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1 **Gut Microbiome Diversity and Composition in Individuals With and Without Extended-**
2 **Spectrum β -Lactamase-Producing *Enterobacterales* Carriage: a Matched Case-Control Study in**
3 **infectious diseases department**

4
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76 **ABSTRACT**

77

78 **Background:** Little is known on the effect of gut microbial and Extended-Spectrum β -Lactamase-
79 Producing *Enterobacterales* (ESBL-E) carriage, particularly in the general population. The aim of this
80 study was to identify microbiota signatures uniquely correlated with ESBL-E carriage.

81 **Methods:** We conducted a case-control study among individuals seeking care at the Sexual Health
82 Clinic or Department of Infectious and Tropical Diseases at Saint-Antoine Hospital, Paris, France.
83 Using coarsened exact matching, 176 participants with ESBL-carriage (i.e., cases) were matched 1:1
84 to those without ESBL-carriage (i.e., controls) based on sexual group, ESBL-E prevalence of countries
85 traveled in <12 months, number of sexual partners in <6 months, geographic origin, and any antibiotic
86 use in <6 months. 16S rRNA gene amplicon sequencing was used to generate differential abundances
87 at the genus level and measures of α - and β -diversity.

88 **Results:** Participants were mostly men (83.2%, $n=293/352$) and had a median age of 33 years
89 (IQR=27-44). Nine genera were found associated with ESBL-E carriage (Figure 2C): *Proteus*
90 ($p<0.0001$), *Carnobacterium* ($p<0.0001$), *Enterorhabdus* ($p=0.0079$), *Catonella* ($p=0.017$),
91 *Dermacoccus* ($p=0.017$), *Escherichia/Shigella* ($p=0.021$), *Kocuria* ($p=0.023$), *Bacillus* ($p=0.040$), and
92 *Filifactor* ($p=0.043$); however, differences were no longer significant after Benjamini-Hochberg
93 correction ($q>0.05$). There were no differences between those with versus without ESBL-E carriage in
94 measures of α -diversity (Shannon Diversity Index, $p=0.49$; Simpson Diversity Index, $p=0.54$; and
95 Chao1 Richness Estimator, $p=0.16$) or β -diversity (Bray-Curtis dissimilarity index, $p=0.42$).

96 **Conclusion:** In this large carefully controlled study, there is lacking evidence that gut microbial
97 composition and diversity is any different between individuals with and without ESBL-E carriage.

98

99 **Keywords:** ESBL- producing *Escherichia coli*, gut microbiome, colonization resistance, 16S
100 sequencing, antibiotic resistance

101 **BACKGROUND**

102

103 The intestinal microbiota is a complex community of microorganisms, which is believed to play a
104 broad range of physiological functions essential for human health. Among the most explored
105 functions, colonization resistance (CR) represents the capacity of the intestinal microbiota to prevent
106 colonization by exogenous aerotolerant or aerobic bacteria, including *Enterobacteriales* [1]. While
107 some of these organisms encompass beneficial commensal microbiota [2] others have a pathogenic
108 potential [3]. Extended-Spectrum β -Lactamase-Producing *Enterobacteracerales* (ESBL-E),
109 particularly *E. coli*, are considered by the World Health Organization among the 12 types of bacteria
110 that pose the greatest threat to human health [4]. In this context, there is an increasing interest in
111 understanding the role of the gut microbiome in ESBL-E carriage.

112

113 CR is the consequence of complex interactions that involve hundreds of species composing the
114 intestinal microbiota [5]. CR results from many mechanisms that include competition for carbon and
115 energy sources, antagonism mediated by small molecules, such as antimicrobial peptides between
116 normal microbiota and competing species, inflammation of the microenvironment, with possible
117 effects from yeasts, viruses or Archaea. As a consequence, any factor that could affect the species
118 equilibrium could result in dysbiosis, and therefore influence the microbiota's function in CR [6,7,8].
119 The most common factor responsible for this dysbiosis is exposure to antibiotics [9,10,11,12].

