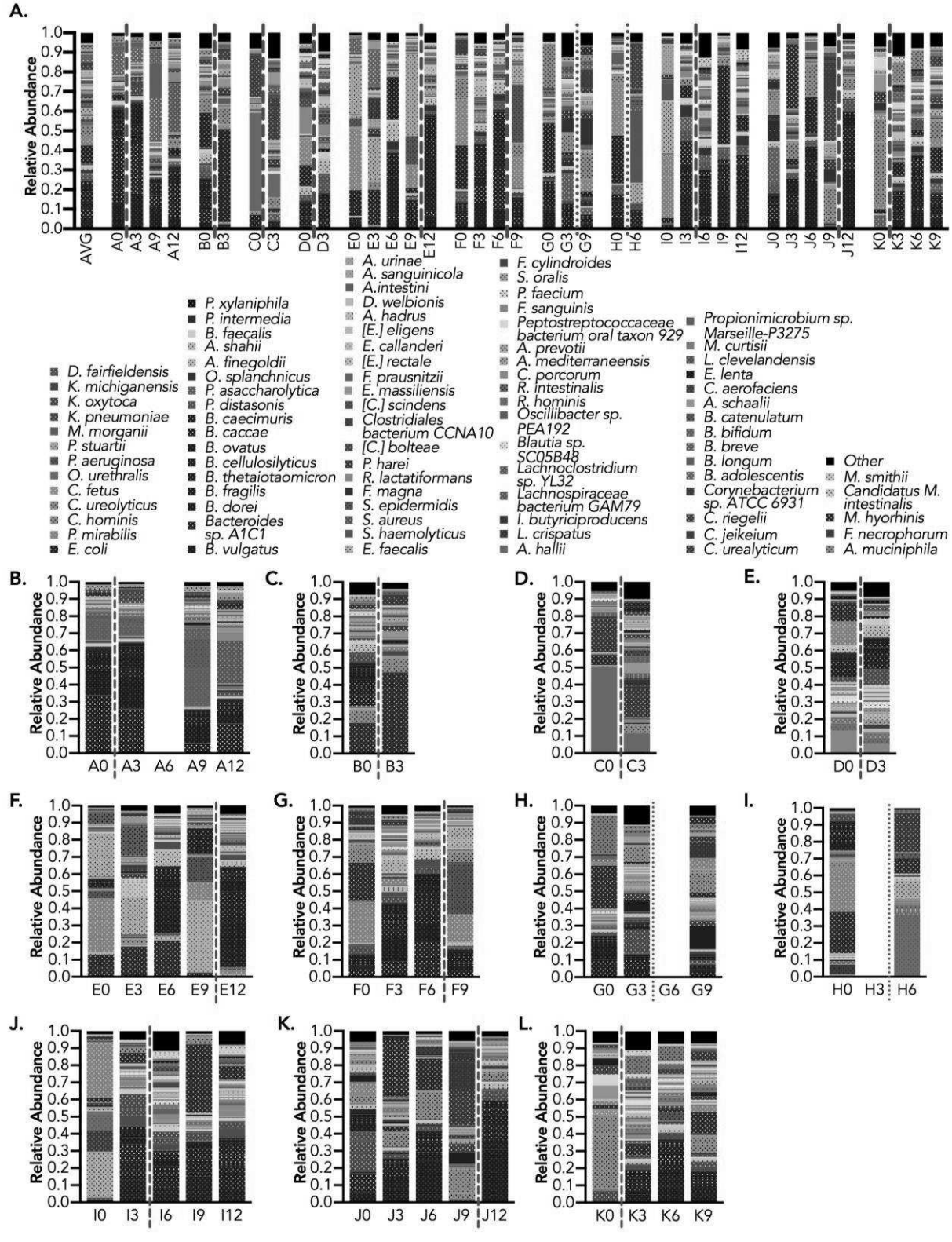
Supplemental Figure 1: Relative Abundances of Phyla Across and Within Subjects, Related to Figure 2

(A) Relative abundance of phyla in all samples, ranked by average relative abundance across all samples. (B-L) Relative abundances of phyla by subject, ranked by average within each subject. See Supplemental Table 3 for underlying taxonomic abundances. Red lines indicate levofloxacin administration; dashed lines indicate usage between consecutive timepoints, while dotted lines indicate usage where the immediately post-levofloxacin sample is missing. See Supplemental Table 3 for underlying taxonomic abundances.



