Antimicrobial resistance gene levels are independent of levofloxacin administration but related to abundance of specific pathobionts in dementia patients

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16 **ABSTRACT**

17 Background: The issue of antimicrobial resistance continues to grow worldwide, and long-term
18 care facilities are significant reservoirs of antimicrobial-resistant organisms, in part due to high
19 frequency of antimicrobial use. Patients with advanced dementia are particularly vulnerable to
20 multidrug-resistant organism acquisition and antimicrobial overuse, which has negative
21 consequences for the gut microbiome and can contribute to the selection and propagation of
22 antimicrobial resistance genes. In this study, we examined the impacts of the fluoroquinolone
23 antimicrobial levofloxacin on the gut microbiome of advanced dementia patients and the
24 correlation between abundance of pathogens and antimicrobial resistance genes.

25 Results: We did not find significant impacts of levofloxacin on the diversity, composition,
26 function, or resistome of the gut microbiota of this population. In fact, we observed significant
27 inter- and intra-subject heterogeneity in the composition of the microbiota, suggesting temporal
28 instability. However, we were able to link the antimicrobial resistance gene burden in a sample
29 with the relative abundance of several pathobionts – particularly *Escherichia coli*, *Proteus*
30 *mirabilis*, and *Enterococcus faecalis*, as well as less-prevalent species including *Providencia*
31 *stuartii* and *Staphylococcus haemolyticus*. Furthermore, we used metagenomic assembly and
32 binning to demonstrate that these species had higher genomic resistance gene levels than common
33 gut commensals, and we were able to predict antimicrobial resistance gene burden from the relative
34 abundances of these species in a separate, larger cohort from the same population.

35 Conclusions: We observe that levofloxacin did not have an impact on the microbiota, potentially
36 due to high host absorption rate of this antimicrobial and high intra-subject temporal variability.
37 However, we found that the relative abundances of several pathobionts were correlated with and
38 were even predictive of the level of antimicrobial resistance genes in corresponding samples, and

that these species carried high levels of resistances genes in their assembled genomes. Given the high frequency with which these species were found at high levels in this population and the underlying vulnerability to infection with multidrug resistant organisms of advanced dementia patients, attention to microbial blooms of these species may be warranted.

Keywords: antimicrobial resistance, pathobiont, dementia, microbiome, metagenome assembly

46 **BACKGROUND:**

47 It is well-recognized that there is a growing threat of antimicrobial-resistant (AMR)
48 bacterial strains that threaten the health and lives of millions worldwide. In the United States alone,
49 the Centers for Disease Control and Prevention estimates that at least 2 million people get an AMR
50 infection each year, and at least 23,000 die as a result[1]. A number of factors have driven the rise
51 in AMR bacteria worldwide, including overprescription of antibiotics in the healthcare setting,
52 over-the-counter access to antibiotics in some countries, and widespread use of antibiotics in
53 animal husbandry for non-veterinary purposes[2-4]. Concerningly, hospitals and other medical
54 institutions are frequent sites of AMR bacteria acquisition, where patients may already be ill or
55 immunocompromised, antimicrobial use is common, and patient-to-patient transmission of AMR
56 isolates can occur via inadequate hygiene or environmental contamination[5-8]. For example,
57 AMR bacteria are highly prevalent in nursing homes, with estimates that over 35% of nursing
58 home residents are colonized with multidrug resistant organisms (MDROs)[9-14]. This is
59 particularly problematic in light of the fact that elderly patients in long-term care facilities may be
60 frequently hospitalized, potentially serving as a mode of bidirectional transport of MDROs
61 between healthcare facilities[15-17]. They are also prone to infections and are frequently treated
62 with antimicrobials[18-20], which has long been associated with acquisition of MDROs and may
63 not always be indicated[12, 17, 20-28].

64 The problem of MDRO prevalence and inappropriate antimicrobial use is of particular
65 relevance in elderly subjects with advanced dementia, a population which receives extensive
66 antimicrobial treatment which becomes more frequent closer to death, calling its benefit and
67 effectiveness into question[28, 29]. Accordingly, advanced dementia specifically has been shown
68 to be a risk factor of MDRO colonization[13, 30]. To examine this issue, the Study of Pathogen

69 Resistance and Exposure to Antimicrobials in Dementia (SPREAD) was undertaken from 2009-
70 2012 in order to analyze MDRO acquisition and appropriateness of antimicrobial prescription in
71 elderly adults with advanced dementia residing in nursing homes[31]. Supporting the widespread
72 nature of MDRO carriage in this population, analysis of SPREAD subjects revealed that there were
73 significant baseline levels and new acquisitions of MDROs, and that there was notable spread of
74 MDRO strains within and even between nursing home facilities[28, 32].

75 In addition to potential facilitation of MDRO acquisition or spread, antimicrobial overuse
76 may also have negative impacts on the diversity, composition, or function of the gut microbiota,
77 which may already be vulnerable in elderly populations. Healthy younger adults tend to have a
78 fecal microbiome characterized by relatively high diversity of species and populated primarily by
79 members of the phyla *Bacteroidetes* and *Firmicutes*, largely obligate anaerobes which exist in
80 homeostasis with the intestinal epithelium[33-37]. However, it has been found that during
81 senescence, the gut tends to have higher levels of *Bacteroidetes* and *Proteobacteria* and harbors
82 higher levels of facultative aerobes and potential pathobionts, including *Enterobacterales* such as
83 *E. coli* [36, 38-45]. These changes become more pronounced as aging progresses, and several
84 studies have indicated that age-related alterations to the gut microbiota are relatively minor in
85 septuagenarians, but become more pronounced over time and are clear in centenarians and
86 supercentenarians[39, 43, 46-48]. This is likely due to a number of factors, including the decline
87 of immune function, onset of age-related diseases (including metabolic disorders), changes to diet
88 and mobility, and the increased likelihood of medication utilization and/or hospitalization[42, 49].
89 However, lifestyle of elderly adults has an important impact, as research suggests that community-
90 resident elderly subjects have a distinct and more diverse microbiome compared with those of their
91 hospitalized or institutionalized peers, which was suggested to be at least in part due to nutritional

92 differences[49, 50]. Furthermore, reduced microbiome diversity has been associated with
93 increased frailty of elderly subjects[49, 51]. Accordingly, given that the microbiomes of
94 institutionalized elderly patients are perhaps already at risk, understanding the impacts of
95 antimicrobial use and MDRO acquisition on this population is of importance.

96 We analyzed the gut microbiomes of eleven subjects from SPREAD to examine the impact
97 of antimicrobial use on the gut microbiota composition, function, and antimicrobial resistance gene
98 (ARG) profile of elderly dementia patients. These subjects were chosen as they were the largest
99 cohort who had received a single antimicrobial (levofloxacin) during the collection period, and we
100 anticipated that this intervention could have an impact on the already-vulnerable microbiota of this
101 elderly, institutionalized cohort. Levofloxacin is an antimicrobial of the fluoroquinolone class with
102 high oral bioavailability[52-54] which has been found to reduce levels of Gram-negative aerobic
103 bacteria – including *Proteobacteria* and particularly *Enterobacterales* – in the fecal microbiota[55-
104 61], although fluoroquinolone resistance among this taxon has been spreading[62-69]. A
105 maximum of five rectal swab samples, collected every three months, were taken from each subject,
106 and both 16S rRNA amplicon and shotgun metagenomics sequencing were performed. We
107 analyzed alpha and beta diversity, taxonomic composition, functional potential, and antimicrobial
108 resistance gene profiles before and after administration of levofloxacin. Interestingly, we did not
109 find a specific impact of levofloxacin on any of these measures. However, we did find an
110 association between blooms of particular enteric species and ARG burden, including in samples
111 where MDRO were not detected by culture, suggesting that certain pathobionts carrying high ARG
112 burdens may frequently colonize this population and that metagenomics may allow detection of
113 resistant bacteria not flagged by culture-based methods.
114

115 **RESULTS**

116 ***Overview of Subjects***

117 Elderly patients in long-term care facilities, and particularly patients with advanced
118 dementia, are frequently exposed to antimicrobials and are at high-risk of acquisition and carriage
119 of MDRO[9-13, 18-20, 27-30, 32]. We were interested in whether levofloxacin, one of the most
120 commonly prescribed antimicrobials, had impacts on the gut microbiota and resistome of these
121 subjects. We analyzed up to five rectal swabs, taken every three months over the course of a year,
122 from eleven subjects in the SPREAD cohort[31], using both 16S rRNA and shotgun metagenomics
123 sequencing (Figure 1A). During their participation in the study, these subjects had received only
124 levofloxacin (average course of eight days), which has previously been shown to decrease the
125 proportion of the *Enterobacterales* order of *Proteobacteria*[55-61]. Of the eleven subjects, all but
126 Subject I were female and all but Subject G were white. They ranged in age from 72 to 101, and
127 six members of the cohort did not survive for the full year of the study (Additional Table 1). All
128 but two subjects (C and G) resided in different nursing homes. Overall, there were 38 samples for
129 metagenomics sequencing (Additional Table 2). Culture-based methods indicated that four of the
130 eleven subjects acquired a MDRO during the study: Subject A acquired methicillin-resistant *S.*
131 *aureus* (MRSA) at the 12-month timepoint, Subject B acquired multidrug-resistant *E. coli* at the
132 3-month timepoint, and Subjects C and D both acquired multidrug-resistant *P. mirabilis* at the 3-
133 month timepoint (Additional Table 1).

