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Leo, Stefano

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UNIVERSITÉ DE GENÈVE
Département de Médecine

FACULTÉ DE MÉDECINE
Professeur Jacques Schrenzel

Dynamiques des changements du microbiote intestinal humain en réponse à la colonisation par des bactéries multirésistantes et à l'administration d'antibiotiques

DYNAMICS OF CHANGES OF HUMAN GUT MICROBIOTA IN RESPONSE TO MULTIDRUG RESISTANT BACTERIA COLONIZATION AND FOLLOWING ANTIBIOTIC TREATMENT

THÈSE

présentée aux Facultés de médecine et des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences en sciences de la vie,
mention Génomique et santé numérique

par

Stefano LEO

de

Italie

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Table of Contents

Figure Index

Table Index

Abstract

Intestinal microbiota is constituted by a multitude of microbial species, mainly bacteria, mostly colonizing the distal part of human digestive tract, *i.e.* the colon. Interspecies interactions within microbiota are often complex and can occur with sophisticated mechanisms which eventually affect the ecological status of the gut and the human health. Moreover, microbiota species can directly or indirectly interact with intestinal epithelial and immune system cells, thus influencing their functioning and regulation mechanisms.

Some microbiota species, in particular, strictly anaerobic microorganisms are not yet culturable and thus require culture-independent approaches to be identified. Next-generation sequencing, *i.e.* whole-genome sequencing, allows a broad characterization of microbiota species and of the ensemble of genes encoding for antibiotic resistance (ARGs).

Correct functioning of intestinal microbiota seems to be dependent on a correct balance of its components. Inflammation and antibiotics lead to dysbiosis –microbiota with unbalanced microbial composition- which in turn can favor pathogen infection. The transition from "healthy" to dysbiotic microbiota is to a certain degree reversible when appropriate interventions are put in place.

The increase in cases of infection caused by multidrug resistant Gram-negative bacteria has alarmed the scientific community. Among Gram-negative pathogens, multidrug resistant Enterobacteriaceae (MRE) have attracted particular attention as they are resistant to various antibiotics, including those from β-lactam class, the major pillar of therapeutic drugs.

The understanding of how gut microbiota composition changes upon intestinal MRE carriage and subsequent infection or during anti-MRE drug therapy could lead to a better clinical management of these pathogens.

I first investigated the microbiota composition before and after travel to tropics which is considered an important risk factor for MRE intestinal acquisition. I analyzed the fecal microbiota of travelers to endemic MRE tropical regions and participating to the VOYAG-R study. I showed that microbiota does not protect from the acquisition of MRE after travel to the tropics but can determine a faster intestinal MRE clearance one month after the return. Furthermore, the species *Prevotella copri* was reported for the first time to be linked to the occurrence of the traveler's diarrhea.

As antibiotics are found detrimental for the intestinal microbial ecosystem, I questioned whether shortening antibiotic treatment would better preserve the microbiota. Thus, in a second project, the PIRATE RESISTANCE, I compared the effects of a short *versus* a longer antibiotic treatment against uncomplicated Gram-negative bacteremia. I found out that short treatment is already sufficient to cause negative effects on microbiota composition by rapidly decreasing its species diversity and richness. Furthermore, the antibiotic therapy resulted in increased abundance of ARGs carriage in the intestine. In particular, although decreased in content, those genes were still detected weeks after interruption of antibiotic therapy.

In a third project, the R-GNOSIS WP3, I analyzed the fecal microbiota of MRE carriers undergoing colistin/neomycin treatment followed by fecal microbiota transplantation (FMT) from healthy donors. I have shown that FMT restored microbiota species diversity following its sharp decrease caused by the colistin/neomycin therapy. Besides, FMT decreased the abundance of Enterobacteriaceae and of the genes encoding resistance against β-lactams. I reported that *Bifidobacterium* spp. and bacteria from Lachnospiraceae and Ruminococcaceae families are fundamental components in preserving microbiota and in avoiding dysbiosis. These bacteria produce butyrate and propionate, two short chain fatty acid molecules that have been shown to modulate intestinal inflammation and the immune system responses in the gut. Thus, besides describing the shift in microbiota composition in response to MRE acquisition, or to short versus longer antibiotic therapy and to FMT, I have also provided information on key microbiota species which could be of interest for translational microbiota research.

Résumé

Le microbiote intestinal est constitué d'une multitude d'espèces microbiennes, essentiellement des bactéries, qui colonisent principalement le côlon, la partie distale du tube digestif humain. Les interactions inter-espèces au sein du microbiote sont souvent complexes et peuvent se produire à travers des mécanismes sophistiqués qui finissent par affecter le statut écologique de l'intestin et affecter la santé humaine. En outre, les espèces du microbiote peuvent interagir directement ou indirectement avec les cellules de l'épithélium intestinal et du système immunitaire, influençant leur fonctionnement et leurs régulations.

Certaines espèces du microbiote, en particulier les micro-organismes strictement anaérobies, ne sont pas encore cultivables et nécessitent donc des approches indépendantes de la culture pour être identifiées. Le séquençage de nouvelle génération, c'est-à-dire le séquençage du métagénome, permet une large caractérisation des espèces du microbiote et de l'ensemble des gènes codant pour la résistance aux antibiotiques (GRA).

Le bon fonctionnement du microbiote intestinal semble dépendre d'un équilibre correct de ses composants. L'inflammation et les antibiotiques entraînent une dysbiose - un microbiote dont la composition microbienne est déséquilibrée - qui peut à son tour favoriser l'infection par des agents pathogènes. Le passage d'un microbiote "sain" à un microbiote dysbiotique est, dans une certaine mesure, réversible lorsque des interventions appropriées sont mises en place.

L'augmentation constante des cas d'infections causées par des bactéries Gram-négatives multirésistantes a alarmé la communauté scientifique. Parmi les agents pathogènes à Gram négatif, les entérobactéries multirésistantes (EMR) ont attiré une attention particulière car elles sont résistantes à divers antibiotiques, y compris ceux de la classe des β-lactamines, la classe de molécules les plus fréquemment utilisées en clinique.

La compréhension de la manière dont la composition du microbiote change lors de la colonisation intestinal d'EMR et de l'infection qui s'ensuit ou pendant le traitement médicamenteux contre les EMR pourrait conduire à une meilleure prise en charge de ces pathogènes.

J'ai d'abord étudié la composition du microbiote avant et après le voyage aux tropiques qui est considéré comme un facteur de risque important d'acquisition intestinale des EMR. J'ai analysé le microbiote fécal de voyageurs, participant à l'essai clinique VOYAG-R et se rendant dans des régions tropicales où les EMR sont endémiques. J'ai également démontré que le microbiote ne protège pas de l'acquisition d'EMR suite à un voyage dans les tropiques mais peut déterminer une perte des EMR plus rapide un mois après le retour. En outre, l'espèce *Prevotella copri* a pour la première fois été liée à l'apparition de la diarrhée du voyageur.

Les antibiotiques ont des conséquences néfastes pour l'écosystème microbien intestinal, donc nous avons investigué si le fait de raccourcir le traitement antibiotique pourrait être bénéfique pour le microbiote. Ainsi, dans un second projet, la PIRATE

RESISTANCE, j'ai comparé les effets d'un traitement antibiotique court par rapport à un traitement plus long pour traiter les bactériémies à Gram négatifs. J'ai découvert qu'un traitement court était déjà suffisant pour causer des effets négatifs sur la composition du microbiote en diminuant sa diversité et sa richesse en espèces. En outre, l'antibiothérapie a entraîné une augmentation de l'abondance des GRA dans l'intestin. Bien que leur quantité ait diminué, ces gènes étaient encore détectés plusieurs semaines après l'interruption de l'antibiothérapie dans les selles de ces patients.