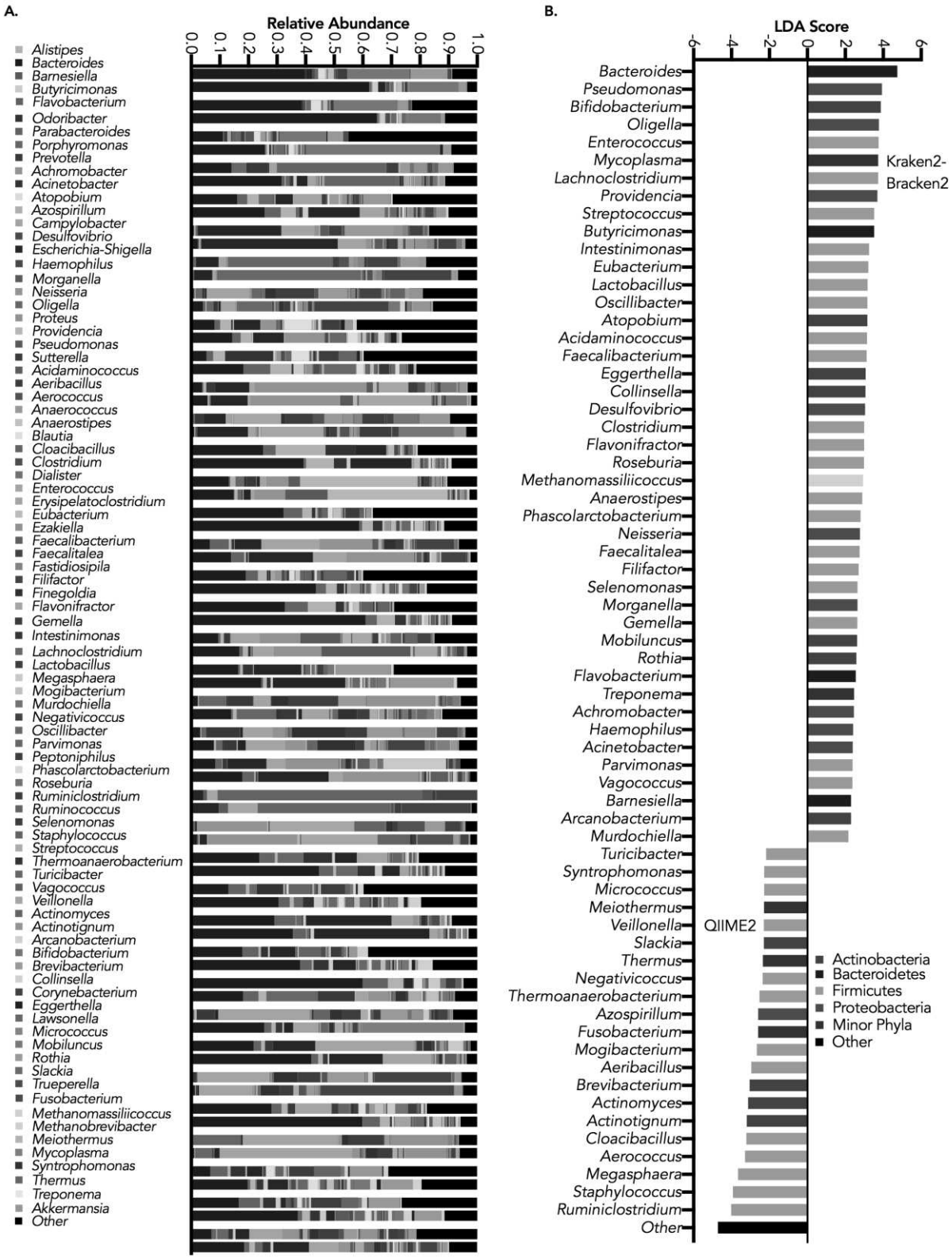
Supplemental Figure 2: Relative Abundances of Species Across and Within Subjects, Related to Figure 2

(A) Relative abundance of species in all samples, grouped by genus and phylum and ranked within those levels by average relative abundance across all samples. (B-L) Relative abundances of phyla by subject, grouped by genus and phylum ranked within those levels by average within each subject. Coloring is the same as in Figure 2A. See Supplemental Table 3 for underlying taxonomic abundances. Red lines indicate levofloxacin administration; dashed lines indicate usage between consecutive timepoints, while dotted lines indicate usage where the immediately post-levofloxacin sample is missing. See Supplemental Table 3 for underlying taxonomic abundances.