120

121 Studies aiming to link intestinal composition with the risk of ESBL-E colonization in the community
122 setting are scarce [13,14,15,16,17] and have yielded heterogeneous results. In a previous cross-
123 sectional study conducted at a sexually transmitted infection (STI) and human immunodeficiency virus
124 (HIV) clinic in Paris, France, it was observed that 10.5% of 2157 participants carried ESBL-E [18].
125 The prevalence of ESBL-E carriage in this study was notably higher as the number of sexual partners
126 increased, suggesting that sexually active individuals are an important key population for harboring
127 ESBL-E. Nevertheless, reasons other than sexual contact could explain the higher prevalence of
128 ESBL-E carriage in this population and given the previous evidence, gut microbiota diversity and

129 composition could be a major driver of carriage. To this end, we aimed to identify microbiota
130 signatures that may be associated with the presence of ESBL-E.

131

132

133 **METHODS**

134

135 **Study design and participants**

136

137 Participants were selected from the *Bactéries MultiRésistantes-Infections sexuellement transmissibles*
138 (BMR-IST) study. The study design and procedures were described previously [18]. In brief, we
139 included individuals who were ≥ 18 years old, were HIV negative (seeking care at the Sexual Health
140 Clinic) or HIV positive (seeking care at the Department of Infectious and Tropical Diseases), and
141 signed informed consent. This study was approved by the *Comité de protection des personnes Ouest II*
142 ethics committee (2017-73), in accordance with the Helsinki Declaration.

143

144 After obtaining written informed consent, we asked participants to fill out a questionnaire on
145 demographic characteristics, ESBL-E risk factors, and sexual behavior with the assistance of a study
146 investigator. The choice of STI tests was at the physician's discretion. Participants provided a self-
147 swab (Copan ESwab), which were later processed at the Microbiology Department of Saint-Antoine
148 Hospital. We gave participants a self-swab (Copan ESwabTM) and asked them to collect a sample in
149 the lavatory of the clinic by inserting the swab into the rectal canal. Only swabs with visible fecal
150 material were included in bacteriological analysis.

151 Heart-brain broths supplemented with 10 $\mu\text{g/mL}$ cefotaxime were inoculated with rectal swabs,
152 incubated for 18 hours at 37°C and plated on ESBL bioMérieux media, as previously described [18].
153 Grown colonies were identified (MALDI-TOF, Bruker, Germany). In the BMR-IST study, to ensure
154 that *Enterobacteracerales* exhibiting antibiotic resistance via indirect pathways (e.g., overproduction
155 of AmpC) were not selected, all *Enterobacteracerales* isolates were subjected to a standard
156 antibiogram using Mueller-Hinton agar (Biorad), according to recommendations from the European

157 Committee on Antimicrobial Susceptibility Testing (<https://www.eucast.org/>). Then, briefly, whole
158 genomes were sequenced on these isolates using Illumina NextSeq and the NextEra XT sequence
159 library (Illumina, San Diego, CA), and were then used to perform ESBL-E Multi-Locus Sequence
160 Typing (MLST) [18].

161
162 In this case-control study, we matched the individuals with ESBL-E carriage (i.e., cases) 1:1 to
163 individuals without ESBL-E carriage (i.e., controls) using coarsened exact matching (details provided
164 in the Supplementary Methods). The following matching criteria were used (Supplementary Methods):
165 sexual group, ESBL-E prevalence of countries traveled in the previous 12 months, number of sexual
166 partners in the previous 6 months [$\log(n+1)$ -transformed], geographic origin, and any antibiotic use in
167 the previous 6 months. These characteristics were selected *a priori* for their putative confounding role
168 in the association between microbiota and ESBL-E carriage. This procedure was carried out using the
169 ‘cem’ ado command in Stata.