134 ***Alpha and Beta Diversity Metrics***

135 We first used the metagenomic sequencing data to compare the alpha diversity, or the
136 diversity within each sample, of samples collected before and after levofloxacin administration.
137 According to Shannon's Diversity Index, which incorporates both richness and evenness of

138 samples, there was no significant difference between the pre- and post-levofloxacin samples
139 (Figure 1B). Furthermore, the alpha diversity was variable over time even within the same subject,
140 and there was no clear trend of recovery in alpha diversity after antibiotic cessation. This suggests
141 a degree of temporal instability, in which the richness and/or evenness of the samples varies
142 changes over time.

143 We then examined beta diversity, or the diversity between samples. We utilized the Bray-
144 Curtis Dissimilarity metric, which considers the identity and abundance of taxa shared between
145 samples. Plotting this metric in a principal coordinate analysis (PCoA) revealed no apparent
146 pattern of clustering based on either subject or sample collection point relative to levofloxacin, and
147 in fact, samples from the same subject were often located quite distantly from one another (Figure
148 1C). We then compared the within-subjects dissimilarity of sequential samples within a subject
149 when both were pre-levofloxacin, both were post-levofloxacin, or one sample was pre- and one
150 was post-levofloxacin; there was no significant difference between any of the groups (Figure 1D),
151 suggesting that levofloxacin was not associated with community disruption. Furthermore, while
152 within-subject dissimilarity was lower than between-subjects dissimilarity, the effect size was low
153 (0.7013 vs. 0.7712, respectively; Figure 1E).

154 ***Taxonomic Composition***

155 We utilized Kraken2 in conjunction the with Bayesian Reestimation of Abundance with
156 KrakEN2 (Bracken2) pipeline to assign taxonomy to our metagenomic sequencing samples[70,
157 71]. Corresponding to the high between-subjects beta-diversity, the taxonomic composition of the
158 gut microbiome varied significantly between subjects. As is typical for the human gut microbiome,
159 most bacteria belonged to the five major phyla of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*,
160 *Actinobacteria*, and *Verrucomicrobia*. However, consistent with the high within-subjects beta

161 diversity, the dominant phyla varied greatly even between samples from the same subject
162 (Additional Figure 1); for example, the most abundant phylum in Subject E was *Bacteroidetes* at
163 two timepoints, *Proteobacteria* at two timepoints, and *Firmicutes* at one (Additional Figure 1F).
164 Overall, the most abundant phylum was *Actinobacteria* in three samples, *Bacteroidetes* in
165 seventeen samples, *Firmicutes* in seven samples, and *Proteobacteria* in eleven samples
166 (Additional Figure 1A-L); averaging across all samples, *Bacteroidetes* was highest at 34.2%,
167 followed by *Proteobacteria* (26.9%), *Firmicutes* (23.3%), and *Actinobacteria* (11.2%) (Additional
168 Figure 1A). Qualitatively, many of the samples from this population represent highly divergent
169 and dysbiotic microbiomes compared with what is typically seen with younger subjects, in which
170 *Proteobacteria* in particular make up a much smaller proportion of the microbiome than in these
171 elderly dementia subjects[33].

172 The genus- and species-level taxonomic composition was also variable. Blooms of
173 potential pathogens[72], including *Campylobacter ureolyticus*[73], *Corynebacterium*
174 *urealyticum*[74], *Enterococcus faecalis*[75, 76], *Escherichia coli* [77, 78], *Oligella urethralis*[79-
175 82], *Proteus mirabilis*[83, 84], *Providencia stuartii*[85, 86], *Pseudomonas aeruginosa*[87, 88],
176 *Staphylococcus aureus*[89-91], and *Staphylococcus haemolyticus*[92-94], were fairly common,
177 both before and after levofloxacin administration (Figure 2A, Additional Figure 2). Across
178 subjects, even baseline samples varied in composition, as expected from beta-diversity analysis.
179 Averaging across all samples, the single most-abundant species was *E. coli*, further supporting the
180 qualitatively dysbiotic nature of the gut microbiome of this cohort (Figure 2A). Despite the high
181 proportion of members of *Enterobacterales* in this cohort, Linear Discriminant Analysis Effect
182 Size (LEfSe) analysis[95] did not reveal biomarkers for pre- or post-levofloxacin samples at the

183 phylum, genus, or species level. Full data on taxonomic composition at the phylum and species
184 levels can be found in Additional Data 1.

185 As we had access to full 16S rRNA and shotgun metagenomics data for our samples, we
186 compared their taxonomic identifications at the genus level. The two methods of analysis were
187 generally consistent, and blooms of prominent genera (including *Escherichia, Proteus,*
188 *Enterococcus, Providencia, Staphylococcus*, and *Bacteroides*) were generally detected by both
189 analysis pipelines (Additional Figure 3A). Metagenomics analysis was unsurprisingly able to
190 detect more distinct genera, and of the genera that were called by both pipelines, LEfSe analysis
191 revealed biases in both methods. For example, metagenomics analysis by Kraken2 and Bracken2
192 detected higher levels of *Bacteroides*, while 16S rRNA analysis with Quantitative Insights Into
193 Microbial Ecology 2 (QIIME2)[96] detected higher levels of *Ruminiclostridium*. Full data on
194 taxonomic abundances at the genus level can be found in Additional Data 1 for metagenomics and
195 Additional Data 2 for 16S rRNA.

196 ***Functional Potential***

197 We used the Human Microbiome Project Unified Metabolic Analysis Network 2
198 (HUMAnN2) pipeline[97] to analyze the genetic content of the metagenomic samples. We utilized
199 LEfSe to compare community function at the Kyoto Encyclopedia of Genes and Genomes (KEGG)
200 ortholog, Gene Ontology (GO) term, and MetaCyc pathway levels. As in the taxonomic analysis,
201 there were no significant biomarkers of either pre-or post-levofloxacin administration samples.
202 However, while the taxonomic profile of the samples varied greatly, the functional capacity of the
203 samples was fairly consistent across samples (Additional Figure 4). Full data on functional
204 potential can be found in Additional Data 3.

205 *Antimicrobial Resistance Gene Profile*

206 We used the DeepARG machine-learning program[98] to detect resistance genes in the
207 metagenomic samples. Across all samples, the most abundant class of ARG was "multidrug",
208 followed by "macrolide-lincosamide-streptogramin" (MLS), and "tetracycline". The most
209 common specific gene detected was the multidrug resistance *rpoB2* variant of the RNA polymerase
210 beta subunit, followed by the MLS resistance gene *macB* and a multidrug ABC transporter (Figure
211 2B). LEfSe analysis revealed no ARG biomarkers of either pre- or post-levofloxacin samples. Full
212 data on ARG composition can be found in Additional Data 4.

213 However, we were able to detect changes in specific ARG classes and genes that
214 corresponded with the detection of antimicrobial-resistant organisms in two subjects. Subject A
215 acquired MRSA at the 12-month timepoint, and a bloom of this species to 25.0% could be detected
216 in the metagenomic taxonomic data (Figure 3A, Additional Figure 2B). While the overall level of
217 ARGs did not notably increase at this timepoint, there was a clear expansion in beta-lactam
218 resistance genes (Figures 3B, Additional Figure 5B), including the *mecA*/*mecR1*/*mecI* operon,
219 which regulates expression of the low-affinity penicillin-binding protein *mecA* (PBP-2A)[99-102]
220 (Figure 3C). This operon is characteristic of MRSA strains[99-102], supporting the culture-based
221 classification of this *S. aureus* isolate as MRSA.

222 Similarly, Subject B acquired multidrug-resistant *E. coli* (resistant to the beta-lactams
223 ampicillin/sulbactam, cefazolin, ceftazidime, and ceftriaxone and to the fluoroquinolone
224 ciprofloxacin) at the three-month timepoint, and the proportion of this species expanded to 47.3%
225 of the population in the corresponding sample (Figure 3D, Additional Figure 2C). Accordingly,
226 this sample showed a notable increase in the relative abundance of ARGs, which was in large part
227 driven by an increase in a number of multidrug resistance genes (Figure 3E); there was also a clear
228 increase in several beta-lactam resistance genes, including the low-affinity penicillin-binding

229 protein genes *PBP-1A, PBP-1B,* and *penA* (*PBP2*) as well as class C beta-lactamase genes[103-
230 108], and several fluoroquinolone resistance genes, including the transporters *patA* and *mdtK* [109-
231 113] (Figure 3F-G).

232 Despite the acquisition of multidrug-resistant *P. mirabilis* at the three-month timepoint in
233 Subjects C and D, there was no corresponding increase in ARGs. ARG levels stayed approximately
234 the same in Subject C (0.372% at baseline and 0.384% at three months) and decreased in Subject
235 D from 0.482% at baseline to 0.364% at the three-month timepoint (Figures 2B, Additional Figure
236 5D-E). However, this corresponds to the taxonomic data; levels of *P. mirabilis* were low and stable
237 in Subject C (0.55% at baseline and 0.61% three months later), and while *P. mirabilis* made up
238 13.8% of the population at baseline in Subject D, it underwent a reduction to 2.3% at the three-
239 month timepoint (Figures 2A, Additional Figure 2D-E). Taken together, this data indicates that
240 our metagenomics pipeline can detect blooms of AMR pathogens and that the corresponding
241 change in ARG levels aligns with culture-based detection of MDROs. At the same time,
242 metagenomic analysis of some samples found blooms of pathogens and ARGs that were not
243 associated with culture-based MDRO detection.