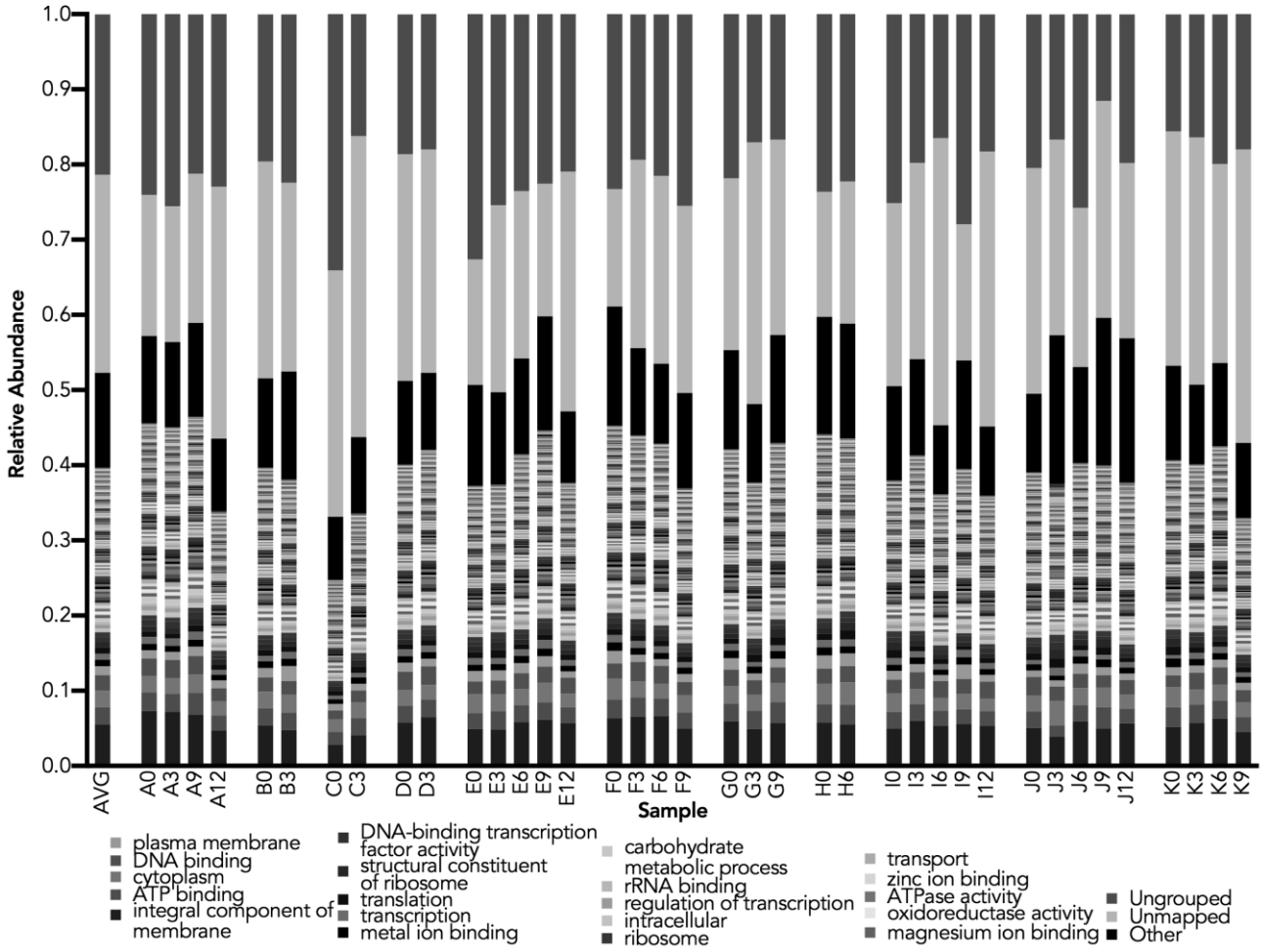
Supplemental Figure 3: Comparison of Genus-level Classifications by Metagenomics and 16S rRNA Analysis, Related to Figure 2

(A) Relative abundances of genera called by both QIIME2 (16S rRNA) and Kraken2/Bracken2 (metagenomics), where pairs of stacked bars indicate the same sample as measured by both methods. The upper bar indicates QIIME2 and the lower bar indicates Kraken2/Bracken2. (B) Genera called by LEfSe as associated with either QIIME2 or Kraken2/Bracken2. Each genus is colored according to its source phylum. See Supplemental Table 3 for underlying taxonomic abundances.



