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

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ABSTRACT

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

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

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

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

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

BACKGROUND

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

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

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

METHODS

Study design and participants

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

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

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

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

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

16S rRNA gene amplicon sequencing

Extraction and controls

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

16S rRNA gene amplification and sequencing

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

Bioinformatic analysis

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

Statistical analysis

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

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

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

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

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

RESULTS

Description of the study population

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

Participant characteristics are described in Table 1. Covariate patterns were similar between cases and controls (Supplementary Methods). Participants were mostly from Europe (86.1%, *n*=303/352) and had a median age of 33 years (IQR=27-44). 78.4% of participants traveled outside France within the past year (*n*=276/352), and 22.7% reported using antibiotics within the past 6 months (*n*=80/352). Two participants carried *Klebsiella pneumoniae,* one participant *Enterobacter cloacae* and all others *Escherichia coli.*

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

There are certain limitations of our study. First, since we used rectal swabs, there might have been some individuals without ESBL-E carriage who were in fact with carriage (i.e., false-negative). The extent of this misclassification depends on bacterial loads, which were unknown in this study. However, this limitation is shared across all studies employing this commonly-used method. Second, there were no data on the use of proton pump inhibitors and antidiabetics, which could have additionally confounded the association between gut microbiota and ESBL-E prevalence [37,38]. Nevertheless, given that our study's population is comprised of mostly young participants, these medications would have been unlikely prescribed. Second, longitudinal data on gut microbiota were not collected, which could provide information on the possible changes of microbiota when ESBL-E carriage either develops or clears. Furthermore, our study was not designed to describe the functional pathways involved in colonization resistance. The use of advanced techniques in microbiological analysis, such as metagenomics, metatranscriptomics, metaproteomics and metabolomics, could have provided a more comprehensive understanding of functional profiling of microbial communities [39].

CONCLUSION

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

SUPPLEMENTARY INFORMATION

Additional file 1: Supplementary materials.

ABBREVIATIONS

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

DECLARATIONS

Ethics approval and consent to participate

The “Comité de protection des personnes Ouest II” ethics committee (2017-73) approved the study. The study was carried out in accordance with the Helsinki Declaration. All patients signed informed consent for participation.

Consent for publication

Not applicable.

Availability of data and material

The microbiome dataset comprising of raw 16S rRNA sequences supporting the conclusions of this article were deposited under the National Center for Biotechnology Information BioProject PRJNA1024378 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1024378). Due to the sensitive nature of participant data, metadata cannot be made publically available. Nevertheless, these data are available upon reasonable request to the author with approval from the scientific committee of the study and appropriate data transfer agreements.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.S. coordinated the study and oversaw data analysis, and data interpretation and performed critical editing and review of the manuscript. A.B. participated to study design, oversaw data analysis and performed critical editing and review of the manuscript. P.L.W. and R.A. contributed to data interpretation and performed critical editing and review of the manuscript. M.E.D performed statistical analysis, created figures, interpreted data and drafted the initial version of the manuscript. J.C. and V.D. performed microbiological analyses. J.M.P. and K.L. contributed to study design and performed critical review of the manuscript. C.R. contributed to data interpretation and drafted parts of the manuscript. G.C. contributed to data interpretation. All authors contributed significantly to the manuscript and approved the final version.

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