244 ***Attribution of ARG Density to Specific Species***

245 While total ARG density within samples did not vary by levofloxacin administration, there
246 was significant variability between samples. In fact, most samples had similar baseline levels of
247 ARGs of 0.3% to 0.4% of the total reads, while only a few samples rose above this value to between
248 0.6% and 0.8%. Close inspection of the taxonomic composition of the samples revealed that
249 samples with higher levels of ARGs tended to have blooms of one or more of the *Proteobacteria*
250 species *E. coli* and *P. mirabilis* and the *Firmicutes* species *E. faecalis*, strains of which are common
251 pathobionts[114-120] (Figure 4A). Confirming this association, correlation analysis between ARG

252 levels and the sum of the relative abundances of these three species showed a strong and significant
253 positive correlation ($r = 0.791$, $R^2 = 0.6254$, $p < 0.0001$, Pearson's correlation; Figure 4B). This
254 suggests that in samples with higher-than-baseline ARG levels, ARG abundance is being driven
255 by high relative abundance of these three species.

256 However, there were two notable exceptions: Samples E9 and H6 had high levels of ARGs
257 without corresponding blooms of these three species. However, *P. stuartii* bloomed to 41.9%
258 relative abundance in Sample E9 and *S. haemolyticus* bloomed to 36.9% in Sample H6 (Figure
259 2A, Additional Figure 2F&I). Both species have long been associated with AMR phenotypes[93,
260 94, 121-127] and were not found at high levels in other samples, but could explain the higher ARG
261 abundance in these samples (Figure 4A). Supporting this possibility, an examination of the ARGs
262 in Sample H6 showed a distinct profile relative to other samples, with high levels of staphylococcal
263 resistance genes including fluoroquinolone resistance gene *norB* and macrolide-streptogramin
264 resistance gene *msrA* [128-131](Figures 2B, 3I-L). Accordingly, addition of *P. stuartii* and *S.*
265 *haemolyticus* abundances to the analysis resulted in a stronger correlation ($r = 0.933$, $R^2 = 0.8706$,
266 $p < 0.0001$, Pearson's correlation; Figure 4C).

267 To more rigorously examine the relationship between the species of interest and ARG
268 levels, we performed metagenomic assembly and binning to compare the levels of ARGs in these
269 organisms to levels in other common and abundant species, including likely commensals and
270 potential pathogens (Figure 4D). Specifically, we analyzed bins that passed various quality
271 controls (Additional Table 3) and corresponded to species identified by Kraken2/Bracken2 to
272 make up greater than 0.1% of their source samples (Additional Table 4).

273 As anticipated, we found that the levels of ARGs in bins from *E. coli* and *P. mirabilis* were
274 consistently high compared to other species analyzed. In fact, *E. coli* had the highest average ARG

275 density of any species analyzed, while *P. mirabilis* was the fifth-highest. Notably, the ARG
276 composition of the bins of these species from samples in which MDROs were detected (B3, C3,
277 and D3) did not appear to be different from those of other samples (Additional Figure 6A-B),
278 although it is possible that some resistance genes were carried on plasmids that were not assembled
279 into genomes. *P. stuartii* had the second-highest average ARG density, reflecting the expansion of
280 ARGs detected in sample E9, where this species bloomed to 41.9% of the population. The third
281 and fourth positions were taken by the single bins constructed for *Klebsiella oxytoca* and
282 *Morganella morganii*, other *Proteobacteria* with pathogenic potential[132-134]. *P. aeruginosa*
283 bins rounded out the top six, with similar levels to the other top species. However, as *K. oxytoca*
284 and *M. morganii* were never present at greater than 3% and *P. aeruginosa* bloomed in only two
285 samples, they did not significantly contribute to overall ARG density in the cohort. Importantly,
286 high ARG density was not a universal feature of *Proteobacteria*, or even of pathogenic
287 *Proteobacteria*; bins constructed for the *Campylobacter* species *C. hominis* and *C. ureolyticus* had
288 universally low ARG levels. Additionally, while we could not construct a high-quality bin for *O.*
289 *urethralis*, the low ARG densities in the samples in which this species bloomed (C0 and C3)
290 suggests that it also has low genomic ARG content. This suggests that high ARG density among
291 the *Proteobacteria* analyzed was restricted to the *Gammaproteobacteria* class, primarily of the
292 order *Enterobacterales* but also including *Pseudomonadales*.

293 We were only able to construct two good-quality bins for *E. faecalis*, which varied in their
294 ARG levels, particularly on the basis of bacitracin resistance. On average, while the two bins did
295 not have ARG levels as high as the *Proteobacteria* of interest, they did rank among the highest of
296 the *Firmicutes* bins tested. We were also able to create a single bin for *S. haemolyticus* from
297 Sample H6 in which it made up 36.9% of the population. This bin had an ARG density higher than

298 the average for any other non-*Proteobacteria* species, supporting its role in the high ARG levels
299 found in the corresponding sample. As expected from the analysis of the total ARG population of
300 that sample (Figure 3G), the staphylococcal resistance genes *norB* and *msrA* were found in this
301 bin. We were also able to create two bins for *S. aureus*, including from sample A12 where MRSA
302 was detected. The A12 bin contained the characteristic MRSA gene *mecA* while the H6 bin did
303 not, suggesting that the *S. aureus* strain found in H6 was not MRSA (Figure S6C). In general, bins
304 from the phyla *Actinobacteria* (including *Bifidobacterium* and *Corynebacterium* species) and
305 *Bacteroidetes* (including *Bacteroides* and *Parabacteroides* species) had low ARG levels. Full data
306 on the ARGs and classes found in species-level bins can be found in Additional Data 4.

307 ***Prediction of ARG Density from Species Abundances***

308 Our initial analysis only considered the eleven subjects for whom we had longitudinal
309 metagenomics data due to their receiving levofloxacin. We also had access to a larger dataset:
310 shotgun metagenomics had been performed on a further 67 samples for a related study. In this
311 case, the data was not longitudinal and encompassed an array of antibiotic treatment conditions
312 across 67 subjects, providing a diverse set of taxonomic and ARG data on which to test whether
313 the relationship between *E. coli*, *P. mirabilis*, and *E. faecalis* and ARG density held true. As an
314 initial test, we performed the same correlation analyses between species of interest and ARG levels
315 as on the levofloxacin dataset, finding that both the simple and complex models showed significant
316 correlation ($r = 0.7179$, $r^2 = 0.5154$, $p < 0.0001$ and $r = 0.7627$, $r2 = 0.5817$, $p<0.0001$, respectively;
317 Pearson's correlation; Figure 5A-B). This provided initial support for the trend being present in
318 the wider dataset.

319 We then created a multiple linear regression model to predict ARG density using the
320 relative abundances (RA) of the three main species of interest in the initial levofloxacin dataset,

321 with the following equation: (ARG density) = 0.003482 + 0.006221(*E. coli* RA) + 0.006248(*P.*
322 *mirabilis* RA) + 0.006920(*E. faecalis* RA) (Figure 5B). We then used this equation to predict the
323 ARG density in the larger metagenomics dataset and found that it was able to accurately predict
324 the true ARG level of those samples, with predicted and actual values correlating significantly (r
325 = 0.7139, r^2 = 0.5096, p<0.0001; Pearson's correlation; Figure 5C). As before, there were a few
326 notable outliers with higher ARG levels than predicted by the model; those three samples
327 contained high levels of *P. stuartii*, *P. aeruginosa*, or *Klebsiella pneumoniae* This maps well to
328 the fact that we observed high levels of ARGs in bins constructed from *P. stuartii*, *P. aeruginosa*,
329 and the related species *K. oxytoca* (Figure 4D).

330 We also created a multiple linear regression model that incorporated the relative
331 abundances of *P. stuartii* and *S. haemolyticus*, which caused outliers from the original species-
332 ARG correlation: (ARG density) = 0.003253 + 0.006715(*E. coli* RA) + 0.006748(*P. mirabilis* RA)
333 + 0.003461(*E. faecalis* RA) + 0.01123(*S. haemolyticus* RA) + 0.007569(*P. stuartii* RA) (Figure
334 5E). As before, we tested this equation against the larger dataset, and found that it slightly
335 increased the accuracy of the predictions; specifically, it eliminated the outlier which had high *P.*
336 *stuartii* levels and slightly improved the correlation between predicted and actual ARG levels (r =
337 0.7753, r^2 = 0.6012, p<0.0001; Pearson's correlation; Figure 5F). However, the simpler model is
338 more broadly applicable, as blooms of *P. stuartii* and *S. haemolyticus* are relatively uncommon.
339 Similarly, while *Klebsiella* spp. and *P. aeruginosa* may also contribute to high ARG density in
340 samples, they do not bloom as commonly in this cohort as the core predictive species of *E. coli*, *P.*
341 *mirabilis*, and *E. faecalis*.