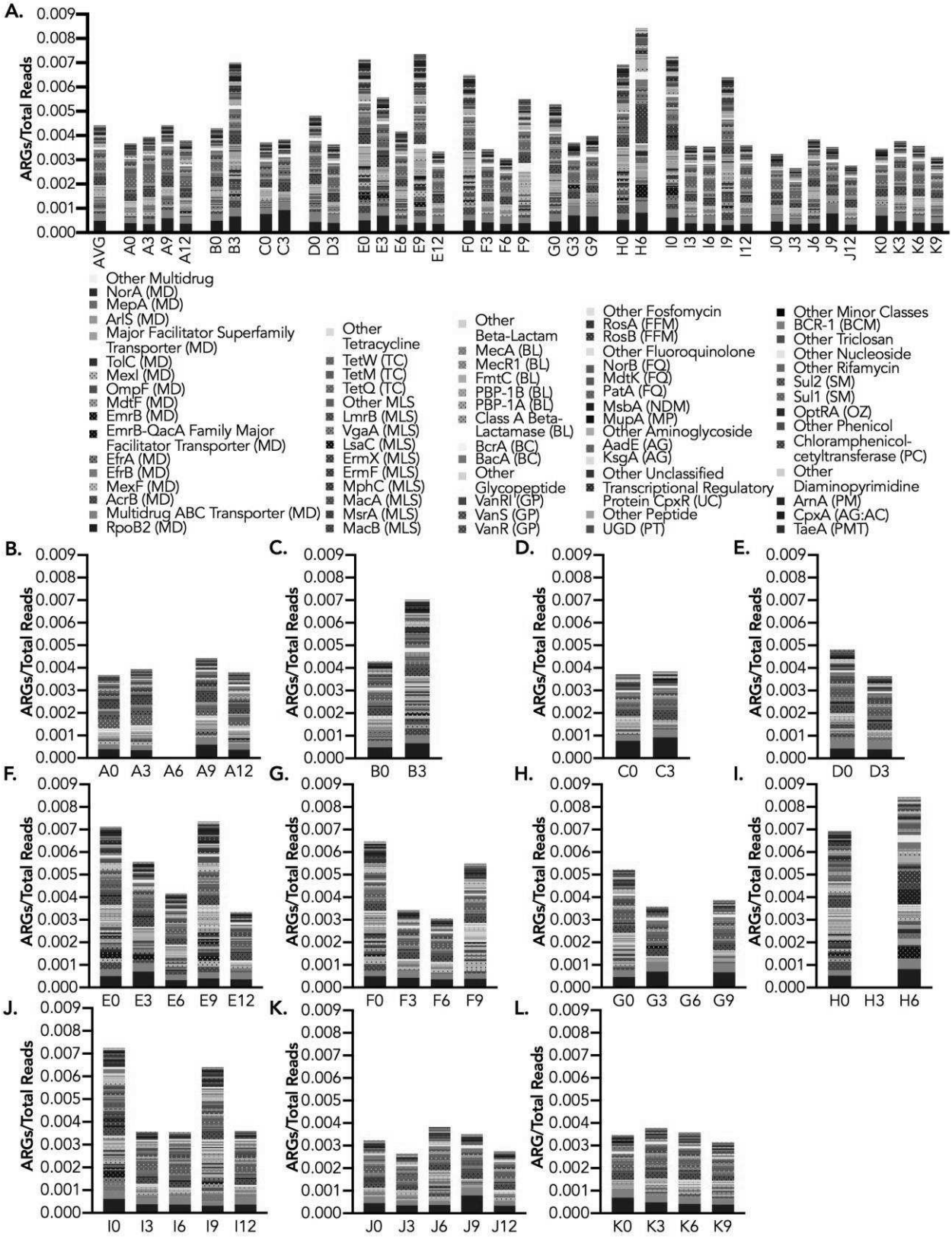
Supplemental Figure 4: Relative Abundance of Gene Ontology Terms Across All Samples, Related to Figure 1

(A) Relative abundances of the top 250 most-abundant GO terms, representing broad functional categories, across all samples. A significant proportion are "unmapped" or "ungrouped", as not all UniRef90 gene families can be mapped to a GO term. See Supplemental Table 4 for underlying metagenomic abundances.



Supplemental Figure 5: Relative Abundance of Resistance Genes Within and Across Subjects, Related to Figure 2

(A) Relative abundance of ARGs in all samples, grouped and ranked within ARG class by average relative abundance (ARGs/total reads) across all samples. (B-L) Relative abundances of ARGs by subject, grouped and ranked within class by average relative abundance within each subject. Coloring is the same as in Figure 2B. See Supplemental Table 5 for underlying ARG abundances.



SUPPLEMENTAL TABLES

(Supplemental Tables 2-6 are provided elsewhere as Microsoft Excel files)

Supplemental Table 1: Metadata on levofloxacin subjects from SPREAD, Related to Figures 1-5

This table lists the age, biological sex, and race of all subjects (F = female, M= male, W = white, NW = non-white), whether a multidrug-resistant organism (MDRO) was detected in the subject at any timepoint, the duration of levofloxacin administration, and the reason for which they were administered levofloxacin. For MDROs, the specific organism detected and the antimicrobial agents it was found to be resistant to are also listed. This table also lists all samples from the levofloxacin cohort that were collected, sequenced, or analyzed for each subject, where T0-T12 indicates the timepoint. Samples that were successfully analyzed are marked with a "yes", while samples that could not be collected, sequenced, or analyzed are marked with a "no". For samples that were not analyzed, a reason is also provided according to the following key: SD = subject deceased at this timepoint, NC = sample was not collected, NS = sample was not sequenced, SP = sample sequenced poorly.