Dans un troisième projet, le R-GNOSIS WP3, j'ai analysé le microbiote fécal de porteurs d'EMR ayant subi un traitement à la colistine/néomycine suivi d'une transplantation de microbiote fécal (TMF) à partir de donneurs sains. J'ai démontré que la TMF a rétabli la diversité des espèces du microbiote dont l'abondance avait été préalablement réduite par le traitement à la colistine/néomycine. En outre, l'abondance, dans le microbiote, des entérobactéries et des gènes codant pour la résistance aux β-lactamines a été diminuée après TMF. J'ai montré que les *Bifidobacterium* spp. et les bactéries appartenant aux familles Lachnospiraceae et Ruminococcaceae sont des composants fondamentaux pour préserver le microbiote et éviter la dysbiose. De manière intéressante, ces bactéries produisent le butyrate et le propionate, des acides gras à chaîne courte qui modulent l'inflammation intestinale et les réponses du système immunitaire dans l'intestin. Ainsi, en plus de décrire le changement de composition du microbiote en réponse à l'acquisition d'EMR, à l'antibiothérapie courte ou plus longue et à la TMF, j'ai également fourni des informations sur des espèces-clés du microbiote qui pourraient être intéressantes pour la recherche translationnelle sur le microbiote.

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I am profoundly in debt with my supervisor Professor Jacques Schrenzel because he gave me the great opportunity to work in his laboratories, first as research assistant and then as PhD student. I would like to thank him for the great guidance, for all the advices on the projects and for the clinical, beyond the scientific, criticism in data interpretation.

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I would like to thank all my past supervisors and teachers. I hope to have made you proud of me. I own a lot from all of you. Thanks for all support and for having encouraging me in pursuing Science.

I would like to devote this thesis work to my parents Antonio and Maria for all the love that they put towards their children and to the memory of my grand-parents Giuseppe and Anna, to their humility and perseverance through all the difficulties that they had to cope with during their lives.

Abbreviations

16S rRNA = 16S ribosomal RNA

23S rRNA = 23S ribosomal RNA

AmpC-bl = Ampicillin class C β-lactamase

AR = Antibiotic resistance

ARG/ARD = Antibiotic resistance encoding genes / determinants

bp = base pairs

CP = Carbapenemases

CP-E = CP-producing Enterobacteriaceae

ESBL = Extended spectrum β-lactamase

ESBL-E = ESBL- producing Enterobacteriaceae

ETEC = Enterotoxigenic *Escherichia coli*

GALT = Gut-associated lymphoid tissue

IBD = Inflammatory bowel disease

IEC = Intestinal epithelial cell

LPS = Lipopolysaccharide

MBL = Metallo-β-lactamase

MIC = Minimum inhibitory concentration

MRE = Multidrug resistant Enterobacteriaceae

PAMP = Pathogen-associated molecular pattern

pAmpC= plasmid-encoded AmpC type cephalosporinases

PERMANOVA = Permutational multivariate analysis of variance

SCFA = Short chain fatty acid

SFB = Segmented filamentous bacteria

TD = Traveler's diarrhea

WMGS = Whole metagenome shotgun sequencing

Introduction

Chapter 1: Microbiota research

With the concept gut microbiota (or also called intestinal microbiota) we define all microorganisms living within the intestinal tract. The term intestinal microbiome is, instead, used to indicate the ecological niche (including all possible molecular pathways) where intestinal microbiota lives and interact with the host [1].
Gut microbiota has been extensively investigated during the last two decades, permitting to link intestinal flora composition to numerous diseases and leading to a deep and ongoing revolution in clinical microbiology and in the field of infectious diseases. How did we come up studying microbiota? Is it really a recent field? Which technologies and bioinformatics tools allow analyzing the composition of intestinal microbiota? In the following paragraphs, we will try to address these questions but this implies first jumping back some centuries ago.

1.1 From basic culturing to new-generation sequencing

Antoine van Leeuwenhoek was the first to report the presence of "little animals", likely *Giardia* spp., observed through a microscope in human feces in the very far 1681 [2]. In 1854 Joseph Leidy affirmed that each animal has its own "flora and fauna", so officially "baptizing" the rise of the microbiota research field [3].
During the same period, the germ theory established itself thanks to the advancements brought by the studies of Robert Koch and Louis Pasteur. L. Pasteur proposed that also non-infectious microorganisms are important for health [4, 5] and R. Koch, who was one of the pioneers of microbial solid cell culture, defined the principles of the association between diseases and pathogens [6]. Ilya Metchnikoff, one of the father founders of immunology, hypothesized that host and microorganisms interact with each other [7]. In 1885, Theodor Escherich, who discovered and cultured for the first time the intestinal bacterium *Escherichia coli* [8], affirmed that intestinal microbes play a crucial role in the physiopathology of the human gut [9].
Since 1885 there has been a gradual increase in the number of recovered cultivable gastrointestinal species (Figure 1). Although already in 1931 intestinal microbes were shown to be mostly strict anaerobes, it was only since 1969 that anaerobic culturing techniques were improved, thus leading to a steep increase in the number of cultivable microbiota species (Figure 1; reviewed in [10]).

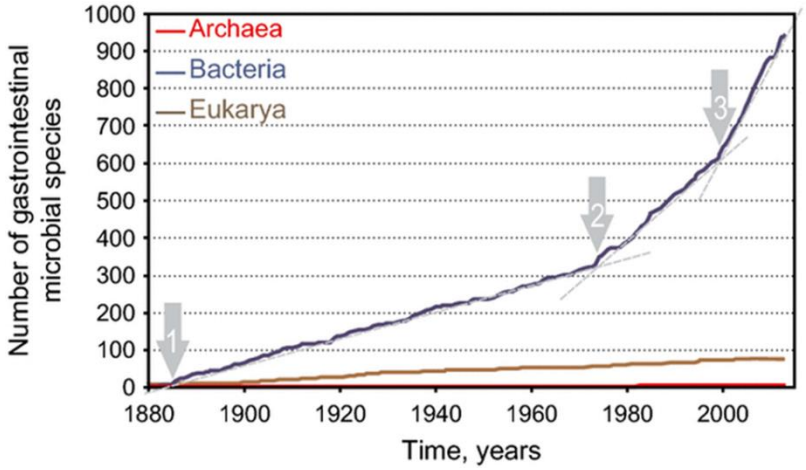

Figure 1. Number of detected gastrointestinal species over the last centuries.
The number of bacterial (blue), archaeal (red) and eukaryotic (brown) gastrointestinal species are reported *versus* time (in years). Arrows represent three important milestones in the microbiota research: 1. the discovery and culturing of *E. coli*; 2. the introduction of techniques allowing culturing under anaerobic conditions; 3. the advent of molecular biology techniques. The graph was taken from Rajilić-Stojanović *et al.*, 2014 [10].

Today, we know that only a fraction of microbiota species are culturable (10-50%) [11, 12]. However, thanks to new culturomics techniques, the number of cultivable species is expected to increase in the coming years [13].
The introduction of molecular biology techniques and of culture-independent approaches, like fluorescent *in situ* hybridization or flow cytometry, permitted to identify unculturable bacteria. An interesting approach was proposed at the end of the 1970s, according to which the presence/absence of bacteria could be linked to microflora associated characteristics (MACs) [14]. For example, the content of short chain fatty acid production, which has been one of the most studied MACs [15], is in relation to the amount, in the intestine, of bacterial species producing these compounds as a result of the bacterial fermentation of dietary fibers.
In the late '70, Carl Woese proposed the gene encoding the 16S ribosomal RNA (16S rRNA) as potential marker for taxonomic classification [16] at the same time when the Sanger sequencing method took its first steps [17]. The combination of such theoretical and technical advances brought a new way of approaching the ecology and classification that was indeed revolutionizing. 16S rRNA gene has become popular in scientific and clinical communities because it is present in all bacteria, includying in multiple copies, and its function during evolution is very well-conserved [18, 19].
At the beginning several DNA-based approaches for the analyses of 16S rRNA gene have been implemented in the field of microbiota research from the most basic like temperature gradient and denaturing gradient gel electrophoresis to more advanced as phylogenetic microarrays. While such methodologies allowed to overcome most limitations of culture techniques (*e.g.* setting anaerobic conditions or particular growth media), they were not able to provide large-scale analyses of microbiota, like high-throughput sequencing [20].