342 These results indicate that in this population, levels of only a few key species could predict
343 the majority of ARG abundance beyond background levels. Both the core predictive species (*E.*

344 *coli*, *P. mirabilis*, *E. faecalis*) and others that are associated with high ARG levels in samples (*P.*
345 *stuartii*, *S. haemolyticus*, *P. aeruginosa*, *Klebsiella* spp.) are pathogens and/or pathobionts.
346 Monitoring levels of these species may be helpful in elderly, institutionalized populations, as these
347 patients may be vulnerable to developing or transmitting AMR infections from high-level carriage
348 of these species.
349

350 **DISCUSSION**

351 Overall, we found that the microbial composition of the gut microbiome of elderly patients
352 with advanced dementia was quite variable, both between subjects and over time within the same
353 subject. Even in the absence of antimicrobial treatment, there was notable fluctuation in the
354 abundance of a number of species, including pathobionts such as *E. coli*, *P. mirabilis*, and *E.*
355 *faecalis*. When comparing the taxonomic composition, functional potential, and resistome of pre-
356 and post-levofloxacin samples, we did not find any significant differences. One potential reason
357 for this finding is that oral levofloxacin is well-absorbed by the host, with greater than 99%
358 bioavailability[52, 53, 135-137], and therefore may not be directly available to the luminal
359 microbiota of the lower gastrointestinal tract at high levels. Furthermore, other studies have
360 suggested that levofloxacin has a relatively minor impact on the gut microbiome, primarily
361 reducing levels of *Enterobacterales*[55-61], and it may be less-associated with *Clostridiodes*
362 *difficile*-associated diarrhea outbreaks than other antimicrobials, including other
363 fluoroquinolones[138].

364 Additionally, in this cohort, levofloxacin was typically administered at least two weeks
365 prior to collected timepoints, potentially allowing sufficient time for the microbiome to recover
366 from or shift away from its immediately post-antibiotic state. Furthermore, the impacts of

367 levofloxacin on the gut microbiome may be dependent upon the initial state upon administration.
368 If the microbiome is initially relatively diverse and healthy, antibiotic administration may be
369 disruptive and allow blooms of atypical dominant species such as members of *Proteobacteria*;
370 such an occurrence might be observed in Subject F, where a diverse *Bacteroides*-dominated
371 microbiome was overtaken by several *Enterobacterales* after levofloxacin treatment (Additional
372 Figure 2G). Alternatively, if the microbiome is initially dominated by one or more pathogens,
373 antimicrobial administration may correct such blooms and allow for the restoration of a diverse
374 community, as might have occurred in Subject E as a *P. stuartii* bloom was eliminated (Additional
375 Figure 2F).

376 Finally, since the pre-existing temporal instability of this community was high,
377 levofloxacin-related changes may not be detectable through the noise of this cohort's general
378 microbiome instability. In contrast to our observations, studies in adults have generally found that
379 the within-subjects dissimilarity is much lower than between-subjects dissimilarity, in line with
380 the fact that the gut microbiome tends to be relatively stable within the same subject over time –
381 including in an elderly cohort[33, 34, 41, 139, 140]. This suggests that the gut microbiomes of the
382 subjects in this study were less stable than that of other cohorts, potentially suggesting that this
383 institutionalized population with advanced functional impairment is more prone to infections or
384 has weaker immune systems than young healthy adults or even community-resident elderly adults.
385 Interestingly, despite the taxonomic variability, the functional composition of the cohort was
386 relatively similar across samples and subjects. This is in line with previous studies of the human
387 gut microbiome, which suggest that variable taxa can fill the same functional niches, resulting in
388 a more similar functional composition across individuals despite inter-individual differences in the
389 taxonomic composition[33, 35, 141].

390 As all of the subjects had been given an antibiotic, we were particularly interested in the
391 antibiotic resistance profile of the subjects before and after levofloxacin administration. However,
392 as observed in the taxonomic and functional data, there was no apparent association of any ARG
393 genes or classes with either pre- or post-levofloxacin status. This may be due to the fact that
394 levofloxacin did not have any specific impacts on the resistome of this cohort, or due to the factors
395 that may have concealed any impacts of levofloxacin, as discussed above. However, we were
396 particularly intrigued by the finding that ARG density in a particular sample could be linked to the
397 abundance of a few key species. *E. coli*, *P. mirabilis*, and *E. faecalis* are all pathobionts that are
398 often found at low levels in a healthy microbiome, but bloomed frequently at various timepoints
399 across a majority of our subjects. All three species can cause severe illness, have been previously
400 observed to colonize nursing home residents, and include well-known multidrug-resistant
401 strains[11-13, 28, 30, 32, 68, 75, 114-120, 142]. In fact, three of the subjects (B, C, and D) are
402 known to have acquired multidrug-resistant strains of *E. coli* and *P. mirabilis* during the study.
403 However, we observed an association between these three species and ARG levels across the entire
404 sample set (Figure 4B), and the ARG composition of the bins of *E. coli* and *P. mirabilis* from
405 samples where MDROs were detected were not distinct from their other bins (Additional Figure
406 6A-B). This suggests that metagenomic sequencing may allow the identification of antimicrobial-
407 resistant organisms that escape detection via culture-based techniques, although it is also possible
408 that the multidrug-resistant isolates contained ARG-carrying plasmids that were not captured by
409 our assembly and binning strategy.

410 A major implication of this finding is that metagenomic analysis could be a particularly
411 useful tool to track antimicrobial resistance in institutions like nursing homes and hospitals,
412 particularly with the capability to construct contigs and bins that allow examination of specific

413 genomes. In this case, it has allowed us to connect the high levels of ARGs in certain samples with
414 correspondingly high levels of specific pathobionts, which had high proportions of ARGs within
415 their genomes even in samples where MDROs were not detected. In a vulnerable population
416 already prone to infections and carriage of MDROs, metagenomics could be a useful surveillance
417 tool to assess the prevalence or transmission of ARGs in long-term care facilities.

418 Importantly, all of the subjects in our study were institutionalized in nursing homes, and
419 there exists significant potential for transfer of bacteria between patients. As all but two of our
420 subjects (C and G) lived in different homes, we could not directly examine this possibility
421 ourselves, but it is possible that the high abundance of pathobionts and/or ARGs in our cohort is
422 related to the spread of isolates within nursing homes. This also raises the possibility that we would
423 not find a similar association between pathobionts and ARG levels in a healthy or community-
424 based elderly cohort, who might be less likely to harbor or transmit such high levels of these
425 bacteria. However, if an association between particular "sentinel" species and ARGs holds true in
426 other elderly institutionalized populations, qPCR detection of the loads of these such pathobionts
427 may allow for prediction of resistant bacterial outbreaks before they occur.

428 In addition to the increased potential for spread of resistant strains through institutions,
429 there are some other potential explanations for the association between ARGs and these particular
430 species. In particular, all of the species that we found to be associated with ARG density are
431 potential human pathogens, can be grown *in vitro*, and have been previously associated with AMR
432 phenotypes. ARGs, as well as mobile genetic elements carrying them, from these species may be
433 better-studied than those from organisms less likely to pose a threat to human health, including gut
434 commensals. If ARGs from these organisms are well-represented in databases, it could potentially
435 bias analyses based on these databases toward detecting pathogen- over commensal-associated

436 ARGs. However, there has been significant work done on the resistome of the human commensal
437 microbiome, including functional metagenomics to detect new ARGs. These have found that
438 commensal anaerobes may serve as significant reservoirs of ARGs, and may in some cases
439 contribute to the transfer of resistance to pathobionts[143-150]. Commensal carriage of
440 antimicrobial resistance genes may correspond to the baseline level of 0.3-0.4% ARGs observed
441 in samples without pathobiont dominance.

442 Some limitations to the findings of this study must be acknowledged. First, as for all
443 database-based methodologies, we are limited by accuracy and completeness of those databases.
444 While the human gut microbiome is fairly well-characterized, there may be so-called microbial
445 dark matter that is not well-represented in the taxonomic database used for species identification.
446 We also used a database composed of bacterial and archaeal genomes, excluding consideration of
447 bacteriophage and microbial eukaryotes from our analyses. As mentioned, database representation
448 is particularly relevant for our ARG analysis, as the genes in this database may be skewed towards
449 easily-culturable and pathogenic source species, and our analysis may have missed ARGs found
450 in commensal or unculturable gut species. Additionally, critics have noted that some genes found
451 in ARG databases used have unclear links to resistance phenotypes, and may perform regulatory,
452 efflux, or other functions not always related to antimicrobial resistance[143, 151].

453 Second, we were limited by the original SPREAD population, in which few subjects
454 received only a single antimicrobial during the course of the study; this makes it difficult to say
455 whether the temporal variability we observed was widespread in the cohort, although the fact that
456 there were frequently high pathobiont levels observed in the larger metagenomics dataset we used
457 to test our multiple linear regression suggests that this may be the case. Third, in this study we
458 worked with rectal swabs, which are similar but not identical to fecal samples and may be

459 susceptible to cross-contamination from urinary pathogens or skin flora, particularly in incontinent
460 advanced dementia patients[152-155]. Fourth, metagenomic assembly has limitations. It cannot
461 create bins of all species found in a given sample, genome reconstruction is based on the isolates
462 present in the database used, and analysis of assembled genomes may exclude consideration of
463 plasmids – which are often sources of ARGs. Finally, as we analyzed metagenomic data, we cannot
464 comment on the actual antimicrobial resistance phenotypes of the communities or individual
465 bacteria that we studied.
466
467 **CONCLUSIONS:**
468 The gut microbiome was highly variable both between and within subjects, with frequent
469 blooms and reductions of bacterial species both before and after levofloxacin treatment. We did
470 not find that levofloxacin had a consistent impact on specific taxa or functions, levels of
471 antimicrobial resistance genes, or overall microbiome diversity in these subjects. This may suggest
472 that administration of levofloxacin to this population does not have a detectable impact on their
473 microbiomes. However, while we could not link levofloxacin to antimicrobial resistance gene
474 levels, there were a number of samples that had higher relative abundances of these genes. In our
475 original metagenomics dataset, we were able to identify that levels of these genes could be linked
476 to blooms of specific bacterial species, including *E. coli*, *P. mirabilis*, and *E. faecalis*. We were
477 able to build a model to predict total ARG levels in a sample from the relative abundance of these
478 species, and confirm the validity of this model in a larger metagenomics dataset from the rest of
479 the SPREAD study, including subjects taking a range of antibiotics. Furthermore, use of
480 metagenomic assembly and binning allowed us to confirm that our species of interest carry greater

481 ARG densities than other abundant members of the microbiome, even in subjects where MDROs
482 were not detected by culturing.