170

171 **16S rRNA gene amplicon sequencing**

172

173 ***Extraction and controls***

174 Only swabs with visible fecal material were included in the bacteriological analysis. Stool swabs
175 (Copan ESwab) obtained by rectal self-swabbing were placed in transport medium and vortexed for 15
176 seconds. DNA-RNA extraction was performed using an unbiased procedure before performing
177 targeted sequencing [19]. Briefly, pre-extraction combining bead beating, chemical cell disruption
178 with detergent, and proteinase K lysis was followed by extraction using the DSP virus-pathogen kit on
179 QiaSymphony (Qiagen, Hilden, Germany). In each run, a negative control and a positive control
180 represented by a microbial community standard (Zymobionics®, Zymo Research, Irvine, CA, USA)
181 were tested to evaluate the performance of the sequencing techniques for the detection of bacteria.

182

183 ***16S rRNA gene amplification and sequencing***

184 16S rRNA gene amplicon sequencing included the study of four amplicon libraries domain V3-V4
185 (16S-V3V4) [20] of the bacterial 16S rRNA gene. As described previously, each amplicon was
186 prepared from 5 μ L extract following the “16S Sequencing Library Preparation Protocol” provided by
187 the manufacturer (Illumina, San Diego, CA, USA) [21]. For each library, the quality was evaluated
188 using a D1000 ScreenTape on a TapeStation (Agilent, Santa Clara, CA, USA) and the quantity using
189 the Quant-it dsDNA Assay kit (ThermoFischer, Waltham, MA, USA) on a Varioskan LUX
190 (ThermoFisher, Waltham, Massachusetts, USA). All libraries were normalized to 4 nM, pooled, and
191 denatured before pair-end sequencing (2*250bp) using MiSeq Reagent Kit v2 (500-cycles) on a
192 MiSeq device (Illumina, San Diego, CA, USA), according to the manufacturer’s instructions [22].

193

194 **Bioinformatic analysis**

195

196 The analysis of bacterial 16S rRNA amplicon data was performed using the USEARCH v11.0.667
197 software pipeline with default parameters and the rdp16s database v16 [23]. Details of this analysis are
198 provided in the Supplementary Methods.

199

200 **Statistical analysis**

201

202 As sample size depended on the availability of ESBL-E-positive samples from a parent study (i.e.,
203 convenience sample), we did not perform any sample size calculation before analysis.

204

205 We calculated the mean \log_{10} difference in relative abundances at the phylum, family, and genus level
206 between cases and controls using targeted maximum likelihood estimation with the ‘tmle’ package in
207 R, while accounting for matched variables (Supplementary Methods) [24]. Variance of the difference
208 was corrected for matched strata and estimated using machine learning techniques with the
209 “SuperLearner” package. We tested whether differences were below or above the null and corrected p-
210 values using the Benjamini-Hochberg procedure with the ‘p.adjust’ function.

211

212 We calculated the α -diversity metrics Shannon diversity index, Simpson diversity index, and Chao1
213 richness estimator. We calculated the median and interquartile range (IQR) of the 3 indices and
214 compared them between cases and controls using the Kruskal-Wallis test.

215
216 We measured β -diversity using the Bray-Curtis dissimilarity index from the relative abundance data.
217 We visualized patterns of compositional similarity or dissimilarity between cases and controls using
218 the non-metric multidimensional scaling (NMDS) ordination and Principal Coordinates Analysis
219 (PCoA) methods [25]. The difference between cases and controls was tested using the PERMANOVA
220 (Permutational Multivariate Analysis of Variance) test under a reduced model with terms added
221 sequentially (first to last) and 1000 free permutations. We carried out these analyses using the “vegan”
222 package in R. In sensitivity analysis, we ran these analyses using the Canberra and robust Aitchison
223 methods to calculate dissimilarity.

224
225 Statistical analyses were conducted using R statistical software (v4.3.1, Vienna, Austria) and Stata
226 (v15.0, College Station, TX).

227

228

229 **RESULTS**

230

231 **Description of the study population**

232

233 In the parent study, 226 and 1931 participants were identified with and without intestinal carriage of
234 ESBL-E, respectively. Participant flow for this study is outlined in Figure 1. In total, 176 cases and
235 176 controls were included in analysis.