The term "metagenomics" was coined by Jo Handelsman in 2004 [21] to indicate the applications of high-throughput sequencing of DNA directly extracted from a microbial community. Amplicon-based sequencing and whole metagenome shotgun sequencing (WMGS; also called metagenomic next-generation sequencing, mNGS) represent the two branches of metagenomics that allow to study and characterize the composition of intestinal microbiota (Figure 2A).

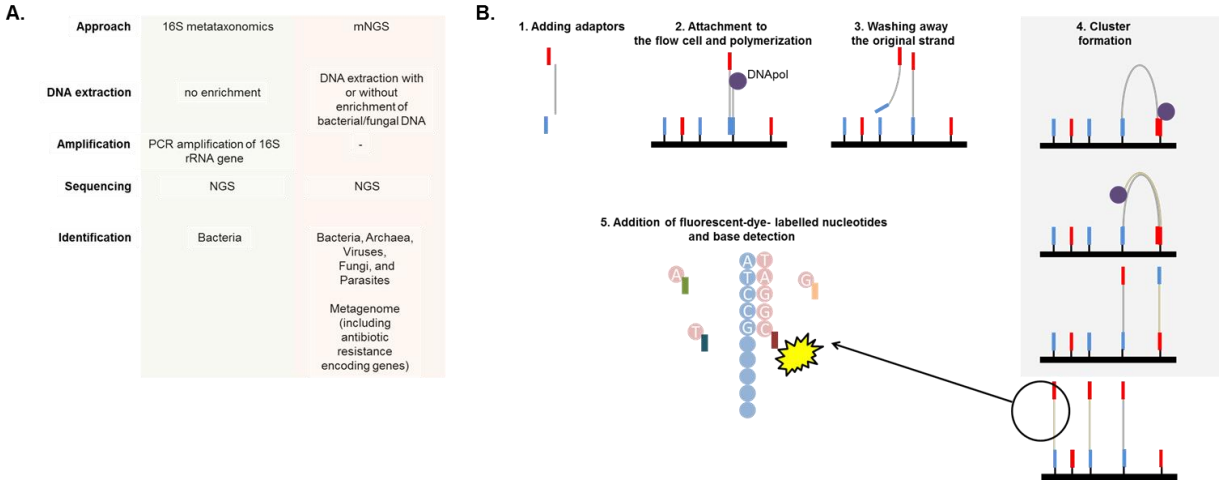

Figure 2. Metagenomics and Illumina sequencing.
A. Main differences between 16S metataxonomics and metagenomic next-generation sequencing (mNGS). B. Principles of Illumina sequencing (redrawn and adapted from Wikipedia, https://en.wikipedia.org/wiki/Illumina_dye_sequencing, and from YouTube Illumina tutorial, https://www.youtube.com/watch?v=fCd6B5HRaZ8&t=11s). 1. A library of small DNA fragments is prepared by attaching adaptor sequences to the ends of the filaments. 2. Adaptors allow the DNA to adhere to a glass flow cell. A polymerization reaction is triggered to create a complementary strand attached to the flow. 3. The double strand is denatured and the original fragment is washed away. 4. The strand binds a neighbouring flow cell sequence to form a brigde-like structure. A polymerization reaction allows to create a complementary strand. This process (in grey shade) is repeated several times to form cluster of identical and complementary sequences. 5. Before sequencing, the reverse strands (represented with red anchors to the flow cells) are cleaved. At this stage, the synthesis of a new strand is carried in presence of the four nucleotides labelled with a fluorescent dye. Once a nucleotide binds to the reference strand, fluorescence is detected, the fluorescent dye cleaved and a new elongation step occurs. This step is repeated for a number of cycles corresponding to the desired length of the read. The sequencing of reverse strands is carried out by denaturing the double strand synthesized during sequencing, repeating step 4. and by cleaving the forward strands before sequencing in step 5.

In amplicon-based sequencing approach (also known as targeted metagenomics or metataxonomics), 16S rRNA gene is conventionally chosen as taxonomic marker (16S sequencing or 16S metataxonomics).
16S rRNA gene is reported to be 1541 bp long in *E. coli* [22] and it is composed of nine clade-specific variable regions (indicated as V1-9) separated by inter-species conserved

regions (Figure 3). Variable regions are characterized by different taxonomically discriminative power (Figure 3). Targeted metagenomics analyzes only some of the 16S rRNA variable regions either individually (*e.g.* V4 or V6) or in groups of neighbouring regions such as V1–V3, V3-V4 or V3–V5 [23-25].

Compared to metataxonomics, WMGS does not necessitate a particular marker to be pre-selected and PCR-amplified. Furthermore, WMGS allows the analyses of a broader spectrum of intestinal microorganisms which includes Archaea, Fungi and Viruses beyond Bacteria. In this case, the whole DNA content is fragmented and processed, thus permitting the analyses of all microbiota genomes and genes (defined together as metagenome [1]), including those encoding for antibiotic resistance.

Illumina represents one of the most used next-generation sequencing technologies [26] (Figure 2B). Illumina sequencing needs the preparation of a library of short DNA fragments attached to adaptor sequences, which allow the DNA filaments to adhere to a glass flow cell. Then, flow cells are incubated with reagents and a polymerase to trigger several cycles of DNA amplifications to form clusters of identical filaments. Sequencing is performed by incorporating each time a nucleotide carrying a fluorescent dye. Fluorescence is, eventually, read by a detector and the dye detached from the newly synthetized strand, thus allowing a new cycle of sequencing to start [27].

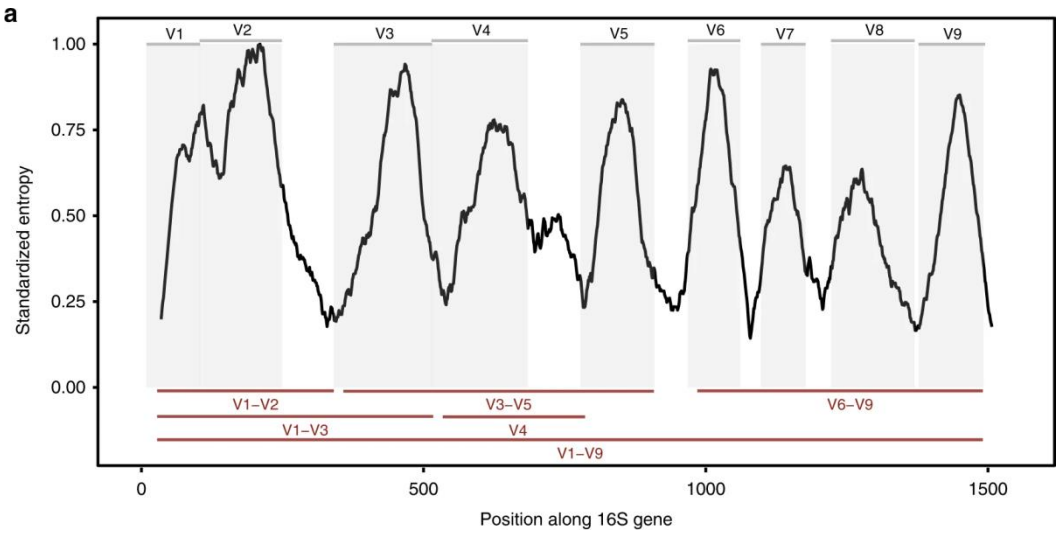

Figure 3. Differential taxonomically discriminative powers of variable regions (V1-9) from 16S rRNA gene.