483 This demonstrates that there is a significant amount of information that can be obtained
484 from metagenomic assembly and binning. With sufficient depth, powerful computational tools
485 allow whole genomes to be assembled from short-read metagenomic sequencing, which permits
486 interrogation of the likely features of species of interest in complex microbial communities. In our
487 case, we were able to confirm the association between pathobiont blooms and ARG levels in the
488 gut, showing that the genomes of pathobionts contained a greater proportion of ARGs than gut
489 commensals such as *Bacteroides* and *Bifidobacterium* species. This suggests that while the
490 commensal microbiota are known to serve as reservoirs of antimicrobial resistance, in this cohort
491 blooms of pathobionts may serve as the driver of ARG levels in the gut microbiome. Given how
492 frequently these blooms occurred, special attention should be paid to these species in dementia
493 patients in long-term care facilities, a vulnerable group which is often immunocompromised,
494 frequently administered medication including antimicrobials, and may carry MDRO at relatively
495 high levels.

496 **METHODS**

497 ***Sample Collection and Preparation***

498 Subjects:

499 Eleven subjects were chosen from the SPREAD cohort based on the following inclusion
500 criteria: at least two consecutive rectal swabs were collected from the subject during the study,
501 subjects had received a single oral course of levofloxacin during the study (average course of 8
502 days), and subjects received no other antimicrobials during the study or in the 3 months prior to
503 the first sample collection. Of the 11 subjects, 10 were female and 10 were white, while ages

504 ranged from 72 to 101. Five subjects lived through the entire sample collection period, while the
505 other six passed away at some point prior to the final collection; between this attrition, one sample
506 that was not collected, and three samples that were not well-sequenced, we had a total of 38 usable
507 metagenomic samples (Figure 1A; Additional Tables S1-2). All samples were collected under
508 SPREAD, which was approved by the Institutional Review Board at Hebrew Life[31].

509 Sample Collection:

510 Samples were collected by insertion of sterile double-tipped swabs (Starswab II; Starplex
511 Scientific Inc., Ontario, Canada) into the anus of the subject. The first swab was used to identify
512 multidrug-resistant organisms (including methicillin-resistant *S. aureus*, vancomycin-resistant
513 enterococci, and multidrug-resistant Gram-negative organisms such as *E. coli*, *P. mirabilis*, *P.*
514 *aeruginosa*, or *P. stuartii*) via culturing techniques as described previously[156]. The second swab
515 was frozen in 20% glycerol at -80°C for DNA extraction and sequencing.

516 Sample Processing:

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

521 ***16S rRNA Amplicon Sequencing***

522 Sequencing:

523 The V4 hypervariable region of the 16S rRNA gene was amplified according to Earth
524 Microbiome Project protocols. Amplification was performed using Illumina-adapted universal 16S
525 primers 515F and 806R under the following conditions: 3 minutes at 94°C, 45 cycles of [45
526 seconds at 94°C, 60 seconds at 50°C, 90 seconds at 72°C], 10 minutes at 72°C. All reactions were

527 prepared using 5 PRIME polymerase 1X HotMasterMix (5PRIME, Gaithersburg, MD) and run in
528 triplicate to alleviate primer bias. Triplicates were pooled before cleaning with a PCR Purification
529 Kit (Qiagen). These products were quantified using the Qubit dsDNA High Sensitivity Assay Kit
530 (Invitrogen, Eugene, OR) and samples were pooled in equimolar amounts. Sequencing was
531 performed using the Illumina MiSeq platform located at the New York University Langone
532 Medical Center Genome Technology Core. Sequences can be found under the BioProject
533 accession number PRJNA573963 (http://www.ncbi.nlm.nih.gov/bioproject/573963).

534 Data Processing:

535 Data processing was performed using the QIIME2 (v 2019.1) pipeline[96]. The Divisive
536 Amplicon Denoising Algorithm 2 (DADA2) method was used to quality-filter sequences and
537 categorize amplicon sequence variants (ASVs)[157], and the SILVA (release 132) 99% identity
538 V4 classifier was used to assign taxonomy to ASVs[158]. See Additional File 1 for more
539 information. Taxonomic relative abundances were exported at the genus level for further analysis.
540 Output data can be found in Additional Data 2.

541

542 ***Shotgun Sequencing***

543 Sample Preparation and Sequencing:

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

550 Technology Core. Sequences can be found under the BioProject accession number PRJNA573963
551 (http://www.ncbi.nlm.nih.gov/bioproject/573963) for the levofloxacin dataset and under the
552 BioProject accession number PRJNA531921 (https://www.ncbi.nlm.nih.gov/bioproject/531921)
553 for the test dataset.

554 Data Processing:

555 Raw shotgun sequencing reads were processed using Kneaddata (v0.6.1) to remove
556 contaminating human sequences from the dataset[159]. Briefly, the *kneaddata* function was used
557 with the Bowtie2 *Homo sapiens* database (v0.1)[160] to remove contaminating host reads from
558 the sequencing files. See Additional File 1 for more information.

559 Taxonomic Classification:

560 Kraken2, a taxonomic classifier that maps shotgun sequencing k-mers to genomic
561 databases, was used to assign taxonomy to kneaddata-processed shotgun sequencing reads[70].
562 Briefly, the *kraken2-build* function was used to create a custom database containing the "bacteria"
563 and "archaea" from NCBI libraries, and the *kraken2* function was used to run the kneaddata-
564 filtered shotgun sequencing reads against this database and assign taxonomy. While Kraken2 does
565 not estimate species abundances, Bracken2 (Bayesian Reestimation of Abundance with KrakEN)
566 uses the taxonomy assigned by Kraken2 to estimate the number of reads per sample that originate
567 from individual species[71]. The Kraken2 database was used to create a Bracken-compatible
568 database using the *bracken-build* function, and the Kraken2 report files for each sample were run
569 against the Bracken database using the *bracken* function for the phylum, genus, and species levels.
570 Phylum- and species-level relative abundance outputs were formatted for biomarker discovery
571 using LEfSe. The *kraken-biom* function was used to convert the Bracken report files into a biom

572 file for import into R. Output data can be found in Additional Data 1. Relative abundance plots
573 were generated in GraphPad Prism v8.

574 Taxonomic Diversity Analysis:

575 Alpha and beta diversity analyses were performed using the phyloseq (v1.27.2)[161, 162]
576 and vegan (v2.5-4)[163] packages in R (v3.4.3). Briefly, the biom file was imported into a
577 phyloseq object. The phyloseq *estimate_richness* function was used to obtain Shannon's Diversity
578 Index values for all samples, while the vegan *phyloseq::distance* and *ordinate* functions were used
579 to generate a Bray-Curtis matrix and PCoA values. See Additional File 1 for more information.
580 Data was exported as csv files for formatting, and plotting was performed in GraphPad Prism v8.

581 Gene and Pathways Analysis:

582 The Human Microbiome Project Unified Metabolic Analysis Network 2 (HUMAnN2)
583 pipeline was used to profile the presence and abundance of genetic pathways in our samples[97].
584 Briefly, the *humann2* function was used with the kneaddata-filtered metagenomic sequences to
585 estimate genes and MetaCyc pathways present in the samples based on the UniRef90 database,
586 files were joined using the *humann2_join_tables* function and the full tables were de-leveled using
587 the *humann2_split_stratified_table* function. The unstratified gene-level abundances were
588 converted to both GO terms and KEGG orthologs using the *humann2_regroup_table* function, and
589 the *humann2_renorm_table* function was used to normalize the MetaCyc pathway, GO term, and
590 KEGG ortholog tables by computing relative abundance. These relative abundance tables were
591 formatted for biomarker discovery with LEfSe. Additionally, the, and LEfSe was also used to
592 analyze pre- and post-treated samples using both outputs. See Additional File 1 for more
593 information. Output data can be found in Additional Data 3. Plots were generated in Graphpad
594 Prism 8.

595 Resistome Analysis:

596 The ARG content of the samples was analyzed using DeepARG-SS, a deep learning model
597 that can predict ARGs from short-read metagenomic data[98]. We first analyzed the data using the
598 *deeparg* function with the *-reads* flag. The mapped ARGs output was then imported into R, where
599 it was processed to obtain tables of the ARGs detected per sample at both the specific gene and
600 antibiotic class levels. The ARGs detected were normalized to the number of reads per sample.

601 Additionally, after metagenomic assembly and binning was performed (see below),
602 individual bins were analyzed using DeepARG-LS, a deep learning model optimized to predict
603 ARGS from gene-level input. The *DNA_features* output from selected bins was analyzed using the
604 *deeparg* function with the *-genes* flag to analyze whether the levels or identity of ARGs could be
605 linked to specific species of interest. The ARGs detected were normalized to the number of features
606 per bin. All output data can be found in Additional Data 4. Plots were generated in GraphPad Prism
607 8.