Subject	Sex	Age	Race	MDRO Detected	Levofloxacin Duration	Reason for Levofloxacin Administration	T0	T3	T6	T9	T12
A	F	94	W	Yes (*S. aureus*; methicillin)	7 days	Urinary tract infection	Yes	Yes	No (NS)	Yes	Yes
B	F	101	W	Yes (*E. coli*; ampicillin/sulbactam, cefazolin, ceftazidime, ceftriaxone, ciprofloxacin)	6 days	Upper respiratory tract infection	Yes	Yes	No (SD)	No (SD)	No (SD)
C	F	88	W	Yes (*P. mirabilis*; ampicillin/sulbactam, ciprofloxacin, gentamicin)	7 days	Urinary tract infection	Yes	Yes	No (SD)	No (SD)	No (SD)
D	F	74	W	Yes (*P. mirabilis*; ampicillin/sulbactam, ciprofloxacin, gentamicin)	10 days	Upper respiratory tract infection	Yes	Yes	No (SD)	No (SD)	No (SD)
E	F	78	W	No	7 days	Upper respiratory tract infection	Yes	Yes	Yes	Yes	Yes
F	F	101	W	No	10 days	Upper respiratory tract infection	Yes	Yes	Yes	Yes	No (NS)
G	F	83	NW	No	11 days	Fever of unknown source	Yes	Yes	No (NC)	Yes	No (SD)
H	F	87	W	No	10 days	Upper respiratory tract infection	Yes	No (SP)	Yes	No (SD)	No (SD)
I	M	89	W	No	8 days	Upper respiratory tract infection	Yes	Yes	Yes	Yes	Yes
J	F	86	W	No	7 days	Fever of unknown source	Yes	Yes	Yes	Yes	Yes
K	F	91	W	No	6 days	Upper respiratory tract infection	Yes	Yes	Yes	Yes	No (SD)

Supplemental Table 7: Metadata for Test Dataset Samples, Related to Figure 5

This table lists the sample names used in this study, the SPREAD IDs, and the BioProject PRJNA531921 sample names for the shotgun metagenomics sequencing files of the 67-sample dataset used to test the multiple linear regression developed from the levofloxacin dataset. It also includes demographic information about the subjects from whom the samples originated, including biological sex, age, and race.

Sample ID	SPREAD ID	BioSample ID	Sex	Age	Race
1_A2	02/007/3/6/R	S02_007_3_6_R	F	79	W
1_A8	02/021/6/8/R	S02_021_6_8_R	F	90	W
1_A11	02/023/3/6/R	S02_023_3_6_R	M	86	W
1_B3	02/032/B/7/R	S02_032_B_7_R	F	88	W
1_B5	02/041/3/7/R	S02_041_3_7_R	F	80	W
1_B11	04/003/6/5/R	S04_003_6_5_R	F	72	W
1_C6	04/011/12/5/R	S04_011_12_5_R	F	85	NW
1_D2	04/059/6/9/R	S04_059_6_9_R	M	80	W
1_D8	06/007/9/6/R	S06_007_9_6_R	F	84	W
1_D12	06/027/6/6/R	S06_027_6_6_R	F	82	W
1_E9	06/048/9/5/R	S06_048_9_5_R	F	73	W
1_F1	06/060/6/5/R	S06_060_6_5_R	F	84	W
1_F8	06/068/6/5/R	S06_068_6_5_R	F	88	W
1_G3	06/083/9/9/R	S06_083_9_9_R	F	85	W
1_G9	06/085/12/9/R	S06_085_12_9_R	F	70	W
1_G10	06/102/B/9/R	S06_102_B_9_R	F	94	W
1_H3	06/107/B/9/R	S06_107_B_9_R	F	70	W
1_H6	06/108/3/9/R	S06_108_3_9_R	F	88	W
2_A2	07/020/3/7/R	S07_020_3_7_R	F	88	W
2_A6	07/056/3/7/R	S07_056_3_7_R	F	105	NW
2_B2	07/059/6/7/R	S07_059_6_7_R	F	93	NW
2_B4	09/018/B/6/R	S09_018_B_6_R	F	89	W
2_B11	09/048/9/5/R	S09_048_9_5_R	F	94	W
2_C1	09/085/3/5/R	S09_085_3_5_R	F	86	W
2_C2	09/086/B/5/R	S09_086_B_5_R	F	93	W
2_C5	09/099/9/9/R	S09_099_9_9_R	M	81	W
2_C9	09/138/9/9/R	S09_138_9_9_R	M	84	W
2_C12	09/143/9/9/R	S09_143_9_9_R	F	94	W
2_D2	09/153/3/9/R	S09_153_3_9_R	M	81	W
2_D6	09/187/3/9/R	S09_187_3_9_R	F	92	W
2_D10	09/192/6/9/R	S09_192_6_9_R	F	90	W
2_E1	09/214/6/9/R	S09_214_6_9_R	F	89	NW
2_E5	10/010/6/6/R	S10_010_6_6_R	F	91	W
2_F1	10/012/6/6/R	S10_012_6_6_R	M	68	NW
2_F11	13/030/3/6/R	S13_030_3_6_R	F	95	W
2_G6	13/035/12/7/R	S13_035_12_7_R	F	89	W
2_G9	13/080/6/7/R	S13_080_6_7_R	F	84	W
2_H2	19/009/6/5/R	S19_009_6_5_R	F	85	W
2_H4	19/031/B/5/R	S19_031_B_5_R	F	84	W
2_H10	21/012/12/7/R	S21_012_12_7_R	F	81	W
3_A7	21/037/6/7/R	S21_037_6_7_R	F	91	W
3_A12	21/060/12/7/R	S21_060_12_7_R	F	91	W
3_B4	23/025/9/9/R	S23_025_9_9_R	F	100	W
3_B8	26/031/6/5/R	S26_031_6_5_R	F	89	W
3_B11	26/038/3/9/R	S26_038_3_9_R	F	88	W
3_C2	29/013/6/9/R	S29_013_6_9_R	M	84	W
3_C4	31/039/B/7/R	S31_039_B_7_R	F	87	W
3_C8	32/019/6/5/R	S32_019_6_5_R	F	93	W
3_D2	32/022/12/5/R	S32_022_12_5_R	F	78	W
3_D8	32/052/9/9/R	S32_052_9_9_R	F	77	W