Shannon entropy, which is a measure of species diversity, is plotted versus nucleotide positions along 16S rRNA gene. The plot was taken from Johnson *et al.* [24].

1.2 The "practice" of metagenomics: bioinformatics tools, databases sources and statistical approaches

Nucleotide alignments are pairwise comparisons of sequences which allow to understand genetic and phylogenetic relationships.
When a taxonomic marker is the object of the alignment, we can analyse the relatedness of a group of species and therefore determine their taxonomy which consists of hierarchical categories that are named from the highest to the lowest as: phylum, class, order, family, genus, species, and strain. Generally a sample sequence, called *query*, is aligned against another one, called *reference*, for which the taxonomy is known.
There are two main approached designed as global [28] or local alignments [29] (Figure 4). While in global alignment sequences are compared with an end-to-end approach, local-alignment-based algorithms (*e.g.* initially BLAST [30]) analyze partial matches between the query and reference sequences. Thus, if the two compared sequences are quite different, we can end up with more gaps in a global alignment than in a local-based approach (Figure 4). The sequence identity is expressed as percentage of nucleotides matching between the two sequences; it is therefore a measurement of their relatedness.

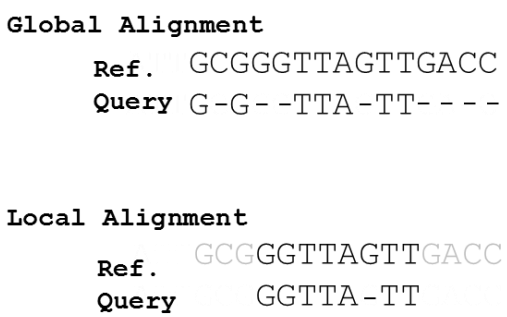

Figure 4. Comparison between global and local alignments.

The output of Illumina technologies consists of very large files, called FASTQ, that can contain millions of short sequences (*i.e.* ≤ 300 bp) that are called *reads*. To gain information from such large data, metagenomic bioinformatics tools align and query reads against sequences that are deposited into reference databases.
Importantly, with metataxonomics, reads obtained from sequenced PCR-amplified 16S rRNA gene are first compared to each other and cluster on the basis of their sequence identity to constitute operational taxonomic units (OTUs). Sequence identity cut-offs used for OTU definition are conventional values (97% [31] or 98.7–99% [32]) that are thought to be able to discriminate species between each other. This means that an OTU could be considered as a bacterial species. Reads from each OTU cluster are then aligned to generate a consensus sequence that will be queried against a reference

database to obtain the taxonomy. Mothur [33], UPARSE [34] and QIIME [35] are the most popular tools for OTU definition in the metagenomic community. Importantly these tools can eliminate chimeric sequences coming from PCR reactions. Among reference databases used in metataxonomics, we can rely on 16S Greengenes [36], SILVA [37] and RDP [38], which can have manually-curated sequences and can count references also from other markers, *i.e.* prokaryotic 23S ribosomal RNA gene and eukaryotic 28S ribosomal RNA gene for Fungi identification. Importantly, taxonomic assignment of some OTUs, especially those representing uncultured bacteria, can stop to the genus or to higher taxonomic ranks.

Another approach to characterize the composition of microbiota, consists in querying WMGS reads against gene catalogues like MetaPhlAn2 [39]. MetaPhlAn2 can analyze FASTQ files and blasts reads against a reference database of non-16S-rRNA genes that are specific of a given group of taxonomically-related bacteria (called *clades*) obtained from roughly 17,000 reference genomes. It then normalizes the abundance of these markers in the sample according to the size of the genome, to which they belong.

There are softwares like CLARK [40] and Kraken2 [41] that are based on the comparison of k-mers from the query and reference genomic sequences. K-mer method decomposes sequences in short fragments through a sliding window of a pre-selected length k (Figure 5A). CLARK and Kraken2 have two different approaches to assign a read to reference genomic taxa. Kraken2 bases its analyses according to a vertical scheme where k-mers of a query sequence are mapped to different taxonomic level of a given clade (Figure 5B). The query read is assigned to the lowest taxonomic rank, called *lowest common ancestor*, with which the read shares the highest number of k-mers.

On the other hand, CLARK first searches for unique k-mers that are specific of each reference species (target-specific k-mers). Then, the k-mers of the query read are mapped against the obtained collection of target-specific k-mers. The read is assigned to the species sharing the highest number of k-mers (Figure 5C).

The advantage of using k-mer-based approaches compared to other traditional methods is in the fact that k-mer-based tools are faster in performing the taxonomic assignment of reads [40, 41].

Irrespective of the methodology used for sequencing and taxonomic identification of microbial community, the output is a table reporting the read counts for each taxon (or each OTU) in each sample. The composition of microbial communities in the dataset is first assessed by analyzing *alpha* and *beta diversity*. While alpha diversity reflects the compositional variability in a given sample, beta diversity allows to compare differences in microbiota composition between samples [42].

Alpha diversity is expressed with ecological indices like richness and Shannon diversity. The former counts the number of different species in a sample while the latter is a mathematical expression that takes in account not only the number of different species but also their abundances. Importantly, to have a fair comparison of alpha diversity between samples, it is important that rarefaction is performed on read count table before computing ecological indices. This step consists in randomly

sampling read counts so that each specimen of the dataset will have the same total number of reads. Beta diversity, instead, is generally expressed by ecological distances or dissimilarities which can be computed with different methods, *i.e.* Bray-Curtis, UniFrac and weighted UniFrac distances [42]. Bray-Curtis dissimilarity is a value between 0 and 1 and that quantifies how much two microbiota profiles are similar to each other (0= equal composition; 1= completely different). Dissimilarities can be used to generate ordination plots, *e.g.* principal component analyses, where differences between microbiota profiles can be visually inspected.