608 Metagenomic Assembly and Binning:

609 To further examine the ARGs present in the samples, kneaddata-filtered reads were
610 uploaded to the web-based Pathosystems Resource Integration Center (PATRIC)[164]. Reads
611 were assembled into contigs using the *auto* option of the Assembly service, which provides both
612 raw output contigs from specific assembly algorithms and contigs of the "best" assembly as judged
613 by the in-house PATRIC script ARAST. We ran the assembly using two different inputs: reads
614 that had been processed by *kneaddata* as pairs, which has the advantage of utilizing mate-pairing
615 information for longer total reads, and reads that had been processed by *kneaddata* after pairs were
616 concatenated into a single file, which has the advantage of keeping reads whose mates failed
617 trimming.

618 Both the raw SPAdes assembly algorithm contigs[165] and the best assembly contigs were
619 then processed using the Metagenomics Binning service, which assigns contigs to specific
620 organisms and annotates the bin's genome. Quality measures were used to define bins as either
621 "good", "acceptable", or "bad" according to the criteria in Additional Table 3, and only "good" or
622 "acceptable" bins were used moving forward. When more than one binning strategy (paired
623 assembly or single assembly, SPAdes contigs or best contigs) called a particular bin as "good" or
624 "acceptable", quality measures from the four strategies were compared and the highest-quality bin
625 for a given species of interest was chosen for ARG analysis. Finally, only bins of species present
626 at 0.1% or greater relative abundance in the corresponding sample were selected for further
627 analysis. A list of bins used, their source, and quality measures can be found in Additional Table
628 4.

629 Taxonomic Biomarker Analysis:

630 LEfSe was used to identify potential biomarkers distinguishing levofloxacin-treated
631 samples[95]. In all cases (taxonomic abundances, MetaCyc pathways, KEGG orthologs, GO
632 terms, ARGs), data was formatted into csv files and uploaded to the Galaxy webserver. LEfSe was
633 run under default parameters for biomarker detection, comparing either all pre-levofloxacin to all
634 post-levofloxacin or immediately pre-levofloxacin to immediately post-levofloxacin. LEfSe was
635 also used to compare genus-level taxonomic abundance outputs from Kraken2/Bracken2 and
636 QIIME2, again under default parameters.

637

638 **DECLARATIONS**

639 Ethics Approval and Consent to Participate: Approval for the SPREAD study was obtained from
640 the Institutional Review Board committee at Hebrew SeniorLife.

641 Consent for Publication: Not applicable

642 Availability of Data and Material: The underlying sequencing data for the current study are
643 available in the NCBI Short Read Archive repository; 16S rRNA and shotgun metagenomics data
644 for the levofloxacin samples can be found at BioProject accession number PRJNA573963
645 (http://www.ncbi.nlm.nih.gov/bioproject/573963) and shotgun metagenomics data for the test
646 dataset can be found at BioProject accession number PRJNA531921
647 (https://www.ncbi.nlm.nih.gov/bioproject/531921). Analysis code and data generated from this
648 study can be found in Additional File 1 and Additional Data 1-4 of this published article,
649 respectively.

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660 and wrote the manuscript. RA collected the data and contributed to manuscript preparation. EMCD
661 collected the data and contributed to manuscript preparation. PB conceptualized the project and
662 wrote the manuscript. All authors read and approved the final manuscript.

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666 this study.

667

668 **ADDITIONAL DATA FILES**

669 *Additional Data 1: Taxonomic Classifications from Shotgun Metagenomics Kraken2/Bracken2*

670 This file includes the relative abundances of the taxonomic classifications at the phylum, genus,
671 and species level for both the initial levofloxacin-treated dataset (tabs 1, 2, and 3) and the second,
672 larger test dataset (tabs 4, 5, and 6). Additional Table 5 links the sample names used for the test
673 dataset in this study with their identifiers in BioProject PRJNA531921.

674

675 *Additional Data 2: Taxonomic Classifications from 16S rRNA Sequencing QIIME2*

676 This file includes the relative abundances of the taxonomic classifications at the phylum (tab 1)
677 and genus (tab 2) level for the initial levofloxacin-treated dataset.

678

679 *Additional Data 3: Metagenomic Classifications from HUMANn2*

680 This file includes the relative abundances of the MetaCyc pathway (tab 1), KEGG ortholog (tab
681 2), and GO term (tab 3) outputs for the initial levofloxacin-treated dataset.

682

683 *Additional Data 4: Antimicrobial Resistance Gene profiles from DeepARG*

684 This file includes the relative abundances of antimicrobial class and specific resistance genes at
685 the phylum, genus, and species level for the initial levofloxacin-treated dataset (tabs 1 and 2), the

686 second, larger test dataset (tabs 3 and 4), and the bins generated by PATRIC (tabs 5 and 6).
687 Additional Table 5 links the sample names used for the test dataset in this study with their
688 identifiers in BioProject PRJNA531921.
689
690 *Additional File 1: Code Used for Analysis*
691 This file includes the analysis code used for QIIME2, Kraken2 and Bracken, Phyloseq,
692 HUMANn2, and DeepARG.
693
694 *List of Abbreviations in Manuscript and Figures*

695 A:A	Aminoglycoside:Aminocoumarin
696 AG	Aminoglycoside
697 AMR	Antimicrobial Resistant
698 ARG	Antimicrobial Resistance Gene
699 BC	Bacitracin
700 BL	Beta-Lactam
701 Bracken2	Bayesian Reestimation of Abundance with KrakEN2
702 DP	Diaminopyrimidine
703 FFM	Fosfomycin
704 FMM	Fosmidomycin
705 FQ	Fluoroquinolone
706 GP	Glycopeptide
707 GO	Gene Ontology
708 HUMANn2	Human Microbiome Project Unified Metabolic Analysis Network 2

KEGG	Kyoto Encyclopedia of Genes and Genomes
LEfSe	Linear Discriminant Analysis Effect Size
MD	Multidrug
MDRO	Multidrug-Resistant Organism
MLS	Macrolide-Lincosamide-Streptogramin
MP	Mupirocin
MRSA	Methicillin-resistant *Staphylococcus aureus*
NM	Nitromidazole
OZ	Oxazolidinone
PATRIC	Pathosystems Resource Integration Center
PC	Phenicol
PMT	Pleuromutilin
PMX	Polymyxin
PT	Peptide
QIIME2	Quantitative Insights Into Microbial Ecology 2
TC	Tetracycline
SPREAD	Study of Pathogen Resistance and Exposure to Antimicrobials in Dementia
UC	Unclassified

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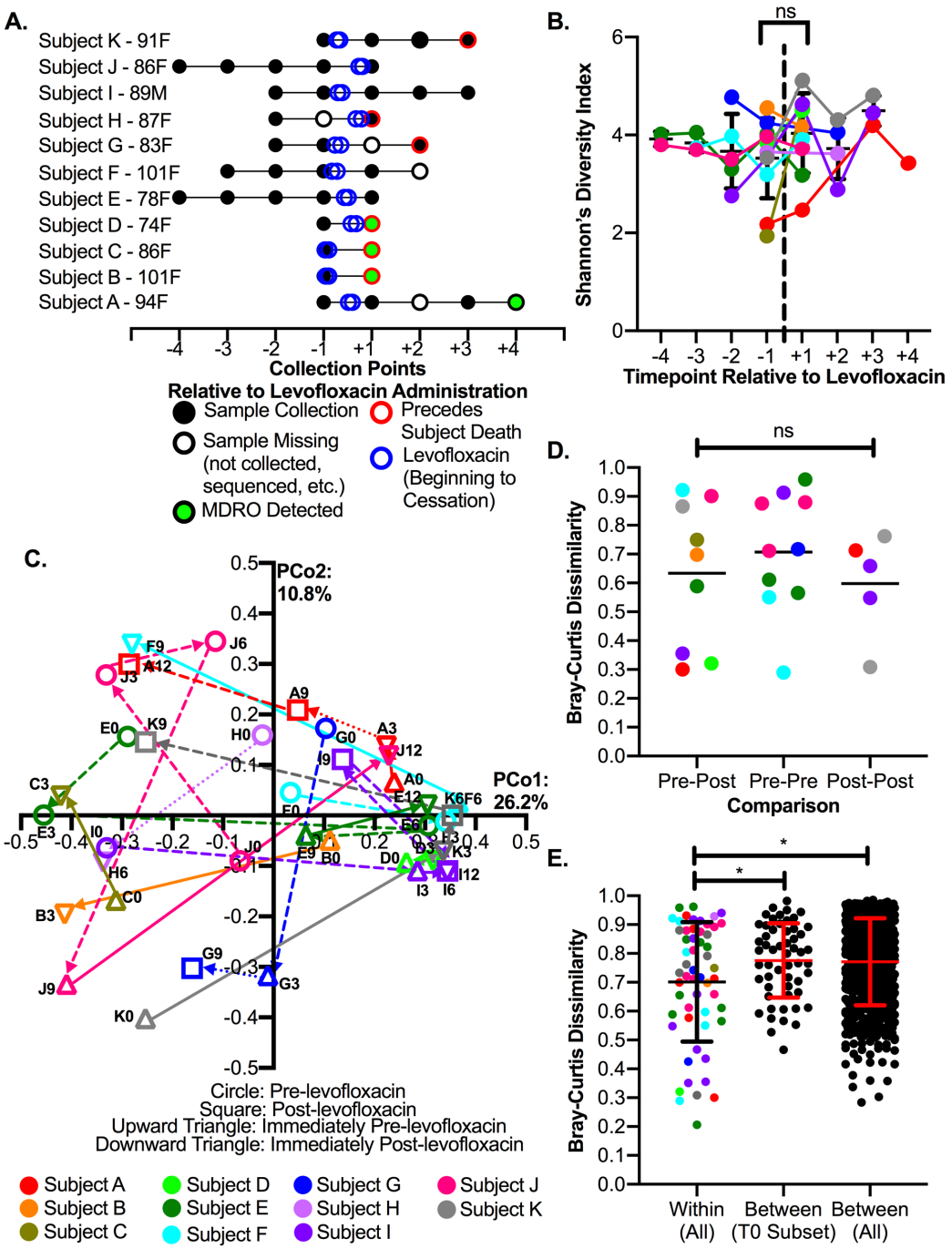