3_D10	34/009/B/2/R	S34_009_B_2_R	M	83	W
3_E4	35/010/B/5/R	S35_010_B_5_R	F	90	W
3_E6	35/031/B/9/R	S35_031_B_9_R	F	83	W
3_E8	36/007/B/7/R	S36_007_B_7_R	F	83	NW
3_F4	38/001/3/5/R	S38_001_3_5_R	F	93	W
3_F10	38/017/B/5/R	S38_017_B_5_R	F	87	W
3_G3	38/024/9/9/R	S38_024_9_9_R	F	88	W
3_G11	39/011/6/7/R	S39_011_6_7_R	F	95	W
3_H6	40/038/9/9/R	S40_038_9_9_R	F	79	W
4_A3	40/044/6/9/R	S40_044_6_9_R	F	91	W
4_A12	42/002/9/7R/2	S42_002_9_7R_2	F	89	W
4_B4	42/014/6/7/R	S42_014_6_7_R	F	97	W
4_B7	42/015/3/7/R	S42_015_3_7_R	F	68	W

TRANSPARENT METHODS

Sample Collection and Preparation

Ethics Approval and Consent to Participate:

Written information about SPREAD was mailed to the healthcare proxies of all eligible subjects. Proxies were then telephoned two weeks later to solicit participation and verbally obtain informed consent for the participation of themselves and the subjects. Approval for SPREAD, including the consent procedures, was obtained from the Institutional Review Board committee at Hebrew SeniorLife.

Subjects:

Eleven subjects were chosen from the SPREAD cohort based on the following inclusion criteria: at least two consecutive rectal swabs were collected from the subject during the study, subjects had received a single oral course of levofloxacin during the study (average course of 8 days), and subjects received no other antimicrobials during the study or in the 3 months prior to the first sample collection. Of the 11 subjects, 10 were female and 10 were white, while ages ranged from 72 to 101. Five subjects lived through the entire sample collection period, while the other six passed away at some point prior to the final collection; between this attrition, one sample that was not collected, and three samples that were not well-sequenced, we had a total of 38 usable metagenomic samples (Figure 1A; Supplemental Tables 1-2). All samples were collected under SPREAD, which was approved by the Institutional Review Board at Hebrew Life(Mitchell et al., 2013).

Sample Collection:

Samples were collected by insertion of sterile double-tipped swabs (Starswab II; Starplex Scientific Inc., Ontario, Canada) into the anus of the subject. The first swab was used to identify multidrug-resistant organisms (including methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and multidrug-resistant Gram-negative organisms such as *E. coli*, *P. mirabilis*, *P. aeruginosa*, or *P. stuartii*) via culturing techniques as described previously(Snyder and D'Agata, 2012). The second swab was frozen in 20% glycerol at -80°C for DNA extraction and sequencing.

Sample Processing:

Frozen rectal swabs were thawed and placed into 96-well plates, before extraction using the PowerSoil DNA Isolation Kit (MOBIO, West Carlsbad, CA) according to the manufacturer's instructions. DNA concentrations were measured using a Nanodrop 1000 (Thermo Scientific, Waltham, MA) and extracted DNA was stored at -20°C until further use.