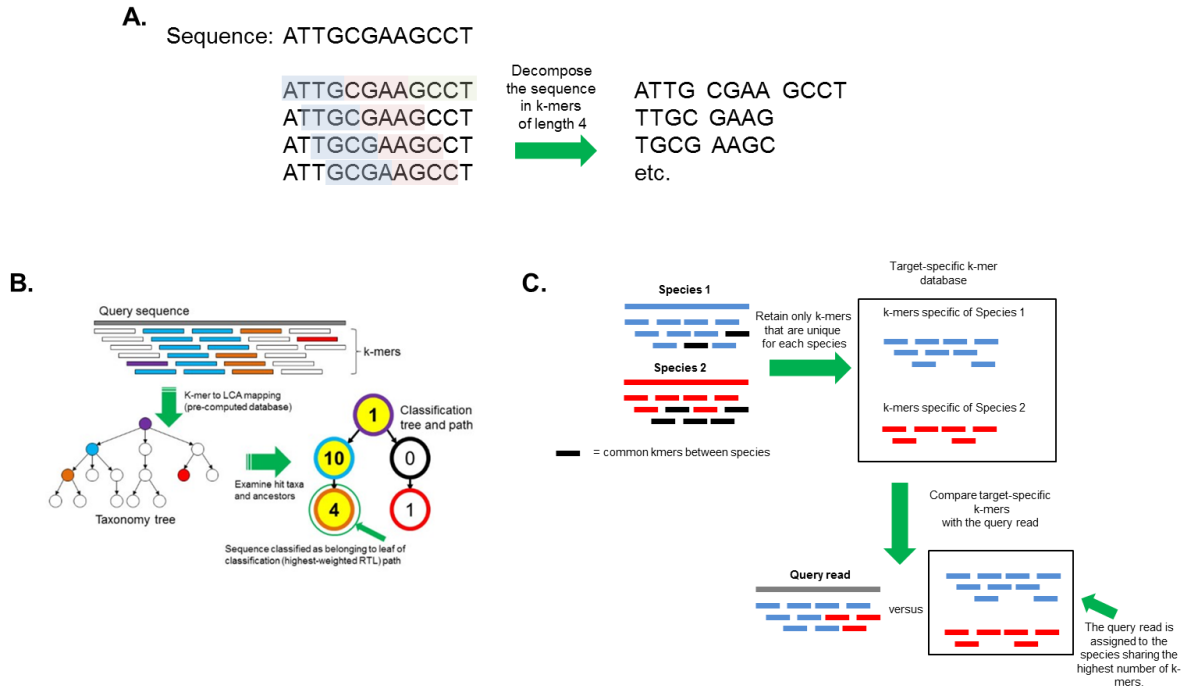

Figure 5. k-mer-based tools for metagenomic analyses.
A. A sequence is decomposed in small fragments of length k (in the example k=4). B. A query read is assigned to a given taxon by Kraken2 according to a vertical scheme. Kraken2 identifies the lowest taxonomic rank (lowest common ancestor) with which the query read had the highest number of k-mers in common. C. CLARK first creates a database of unique species-(or target-)specific k-mers from the reference sequences. Second, it maps query reads against such database and assign them to a given species based on the number of shared k-mers. Panel B is taken from Wood *et al.* [41] (License number 5021850040555 released by Spring Nature on March 04, 2021).

Apart from species identification, WMGS offers also the opportunity to perform functional analyses as for example to investigate the content of antibiotic resistance encoding genes. ResFinder is a public database containing more than 1,000 genes encoding resistance against 12 different antibiotic classes [43]. Other ARG databases include CARD (Comprehensive Antibiotic Resistance Database) [44] and ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) [45].

Inferential statistics, generally non-parametric, is applied to find significant association of microbiota composition with the occurrence of a given disease (*e.g.* Permutational multivariate analysis of variance; PERMANOVA [46]) or more in details to detect taxa that are differentially abundant between two conditions (*e.g.* Wilcoxon tests, DeSeq2 [47], ANCOM2 [48], or LEfSe [49]). Machine-learning algorithms, *i.e.* random forest, have been applied to predict the composition of intestinal microbiota after fecal transplantation intervention [50].

Chapter 2: Intestine and gut microbiota

Advances in culture-independent techniques, together with the development of sophisticated bioinformatics tools, allow to study microbiota and its composition in the intestinal tract. However, to understand the functioning of microbiota, we need to know the identity and roles of its components but also the characteristics of its environment that is the gut.

2.1 The intestinal tract: anatomy, histology and physiology

Intestines are the lower part of the digestive tract and can be divided in small and large intestines (Figure 6).
Small intestine extends for 6 to 7 meters from the pylorus of the stomach to the ileocecal valve and is subdivided in three regions, from the most proximal to the most distal: duodenum, jejunum, and ileum [51]. 90% of water and most nutrients, including proteins and carbohydrates are digested and absorbed in the small intestine [52].
The large intestine, or also called colon, is roughly 1.5 meter long [52] and from an anatomic point of view, is characterized by four sections: 1) the ascending colon with cecum and appendix, 2) the transverse colon, 3) the descending colon and 4) the sigmoid colon. The intestinal tract ends with rectum, through which feces are collected and expelled. The role of the large intestine is to complete the absorption of nutrients, including K and B vitamins produced by colonic bacteria, and to finalize the absorption of water, started in the small intestine, to transform the chyme (indigested food material by the small intestine) into feces.
As a reflection of the different roles that they have in the digestion, the two intestines have also different environmental characteristics. For example, the large intestine, where there is slower transit of food and less secretion of enzymes is characterized by a neutral to mildly acidic pH and decreased oxygen concentrations as compared to the small intestine.
Looking at the cross-sectional structure of the intestines (Figure 6), we can distinguish four layers represented by mucosa, submucosa, muscular layer, and adventitia. Submucosa is a connective tissue formed by a thin matrix enriched in collagen that connects the mucosa to the muscle tissue which allows the food bolus to transit by the gut movements called *peristalsis*. Finally, adventitia is fibrous connective tissue consisting of a visceral membrane and a parietal layer.
Mucosa consists of specialized intestinal epithelial cells (IECs) of different types (*e.g.* enterocytes, Paneth cells, goblet cells, endocrine and exocrine cells) and of a vascularized lamina propria that allows oxygen exchange [51].
Enterocytes have a columnar polarized structure and they are directly involved in the absorption of nutrients thanks to membrane extensions called *microvilli*. The function of microvilli is to enlarge the surface for nutrient absorption. Goblet cells secrete

mucus that has a protective function against pathogens and digestive enzymes. Paneth cells, which are present only in the mucosa of the small intestine, are located below the stem cells layer of the mucosa and can detect invading microbes and secrete peptides with antimicrobial activity. Neuroendocrine cells are important in the neuronal regulations of the digestion. Exocrine cells contribute to the formation of mucus and secrete different types of digestive enzymes, while endocrine cells can secrete the hormones *cholecystokinin* and *secretin* and their activity is directly controlled by the amount of chyme. The turn-over in the replacement of the mucosa with new IECs is reported to be of 4-5 days [51].

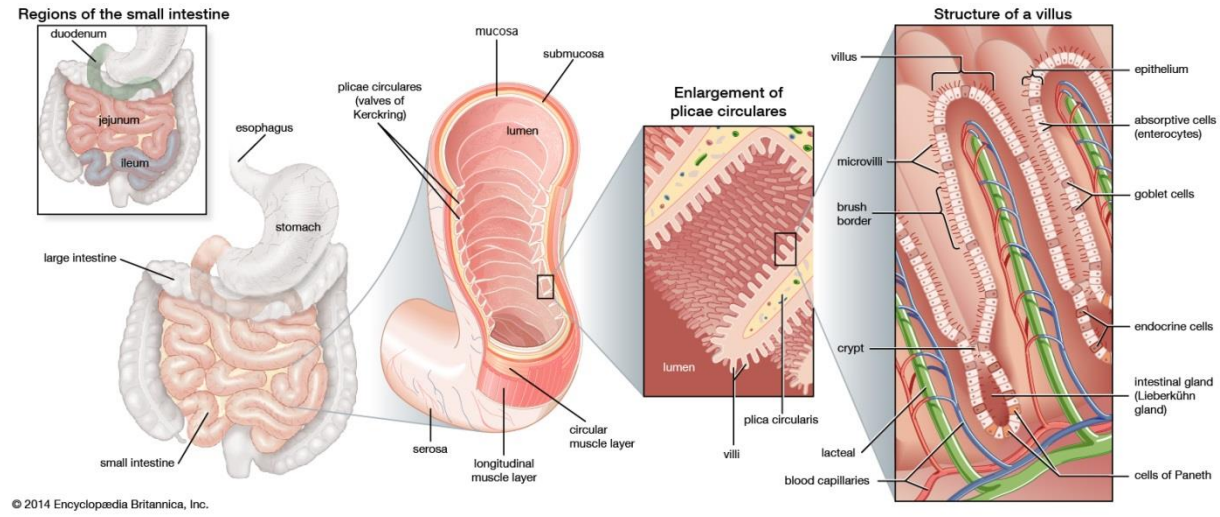

Figure 6. Small intestine: anatomy, histology and cytology.
The scheme recapitulates also the main histological and cytological characteristics of human colon (the picture was taken from Encyclopedia Britannica, https://www.britannica.com/science/small-intestine).