Figure 1: Subject Overview and Diversity Metrics

(A) Metagenomics 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. (B) Shannon diversity over time of all subjects. The dashed line indicates administration of levofloxacin. $p = 0.175$ for immediately pre-levofloxacin vs. immediately post-levofloxacin samples and $p = 0.1006$ for all pre-levofloxacin vs. all post-levofloxacin samples; Mann-Whitney test. (C) PCoA analysis of Bray-Curtis Dissimilarity. 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. $p = 0.6248$ 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. $p = 0.0262$ for overall within-subjects vs. T0 between-subjects and $p = 0.0175$ for overall within-subjects vs. overall between-subjects; t-test with Welch's correction.

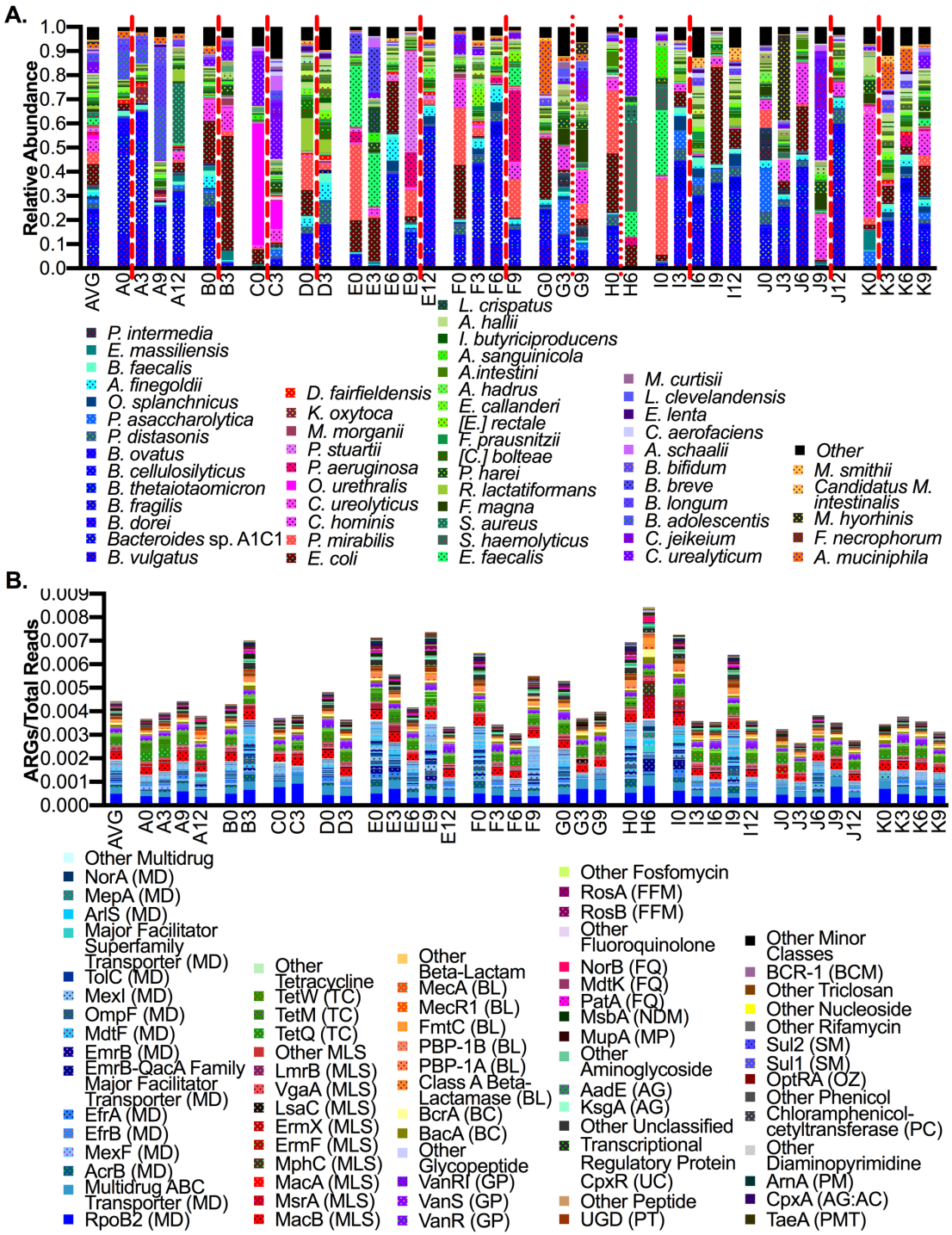

Figure 2: Relative Abundances of Species and Antimicrobial Resistance Genes

(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), while different species of the same genus are given the same specific background color. 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 (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.), while related gene categories (ex: the *mec* operon or *mex* efflux proteins) are given the same specific background color. All ARGs were normalized to the total number of reads.

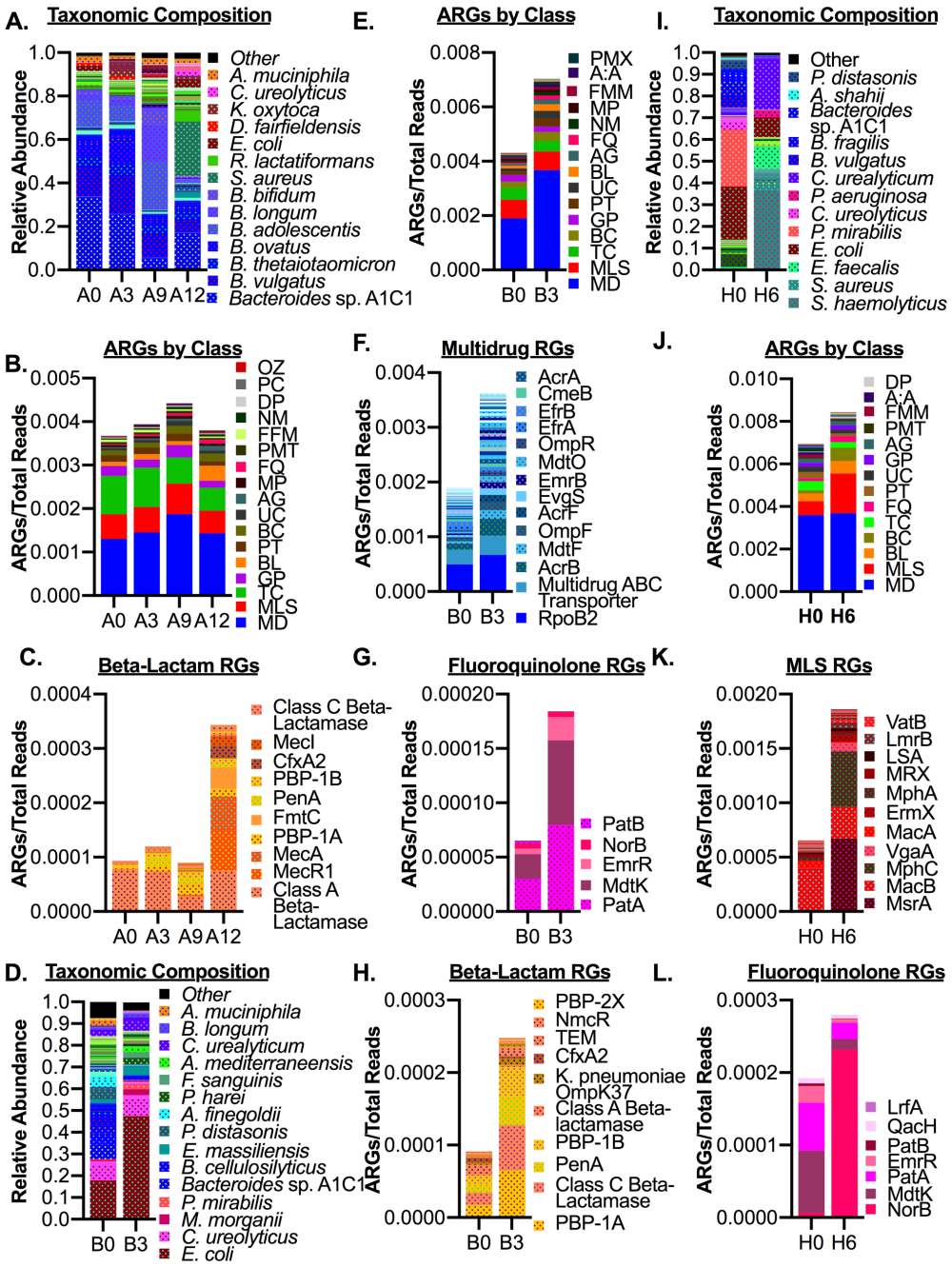

Figure 3: Antimicrobial Resistance Gene Profiles Reflect Taxonomic Observations
(A) Relative abundance of species in Subject A, showing a bloom in *S. aureus* at T12. (B) Relative abundance of ARG classes in Subject A, showing an expansion in beta-lactam resistance genes at T12. (C) Relative abundance 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. (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.