16S rRNA Amplicon Sequencing

Sequencing:

The V4 hypervariable region of the 16S rRNA gene was amplified according to Earth Microbiome Project protocols. Amplification was performed using Illumina-adapted universal 16S primers 515F and 806R under the following conditions: 3 minutes at 94°C, 45 cycles of [45 seconds at 94°C, 60 seconds at 50°C, 90 seconds at 72°C], 10 minutes at 72°C. All reactions were prepared using 5 PRIME polymerase 1X HotMasterMix (5PRIME, Gaithersburg, MD) and run in triplicate to alleviate primer bias. Triplicates were pooled before cleaning with a PCR Purification Kit (Qiagen). These products were quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, Eugene, OR) and samples were pooled in equimolar amounts. Sequencing was performed using the Illumina MiSeq platform located at the New York University Langone Medical Center Genome Technology Core. Sequences can be found under the BioProject accession number PRJNA573963 (http://www.ncbi.nlm.nih.gov/bioproject/573963).

Data Processing:

Data processing was performed using the QIIME2 (v 2019.1) pipeline(Caporaso et al., 2010). The Divisive Amplicon Denoising Algorithm 2 (DADA2) method was used to quality-filter sequences and categorize amplicon sequence variants (ASVs)(Callahan et al., 2016), and the SILVA (release 132) 99% identity V4 classifier was used to assign taxonomy to ASVs(Quast et al., 2013). See Data S1 for more information. Taxonomic relative abundances were exported at the genus level for further analysis. Output data can be found in Supplemental Table 3.

Shotgun Sequencing

Sample Preparation and Sequencing:

Extracted DNA (2 ng DNA in 50 uL buffer) was sheared to 450bp using a Covaris LE220 system. Library preparation was performed using a Biomek FXP Automated Liquid Handling Workstation (Beckman Coulter) with the KAPA HyperPrep Kit (Roche), with 12 cycles of PCR. Final libraries were normalized and pooled, with 20 samples per poor. Each pool was run on 2 lanes of an Illumina HiSeq 4000 using the paired-end 2x150bp protocol. Library preparation and sequencing was

performed at the New York University Langone Medical Center Genome Technology Core. Sequences can be found under the BioProject accession number PRJNA573963 (http://www.ncbi.nlm.nih.gov/bioproject/573963) for the levofloxacin dataset and under the BioProject accession number PRJNA531921 (https://www.ncbi.nlm.nih.gov/bioproject/531921) for the test dataset.

Data Processing:

Raw shotgun sequencing reads were processed using Kneaddata (v0.6.1) to remove contaminating human sequences from the dataset(McIver et al., 2018). Briefly, the *kneaddata* function was used with the Bowtie2 *Homo sapiens* database (v0.1)(Langmead et al., 2009) to remove contaminating host reads from the sequencing files. See Data S1 for more information. Initial and knead-processed read counts can be found in Supplemental Table 2.

Taxonomic Classification:

Kraken2, a taxonomic classifier that maps shotgun sequencing k-mers to genomic databases, was used to assign taxonomy to kneaddata-processed shotgun sequencing reads(Wood and Salzberg, 2014). Briefly, the *kraken2-build* function was used to create a custom database containing the "bacteria" and "archaea" from NCBI libraries, and the *kraken2* function was used to run the kneaddata-filtered shotgun sequencing reads against this database and assign taxonomy. Samples where fewer than 500,000 read pairs were assigned were not further analyzed. The proportion of reads assigned by Kraken2 can be found in Supplemental Table 3. While Kraken2 does not estimate species abundances, Bracken2 (Bayesian Reestimation of Abundance with KrakEN) uses the taxonomy assigned by Kraken2 to estimate the number of reads per sample that originate from individual species(Lu, 2017). The Kraken2 database was used to create a Bracken-compatible database using the *bracken-build* function, and the Kraken2 report files for each sample were run against the Bracken database using the *bracken* function for the phylum, genus, and species levels. Phylum- and species-level relative abundance outputs were formatted for biomarker discovery using LEfSe. The *kraken-biom* function was used to convert the Bracken report files into a biom file for import into R. Output data can be found in Supplemental Table 4. Relative abundance plots were generated in GraphPad Prism v8.

Taxonomic Diversity Analysis:

Alpha and beta diversity analyses were performed using the phyloseq (v1.27.2)(McMurdie and Holmes, 2013, McMurdie and Holmes, 2012) and vegan (v2.5-4)(Dixon, 2009) packages in R (v3.4.3). Briefly, the biom file was imported into a phyloseq object. The phyloseq *estimate_richness* function was used to obtain Shannon's Diversity Index values for all samples, while the vegan *phyloseq::distance* and *ordinate* functions were used to generate a Bray-Curtis matrix and PCoA values. See Data S1 for more information. Data was exported as csv files for formatting, and plotting was performed in GraphPad Prism v8.