236

237 Participant characteristics are described in Table 1. Covariate patterns were similar between cases and
238 controls (Supplementary Methods). Participants were mostly from Europe (86.1%, $n=303/352$) and
239 had a median age of 33 years (IQR=27-44). 78.4% of participants traveled outside France within the

240 past year ($n=276/352$), and 22.7% reported using antibiotics within the past 6 months ($n=80/352$). Two
241 participants carried *Klebsiella pneumoniae*, one participant *Enterobacter cloacae* and all others
242 *Escherichia coli*.

243

244 **Composition of the gut microbiome between individuals with and without ESBL-E carriage**

245

246 The total number of paired-end reads obtained was 43,321,028 with an average of 98,622
247 (SD=22,773) per sample. Results of the negative and positive controls are presented in Supplementary
248 Figure 1.

249

250 The phyla *Firmicutes* (72.85%) was the most abundant, followed by *Actinobacteria* (10.10%),
251 *Bacteroidetes* (10.5%) and *Proteobacteria* (0.70%). There were no differences between individuals
252 with and without ESBL-E carriage in relative abundances of phyla (Figure 2A). Three families were
253 found associated with ESBL-E carriage (Figure 2B): *Intrasporangiaceae* ($p=0.0098$),
254 *Flavobacteriaceae* ($p=0.039$), and *Enterobacteriaceae* ($p=0.048$). These differences were non-
255 significant following the Benjamini Hochberg correction ($q=0.69$, 0.99 and 0.99 , respectively). Nine
256 genera were found associated with ESBL-E carriage (Figure 2C): *Proteus* ($p<0.0001$),
257 *Carnobacterium* ($p<0.0001$), *Enterorhabdus* ($p=0.0079$), *Catonella* ($p=0.017$), *Dermacoccus*
258 ($p=0.017$), *Escherichia/Shigella* ($p=0.021$), *Kocuria* ($p=0.023$), *Bacillus* ($p=0.040$), and *Filifactor*
259 ($p=0.043$). All differences were no longer significant after using the Benjamini-Hochberg correction
260 ($q>0.05$).

261

262 **Comparison of the gut microbiome diversity between individuals with and without ESBL-E** 263 **carriage**

264

265 Results from analysis on α -diversity are shown in Figure 3. The median Shannon diversity index
266 (Figure 3A), Simpson diversity index (Figure 3B) and Chao1 richness index (Figure 3C) were no
267 different between individuals with and without ESBL-E.

268

269 Results from analysis on β -diversity are shown in Figure 4. One participant with outlying values was
270 removed from this analysis. Both the NMDS ordination (Figure 4A) and PCoA plots (Figure 4B)
271 demonstrated no difference in Bray-Curtis dissimilarities between individuals with and without ESBL-
272 E carriage (PERMANOVA, $p=0.25$). Results were comparable when using the Canberra and robust
273 Aitchison methods to calculate dissimilarity (Supplementary Figures 2 and 3, respectively).

274

275

276 **DISCUSSION**

277

278 In the present study, the gut microbial diversity and composition was similar between individuals with
279 and without ESBL-E carriage. To the best of our knowledge, this is the largest study to explore the gut
280 microbiome, namely in terms of diversity and composition, in a specific population comprising
281 predominately younger, healthy individuals.