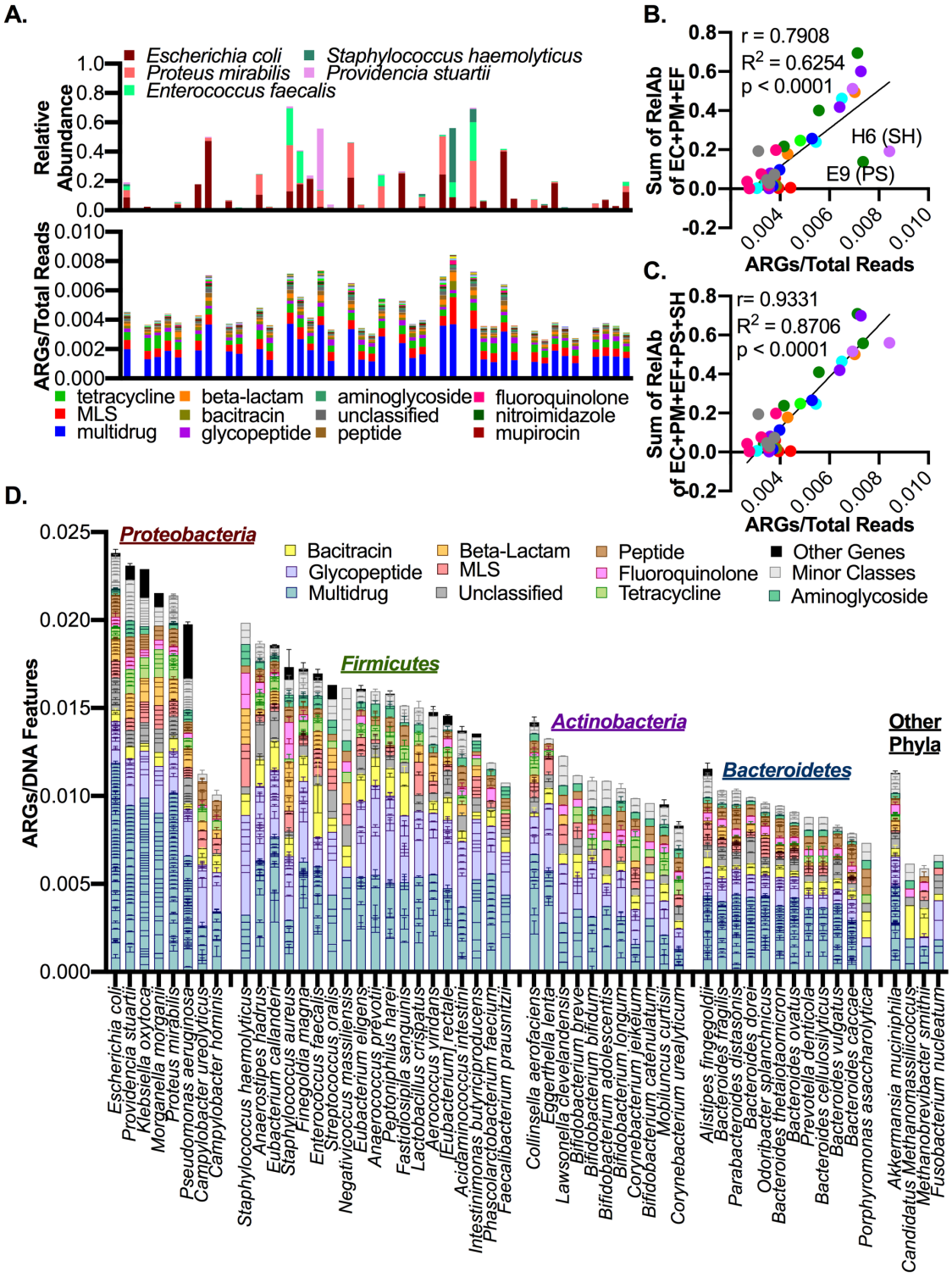

Figure 4: Relationship of ARG Levels to the Relative Abundance of Specific Pathobionts
(A) Correspondence between the relative abundances of key species of interest (*E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus*) and total ARG density in each sample. (B) Correlation between the sum of the relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis* and the total ARG density in each sample ($r = 0.791$, $R^2 = 0.6254$, $p < 0.0001$; Pearson's correlation). (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.

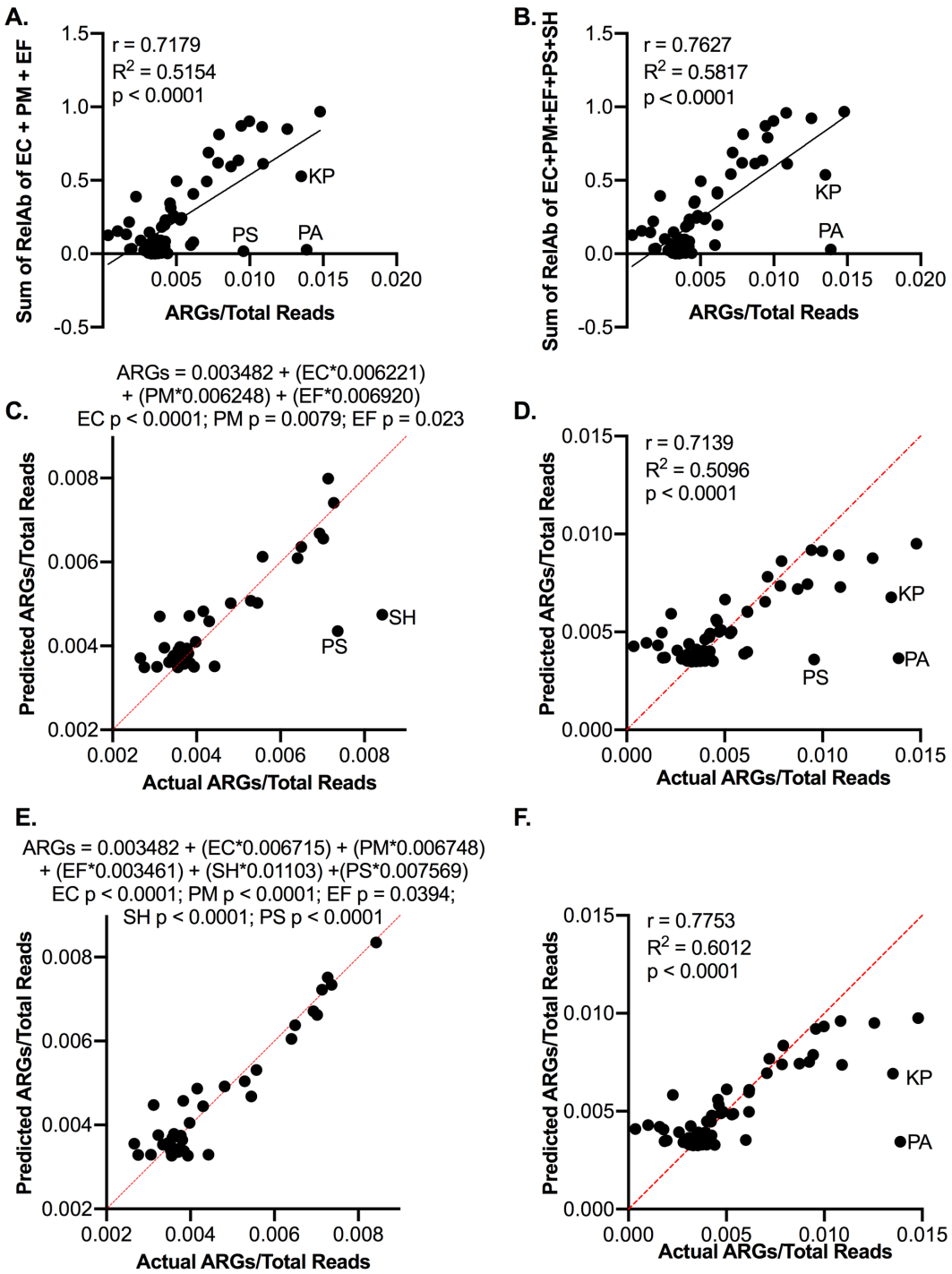

Figure 5: Prediction of ARG Density From Relative Abundance of Specific Pathobionts
(A) Correlation between the sum of the relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis* and the total ARG density in each sample in the test dataset ($r = 0.7139$, $r^2 = 0.5096$, $p<0.0001$; Pearson's 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 (38 samples). (D) Correlation between the predicted ARG density and actual ARG density in the test dataset (67 samples) based on the relative abundances of *E. coli*, *P. mirabilis*, and *E. faecalis*. (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 (38 samples). (F) Correlation between the predicted ARG density and actual ARG density in the test dataset (67 samples) based on the relative abundances of *E. coli*, *P. mirabilis*, *E. faecalis*, *P. stuartii*, and *S. haemolyticus*.