2.2 Stools and gut microbiota composition

Human intestinal microbiota is typically analyzed on stools and rectal swabs. Stools represent the final product of food digestion. In clinical practise, feces are divided in seven classes based on their consistency with the Bristol stool form scale. The inorganic characteristics as pH and water content and overall composition of organic components are dependent mostly on diet and presence/absence of diarrhea [53]. On average, stools from healthy individuals have a moderately acidic pH (*e.g.* 6.64) and are made of more than 70% of water [53]. The fecal organic fraction includes mostly bacteria (corresponding to 25–54% of the biomass of dry stools) but also macromolecules (*e.g.* proteins, carbohydrates and lipids) that are not digested in the intestine [53]. Although bacteria are found in several sites of the human body, the colon contains ~99% of them. The amount of bacterial cells in the colon is estimated between 10^{13} and 10^{14} [54]. This is likely due to the optimal microenvironment

conditions favoring microbial growth (*i.e.* longer permanence of chyme due to reduced peristalsis [54]). Microbiota microorganisms have co-evolved with humans [55] and diet represents the main driving factor shaping microbiota composition [56].
To date, it has still to be clarified whether density of bacteria in feces is comparable to that present in colon [54]. A study performed on chickens revealed that stools (*fecal microbiota*) only partly reflects the intestinal microbial composition [57]. In this thesis, the term fecal microbiota is used as proxy of intestinal microbiota.
Based on the whole gene content, gut microbiota is prevalently composed by Bacteria (99.1%), by few eukaryotic microorganisms and Archaea (0.1%) [58] and by viruses (<0.1%). The human intestinal microbiota gene collection contains ~3.3M genes, which is estimated to correspond to roughly 1,000 bacterial species, each containing 3,364 non-redundant genes [58]. The number of bacterial species per individual was, instead, estimated to be around 160 [58].
The bacterial fraction of intestinal microbiota is constituted by two major phyla, Bacteroidetes and Firmicutes, which together with minor phyla (Actinobacteria, Tenericutes, Fusobacteria, Proteobacteria, Verrucomicrobia, Synergistetes and TM7 candidate phylum) represent the *core* microbiota. About 90% of intestinal bacterial species belong to Firmicutes and Bacteroidetes [59]. The ratio Firmicutes-to-Bacteroidetes is often cited in numerous works as important parameter associated for example to obesity but also to other conditions [60].
In the following sub-paragraphs I will summarize the most important characteristics of each phylum and briefly describe their most important species. I would like to mention that my aim in writing these paragraphs is far from giving a detailed and full description of all bacterial species present in the digestive tract but rather to highlight some important microorganisms that will be useful to know to understand the results reported in this manuscript. The content of the following sections is partly based on the work by Rajilić-Stojanović *et al.* [10]. I would like to underline that to date it represents the most accurate, informative and complete review on the human intestinal microbiota species.

2.2.1 Firmicutes

This phylum includes prevalently Gram-positive species and more than 200 different genera [59], which are mostly anaerobic. The high diversification of Firmicutes explains why this phylum does not only include important commensals, but also difficult-to-treat pathogens like *Clostridioides difficile*. In gut microbiota we can find four major Firmicutes classes: Bacilli, Clostridia, Erysipelotrichia and Negativicutes.
Bacilli include lactic acid producing *Lactobacillus* spp., while Clostridia are characterized by a more diversified group of bacteria belonging to the families of Clostridiaceae, Eubacteriaceae, Lachnospiraceae, Ruminococcaceae and Peptostreptococcaceae.

Coprococcus spp., *Roseburia* spp., *Eubacterium rectale*- and *Eubacterium hallii*-related species from Lachnospiraceae and *Fecalibacterium prausnitzii* from Ruminococcaceae are all producers of butyrate, a short chain fatty acid that we will see to be implicated in the homeostasis of intestinal epithelium.

2.2.2 Bacteroidetes

Bacteroidetes are constituted prevalently by Gram-negative microorganisms and include *Alistipes*, *Bacteroides*, *Prevotella*, and *Barnesiella* genera. They can metabolize carbohydrates as well as proteins. Bacteroides is the most abundant genus (Figure 7) and includes species which can grow in the mucus secreted by the intestinal epithelium and metabolize proteins through a surface-bound proteolytic enzyme.

2.2.3 Actinobacteria

The Actinobacteria phylum is characterized by Gram-positive species and include the first colonizers of human intestinal lumen in infants [10]. The phylum contains species from *Bifidobacterium*, *Propionibacterium*, *Corynebacterium*, and *Rothia* genera. *Bifidobacterium* species are anaerobic and lactate- and acetate-producers which are part of the core microbiota and which are known to have a beneficial effect on human intestinal health; they are also administered as probiotics [10, 61].
These bacteria have a peculiar way to digest dietary or host-derived complex carbohydrates, which are resistant to the degradation performed in the upper digestive tract. This metabolic pathway is called *bifido shunt* and includes the fructose-6-phosphoketolase enzyme which is used as a taxonomic marker for the Bifidobacteriaceae family, to which *Bifidobacterium* genus belongs [62]. The *bifido shunt* pathway allows *Bifidobacterium* members to increase their ATP production as compared to a classic lactic fermentation.
Propionibacterium and *Corynebacterium* spp. are commensals of skin microbiota and their intestinal colonization is reported in infants born with Caesarean section (C-section) [10]. *Propionibacterium* spp. exert the major proteolytic activity in the intestine and they are the major producers of B12 vitamin.

2.2.4 Minor bacterial phyla and non-bacterial components of intestinal microbiota

Minor bacterial phyla of intestinal microbiota include: Proteobacteria, Verrucomicrobia, Tenericutes, TM7 candidate phylum. Proteobacteria includes the Enterobacteriaceae family, which will be discussed in further details later. The abundance of Enterobacteriaceae in the intestine increases with aging; however, it is reported to constitute less than 1% of the biomass in healthy individuals. We will see that this family includes clinically important diarrheagenic pathogens among which

some *E. coli* strains. Verrucomicrobia includes *Akkermansia muciniphila* which is considered as part of the core microbiota [10].

Non-bacterial components include Archaea, Viruses and eukaryotic microorganisms. Among archeal species, the most abundant are methanogenic bacteria *Methanobrevibacter ruminantium* and *M. smittii*. The viruses detected from the gut microbiota (collectively called "virome") are mostly double-stranded (ds) or single-stranded (ss) DNA or RNA phages. Most intestinal phages are dsDNA phages belonging to Caudovirales order (Siphoviridae, Myoviridae, and Podoviridae) and ssDNA phages from Microviridae family [63, 64]. The vast majority of sequences obtained from intestinal phages (75% to 99%) are poorly related to known genomes in terms of sequence similarity [65]. Phages seem to affect the composition of intestinal bacteria; for example in patients suffering from inflammatory bowel disease (IBD), loss of *F. prausnitzii* is accompanied by a significant increase in the abundance of *F. prausnitzii*-specific phages [66].

Eukaryotic microorganisms ("eukaryome") consist of Fungi ("mycobiome"), protozoans and can also include metazoan parasites [67, 68]. Fungi are manly represented by *Saccharomyces*, *Candida* and *Malassezia* genera [67]. The protist *Blastocystis* is frequently found in the intestinal microbiota of healthy individuals [68, 69].