282

283 Previous studies have clearly demonstrated higher prevalence of ESBL-E carriage in older individuals,
284 those coming from or traveling to regions with high ESBL-E prevalence, and those with recent
285 antibiotic use or increased sexual contacts [26, 27]. These factors must then be appropriately
286 considered when examining differences in gut microbiota between those with and without ESBL-E
287 carriage. Indeed, our matched case-control study design provides rigorous matching for factors
288 potentially modifying microbiota composition as well as risk factors for ESBL-E carriage, which was
289 lacking in previous studies [13,14,15]. A recently published study found that ESBL-producing
290 *Escherichia coli* carriage in dogs is associated with a distinct microbiome and resistome composition
291 without adjustment for potential microbiota-modulating confounding factors (i.e., age, breed, diet,
292 hygienic measures, antibiotic use) [28]. One study conducted in an Amerindian community was
293 matched for antibiotic exposure within the year preceding sampling, and revealed an increased
294 abundance of members of two anaerobic taxa, belonging to the genera *Desulfovibrio*
295 (*Desulfovibrionaceae*) and *Oscillospira* (*Ruminococcaceae*) in the microbiota of non-carrier controls

296 [16]. These differences were not corroborated in our study or another recent, more well-designed,
297 case-control study from the Netherlands, which also found similarity in diversity parameters and in
298 relative abundance between ESBL-positive and ESBL-negative groups at the bacterial species-level
299 [17].

300

301 Historically, ESBL-E dissemination was more common in hospital settings. Over-time, emergence of
302 ESBL-E infections has been more frequently reported in community settings. In the United States, an
303 increase of 53% in the incidence of ESBL-E infections, between 2012 and 2017, has been largely
304 attributed to community-onset cases [29]. Growing evidence suggests that the successful spread of
305 ESBL encoding genes in the community setting owes to the ability of plasmids, notably IncF, to adapt
306 to *E. coli* species [30]. Many of these strains also contained ESBL-encoding genes on pre-existing
307 plasmids before newly acquiring other ESBL-containing plasmids [31]. Our results, together with
308 those obtained by Ducarmon et al. [17] clearly indicate that ESBL-E circulating in the community are
309 well adapted to the human intestinal microbiota and seem unaffected by the CR. Instead, ESBL-E
310 dissemination may be driven by specific lineages, as demonstrated by Connor et al. in a gnotobiotic
311 mouse model where a multidrug-resistant (MDR) ST131 *E. coli* was found capable of out-competing
312 and displacing non-MDR *E. coli* from the gut, in absence of antibiotic exposure [32]. According to
313 these authors, these high capacities to colonize human gut could be associated with increased diversity
314 in carbohydrate metabolism genes of the MDR strains, as suggested by functional pangenomic
315 analysis they performed.

316

317 The role of SCFAs in conferring colonization resistance against ESBL-E has been examined
318 extensively. In a study conducted in mice, the total level of SCFAs production was found to be
319 correlated to a decrease in *Enterobacterales* colonization in the intestinal lumen [33]. Another study
320 conducted in 144 residents living in two different nursing homes found that the gut microbiome of
321 individuals with ESBL-E carriage was depleted in butyrate-producing species compared to individuals
322 without ESBL-E carriage, yet this comparison was not adjusted for microbiota-modulating factors
323 [13]. In our study, the abundance of anaerobic bacteria identified as producers of SCFAs

324 (*Lachnospiraceae*, *Ruminococcaceae* families and *Butyrivibrio*, *Roseburia*, *Veillonella*, *Coprococcus*,
325 *Megasphaera*, *Eubacterium*, *Faecalibacterium*, *Ruminococcus*, *Blautia* genera) was similar between
326 individuals with and without ESBL-E carriage [34,35].

327

328 The findings need to be interpreted within the context of our study population. Individuals were
329 seeking care for diverse reasons, possible because of increased sexual activity and risk of STIs for
330 those without HIV, or routine care for those with HIV. The study population also mostly comprised
331 men, mainly because of the low prevalence of women with ESBL-E carriage and the fact that women
332 consult sexual health clinics less frequently than men in France [18,36]. Few participants were also
333 from regions outside Europe. Although these characteristics were part of the matching criteria, the
334 limited numbers of women and those born in a region outside of Europe do not allow us to determine
335 whether the lack of association holds in these populations.

336

337 There are certain limitations of our study. First, since we used rectal swabs, there might have been
338 some individuals without ESBL-E carriage who were in fact with carriage (i.e., false-negative). The
339 extent of this misclassification depends on bacterial loads, which were unknown in this study.