Gene and Pathways Analysis:

The Human Microbiome Project Unified Metabolic Analysis Network 2 (HUMAnN2) pipeline was used to profile the presence and abundance of genetic pathways in our samples(Franzosa et al., 2018). Briefly, the *humann2* function was used with the kneaddata-filtered metagenomic sequences to estimate genes and MetaCyc pathways present in the samples based on the UniRef90 database, files were joined using the *humann2_join_tables* function and the full tables were de-leveled using the *humann2_split_stratified_table* function. The unstratified gene-level abundances were converted to both GO terms and KEGG orthologs using the *humann2_regroup_table* function, and the *humann2_renorm_table* function was used to normalize the MetaCyc pathway, GO term, and KEGG ortholog tables by computing relative abundance. These relative abundance tables were formatted for biomarker discovery with LEfSe. Additionally, the, and LEfSe was also used to analyze pre- and post-treated samples using both outputs. See Data S1 for more information. Output data can be found in Supplemental Table 4. Plots were generated in GraphPad Prism 8.

Resistome Analysis:

The ARG content of the samples was analyzed using DeepARG-SS, a deep learning model that can predict ARGs from short-read metagenomic data(Arango-Argoty et al., 2018). We first analyzed the data using the *deeparg* function with the *-reads* flag. The mapped ARGs output was then imported into R, where it was processed to obtain tables of the ARGs detected per sample at both the specific gene and antibiotic class levels. The ARGs detected were normalized to the number of reads per sample.

Additionally, after metagenomic assembly and binning was performed (see below), individual bins were analyzed using DeepARG-LS, a deep learning model optimized to predict ARGS from gene-level input. The *DNA_features* output from selected bins was analyzed using the *deeparg* function with the *-genes* flag to analyze whether the levels or identity of ARGs could be linked to specific species of interest. The ARGs detected were normalized to the number of features per bin. All output data can be found in Supplemental Table 5. Plots and regression models were generated in GraphPad Prism 8.

Metagenomic Assembly and Binning:

To further examine the ARGs present in the samples, kneaddata-filtered reads were uploaded to the web-based Pathosystems Resource Integration Center (PATRIC)(Wattam et al., 2017). Reads were assembled into contigs using the *auto* option of the Assembly service, which provides both raw output contigs from specific assembly algorithms and contigs of the "best" assembly as judged by the in-house PATRIC script ARAST. We ran the assembly using two different inputs: reads that had been processed by *kneaddata* as pairs, which has the advantage of utilizing mate-pairing information for longer total reads, and reads that had been processed by *kneaddata* after pairs were concatenated into a single file, which has the advantage of keeping reads whose mates failed trimming.

Both the raw SPAdes assembly algorithm contigs(Nurk et al., 2017) and the best assembly contigs were then processed using the Metagenomics Binning service, which assigns contigs to specific organisms and annotates the bin's genome. Quality measures were used to define bins as either "good", "acceptable", or "bad", and only "good" or "acceptable" bins were used moving forward. "Good" bins had to have a coarse consistency, fine consistency, and completeness of at least 87%, less than 10% contamination, and a single copy of the PheS gene. "Acceptable" bins had to meet at least 3 of these criteria, as long as coarse consistency, fine consistency, and completeness were at least 80% and contamination was 20% or less. Any bins that had coarse consistency, fine consistency, or completeness of less than 80%, contamination of greater than 20%, or had 3 or more criteria at the "acceptable" level were considered "bad".

When more than one binning strategy (paired assembly or single assembly, SPAdes contigs or best contigs) called a particular bin as "good" or "acceptable", quality measures from the four strategies were compared and the highest-quality bin for a given species of interest was chosen for ARG analysis. Finally, only bins of species present at 0.1% or greater relative abundance in the corresponding sample were selected for further analysis. A list of bins used, their source, and quality measures can be found in Supplemental Table 6.

Taxonomic Biomarker Analysis:

LEfSe was used to identify potential biomarkers distinguishing levofloxacin-treated samples(Segata et al., 2011). In all cases (taxonomic abundances, MetaCyc pathways, KEGG orthologs, GO terms, ARGs), data was formatted into csv files and uploaded to the Galaxy webserver. LEfSe was run under default parameters for biomarker detection, comparing either all pre-levofloxacin to all post-levofloxacin or immediately pre-levofloxacin to immediately post-levofloxacin. LEfSe was also used to compare genus-level taxonomic abundance outputs from Kraken2/Bracken2 and QIIME2, again under default parameters.

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