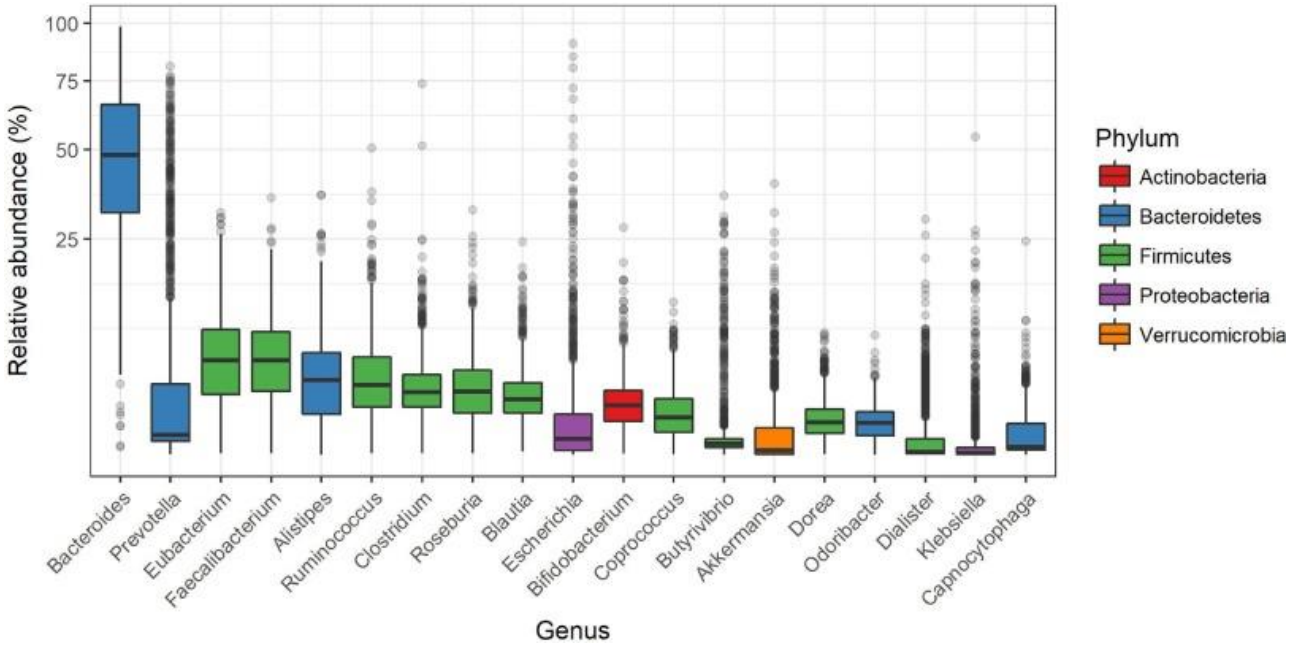

Figure 7. Most abundant intestinal bacterial genera detected over ≥ 1000 individuals.
Relative abundance for each genus is reported with boxplots coloured according to phyla. The plot was taken from Woerther *et al.* [70] (License number 5000210352032 released by Elsevier on Feb. 01, 2021).

2.3 Development of human intestinal microbiota from birth to the adult age

In Mammals, the acquisition of microbiota occurs from mother to offspring through routes of birth delivery and lactation [71].
Importantly, routes of birth delivery - vaginal or C-section - were shown to affect early gut microbiota composition. Vaginal delivery is associated with the presence in different body sites, including meconium, of species typical of vaginal microbiota (*e.g. Lactobacillus, Prevotella*, or *Atopobium* spp.), whereas children born by C-section have increased proportions of skin microbiota species *e.g. Staphylococcus* spp. [72].
From birth to adulthood, microbiota undergoes several changes through three main shifts in composition [56].
Diversity of early microbiota of vaginal delivered babies is poor and it consists of facultative anaerobes like *Streptoccoccus*, *Escherichia* and *Staphylococcus* species which are later substituted by obligate anaerobes from Firmicutes and Actinobacteria [56]. Lactic-acid producers such as *Lactobacillus, Enterococcus* and *Clostridium* species are the most abundant among Firmicutes whereas Actinobacteria mostly consist of *Bifidobacterium* spp. (Actinobacteria) which digest the oligosaccharides provided in breast milk.
The second shift occurs at 1st-2nd year, when solid food is introduced in the diet. At this point *Bifidobacterium* species decrease and leave space to Bacteroidetes species. Eventually when the baby is 18-36 months old, microbiota undergoes its final shift and at this stage is mainly constituted by Firmicutes and Bacteroidetes as a consequence of a more varied solid diet [56].
Adult microbiota is relatively stable in composition and resilient to perturbations. Accordingly, it was estimated that 60-70% of the most abundant bacterial strains remained unchanged during a 5-year study conducted on 37 adults [73]. The abundance and diversity of Actinobacteria and Bacteroidetes were also reported to be more stable than those of Firmicutes and Proteobacteria [73].

2.4 Enterotype, dysbiosis, pathogen colonization and infection

In the 2010s Arumugam *et al.* [74] classified individuals based on their intestinal bacterial composition in three main clusters, which were called enterotypes, each characterized by a dominant genus: *Bacteroides*, *Prevotella* and *Ruminicoccus.* While *Bacteroides* was found more frequently associated to fat rich Western diet, *Prevotella* was associated to plant fiber consumption [75]. However, a study published later demonstrated only marginal segregation between *Bacteroides* and *Prevotella* enterotypes [76]. According to Koren *et al.* [77], microbiota profiles span along a gradient with increasing abundance in *Bacteroides*.
Healthy individuals with a similar body mass index were found with a similar fecal metabolomic profile but with a distinct fecal taxonomic composition, meaning that, to some extent, microbiota species are likely to be functionally redundant [78, 79]. Thus,

we can intuitively understand that in reality the definition of a healthy microbiota is difficult if this is based exclusively on species abundances and composition which vary from person to person.
Therefore, intestinal microbiota could be thought as a dynamic steady-state system where there is an ecological balance between microbial species. Shifts in composition from this steady-state system can lead to *dysbiosis*, a condition characterized by unbalanced microbiota components and changes in microbial functional processes at transcriptomic and metabolomic level (reviewed in [80]). A *dysbiotic* microbiota can lead to numerous disorders as IBD, infection, obesity and so on. The mechanisms leading to dysbiosis are multiple and include pro-inflammatory events, antibiotic treatment and changes in diet [80].
With the term *colonization*, we define the presence of microorganisms which were not previously part of *resident* intestinal microbiota [81]. Intestinal colonization of a pathogen can lead to an *asymptomatic carriage* for a variable duration and can depend on the physiological circumstances which might favor (or not) pathogen survival and growth. Importantly, dysbiosis can be the effect but also the cause of intestinal colonization by pathogens [80]. Following intestinal colonization, asymptomatic carriage can represent a *reservoir* of the pathogen in a given population or community and therefore also being a fuel for pathogen spreading.
Infection occurs with the invasion of the pathogenic microorganisms into the human body and with the intervention of immune system defences [81]. According to some authors, infection first requires colonization to occur, according to others, these should be considered as two independent and distinct events [81].

2.5 Species producing short chain fatty acids

Short chain fatty acids (SCFAs) are one of the most important compounds produced by microbiota bacteria which have a fundamental role in the maintenance of intestinal epithelium and colonization resistance (discussed in the next Chapter).
Three major SCFAs are synthetized from dietary fibers by bacterial microbiota and they are acetate, propionate, and butyrate. They can be chemically described as carboxylic acids having an aliphatic tails of two (acetate), three (propionate) or four carbons (butyrate) [82].
While acetate is produced from fermentation or acetogenesis from a large amount of bacteria, propionate and butyrate are generated only by a particular subset of species (Table 1).
Importantly, the use and the tissue distribution for these three SCFAs vary: butyrate is present in the colon and used as source of energy by colonic mucosa; propionate is a substrate of gluconeogenesis occurring in the liver; acetate is highly abundant in blood (reviewed in [83]). Moreover, the concentration of a given SCFA is also dependent on the accumulation of precursor compounds (some examples are given below).