340 However, this limitation is shared across all studies employing this commonly-used method. Second,
341 there were no data on the use of proton pump inhibitors and antidiabetics, which could have
342 additionally confounded the association between gut microbiota and ESBL-E prevalence [37,38].

343 Nevertheless, given that our study's population is comprised of mostly young participants, these
344 medications would have been unlikely prescribed. Second, longitudinal data on gut microbiota were
345 not collected, which could provide information on the possible changes of microbiota when ESBL-E
346 carriage either develops or clears. Furthermore, our study was not designed to describe the functional
347 pathways involved in colonization resistance. The use of advanced techniques in microbiological
348 analysis, such as metagenomics, metatranscriptomics, metaproteomics and metabolomics, could have
349 provided a more comprehensive understanding of functional profiling of microbial communities [39].

350

351

352 **CONCLUSION**

353

354 In conclusion, our results showed that the gut microbiome diversity and composition did not differ
355 between individuals with and without ESBL-E carriage. Future longitudinal studies should examine
356 the duration of ESBL-E carriage in community settings and factors from the intestinal microbiome that
357 could influence the persistence or clearance of ESBL-E.

358

359

360 **SUPPLEMENTARY INFORMATION**

361

362 **Additional file 1:** Supplementary materials.

363

364

365 **ABBREVIATIONS**

366

367 **ESBL-E:** Extended-Spectrum β -Lactamase-Producing *Enterobacterales*; **CR:** Colonization
368 resistance; **SCFAs:** Short Chain Fatty Acids; **HIV:** Human Immunodeficiency Virus; **STI:** Sexually
369 Transmitted Infection; **BMR-IST:** Bactéries MultiRésistantes-Infections Sexuellement
370 Transmissibles; **MSM:** Men who have Sex with Men; **MSW:** Men who exclusively have Sex with
371 Women; **WSM:** Women who have Sex with Men; **PrEP:** Pre-exposure prophylaxis for HIV; **DOM:**
372 Département Outre-Mer; **TOM:** Territoire Outre-Mer; **OTU:** Operational Taxonomic Unit; **IQR:**
373 Interquartile Range; **NMDS:** Non-Metric Multidimensional Scaling; **PCoA:** Principal Coordinates
374 Analysis; **PERMANOVA:** Permutational Multivariate Analysis of Variance; **MDR:** multidrug-
375 resistant.

376

377 **DECLARATIONS**

378

379 **Ethics approval and consent to participate**

380

381 The “Comité de protection des personnes Ouest II” ethics committee (2017-73) approved the study.

382 The study was carried out in accordance with the Helsinki Declaration. All patients signed informed

383 consent for participation.

384

385 **Consent for publication**

386

387 Not applicable.

388

389 **Availability of data and material**

390

391 The microbiome dataset comprising of raw 16S rRNA sequences supporting the conclusions of this

392 article were deposited under the National Center for Biotechnology Information BioProject

393 PRJNA1024378 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1024378>). Due to the

394 sensitive nature of participant data, metadata cannot be made publically available. Nevertheless, these

395 data are available upon reasonable request to the author with approval from the scientific committee of

396 the study and appropriate data transfer agreements.

397

398 **Competing interests**

399

400 The authors declare that they have no competing interests.

401

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403

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406

407 **Authors' contributions**

408

409 L.S. coordinated the study and oversaw data analysis, and data interpretation and performed critical
410 editing and review of the manuscript. A.B. participated to study design, oversaw data analysis and
411 performed critical editing and review of the manuscript. P.L.W. and R.A. contributed to data
412 interpretation and performed critical editing and review of the manuscript. M.E.D performed statistical
413 analysis, created figures, interpreted data and drafted the initial version of the manuscript. J.C. and
414 V.D. performed microbiological analyses. J.M.P. and K.L. contributed to study design and performed
415 critical review of the manuscript. C.R. contributed to data interpretation and drafted parts of the
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