SCFAs produced by anaerobic bacteria are then absorbed by intestinal mucosa [84]. The interaction of these three SCFAs with receptors present in different types of tissue (*e.g.* adipose, intestine and immune cells) can vary [85].
Butyrate and propionate can be produced by degradation of carbohydrates but also from amino acids, lactate and succinate [83].
The synthesis of butyrate from carbohydrates occurs after glycolysis when two molecules of acetyl-CoA are bound to form acetoacetyl-CoA which is then transformed into butyryl-CoA [83]. The final step occurs then with two different mechanisms: either the intervention of a CoA-transferase or of a butyrate kinase. Butyrate production via acetoacetyl-CoA is typical of *F. prausnitzii* (Ruminococcaceae; Firmicutes), *Eubacterium rectale* and *Roseburia* species (Lachnospiraceae; Firmicutes) (reviewed in [83]). *Anaerostipes hadrus* and *Coprococcus catus* from Lachnospiraceae can also produce butyryl-CoA:acetate CoA-transferase [83, 86].
Butyrate formation via butyrate kinase was found in two species of *Coprococcus* [87, 88], thus butyrate production in Lachnospiraceae can occur with both the above-mentioned mechanisms (*e.g. via* butyrate kinase or *via* CoA-transferase). Propionate synthesis from succinate is found prevalently in Bacteroidetes and in the Negativicutes class of Firmicutes. Importantly, succinate accumulation in the gut is due to the presence of *Prevotella copri* (Bacteroidetes) and some Ruminococcaceae, like *Ruminococcus flavefaciens*. In fact, these bacteria use fermentation to produce succinate rather than propionate as end product (reviewed in [83]). Succinate is then converted in propionate by some Negativicutes, like *Phascolarctobacterium succinatutens*. Propionate can also be synthetized from 1,2-propanediol by various Firmicutes species: *Roseburia inulinivorans* and *Blautia* species (Lachnospiraceae); *E. hallii*, *Lactobacillus reuteri*, *Flavonifractor plautii*, *Intestinimonas butyriproducens* and *Veillonella* spp. [83]. Intestinal accumulation of 1,2-propanediol is also due to its production from *Bacteroides* species, *E. coli* and *Anaerostipes rhamnosivorans*, *Clostridium sphenoides* and the yeast *Saccharomyces cerevisiae* [83]. The synthesis of butyrate and propionate from amino acids has been reported in some *Clostridium* species and several Bacteroidetes and Firmicutes [83].

Table 1. Most important microbiota species that produce butyrate and propionate.
The table, including its legend, was taken from Louis *et al.* [83]. Permission obtained by "John Wiley and Sons" publisher (license number 4996400228667 released on Jan. 26, 2021).

Phylum (family)	Species	Butyrate	Propionate
Bacteroidetes (Bacteroidaceae)	*Bacteroides uniformis*	−	+ (Suc)
	Bacteroides vulgatus	−	+ (Suc)
Bacteroidetes (Prevotellaceae)	*Prevotella copri*	−	+ (Suc)
Bacteroidetes (Rikenellaceae)	*Alistipes putredinis*	−	+ (Suc)
Firmicutes (Lachnospiraceae)	*Eubacterium rectale*	+ (CoAT)	−
	Roseburia inulinivorans	+ (CoAT)	+ (Pdu)
	Roseburia intestinalis	+ (CoAT)	−
	Dorea longicatena	−	−
	Eubacterium hallii	+ (CoAT)	+ (Pdu)
	Anaerostipes hadrus	+ (CoAT)	−
	Ruminococcus torques	−	−
	Coprococcus eutactus	+ (ButK)	−
	Blautia obeum	−	+ (Pdu)
	Dorea formicigenerans	−	−
	Coprococcus catus	+ (CoAT)	+ (Acr)
Firmicutes (Ruminococcaceae)	*Fecalibacterium prausnitzii*	+ (CoAT)	−
	Subdoligranulum variabile	+ (ButK)	−
	Ruminococcus bromii	−	−
	Eubacterium siraeum	−	−
Firmicutes (Veillonellaceae)	*Dialister invisus*	−	+ (Suc)
Firmicutes (Acidaminococcaceae)	*Phascolarctobacterium succinatutens*	−	+ (Suc)
Firmicutes (Erysipelotrichaceae)	*Eubacterium biforme*	+ (CoAT)	−
Actinobacteria (Bifidobacteriaceae)	*Bifidobacterium adolescentis*	−	−
	Bifidobacterium longum	−	−
Actinobacteria (Coriobacteriaceae)	*Collinsella aerofaciens*	−	−
Verrucomicrobia (Verrucomicrobiaceae)	*Akkermansia muciniphila*	−	+ (Suc)

− = absent; + = present, ButK= butyrate kinase; CoAT = butyryl-CoA:acetate CoA-transferase route; Acr = acrylate pathway; Pdu = 1,2-propanediol pathway; Suc = succinate pathway (succinate may be the major product formed instead of propionate in some species and/or under some growth conditions).

Chapter 3: Interactions between intestinal microbiota and immune system and colonization resistance

The digestive tract represents a site for pathogens to enter our body and cause infection. Therefore, systems of protection need to be in place in the gut. What is the role of the intestinal microbiota? How does it behave when a pathogen colonizes and invades the intestine? In this chapter, I will describe how the human immune system acts in the intestine and how microbiota participates to the immune defence.

3.1 Immune responses in the intestinal tract

Intestines are characterized by the presence of gut-associated lymphoid tissues (GALTs) which are follicle-like structure mediating adaptive immune responses [89].
GALTs are composed by microfold cells (M cells), dendritic cells and adaptive immune cells B and T. M cells and dendritic cells mediate the uptake from the lumen and presentation of antigens to B and T cells. In the small intestine, GALTs are called Peyer's patches and are formed mostly by B cell lymphoid follicles interspaced by areas containing T cells. Structures similar to Peyer's patches are also found in the human colon [90, 91].
Immune cells are distributed differently between the mucosal epithelial layer and lamina propria. In fact, the intestinal mucosal layer contains only T cells, whereas vascularized lamina, beyond T and B cells, has also innate immune system cells like macrophages, mast cells and eosinophils.
Intestinal epithelial cells play a pivotal role in the defence from pathogens and in the overall maintenance of gut homeostasis [92]. Through the production of mucus, IECs physically separate and mediate communication between commensal microbiota and human immune cells.
Besides production of mucus, which is thicker in the colon than in the small intestine [92], intestinal epithelial cells are also involved in innate immune responses. They can phagocyte pathogens and regulate inflammation *via* inflammasome [93]. Inflammasome, which is very well studied in macrophages, is constituted by protein complexes activated by cytoplasmatic receptors that can recognize highly conserved structures called pathogen-associated molecular patterns (PAMP), *e.g.* bacterial lipopolysaccharides (LPS), peptidoglycan and flagellin [93]. Once the inflammasome is activated, it induces the activation of pro-inflammatory cytokines and a particular type of immune-induced programmed cell death called *pyroptosis* [94]. Cytokines recruit immune cells to the site of inflammation and induce T cell activation [94].

3.2 Microbiota and intestinal inflammation

Microbiota commensals are not necessarily beneficial and might contribute to enhance inflammatory processes. For instance, microbiota enriched in Prevotellaceae family members (Bacteroidetes), have been shown to induce the production of the pro-inflammatory cytokine CCL5 [95]. *E. coli* uses the nitrates generated in inflammatory processes as substrate for its growth-promoting pathways [96].
On the other side, *Fecalibacterium prausnitzii* (Firmicutes) was shown to produce *in vitro* not-yet characterized compounds that inhibit the NF-κB activation and pro-inflammatory cytokine synthesis [97].
The production of SCFAs from the digestions of alimentary fibers is another essential mechanism through which microbiota tunes the inflammation state of intestinal epithelium.
Several SCFAs receptors exist. For example, GPR43 is present in IECs and intestinal immune system cells while GPR109a is found in various types of immune cells [85]. Activation potency of GPR43 varies according to SCFA: it is high for acetate and moderate for butyrate [85]. Importantly, butyrate prompts a strong activation of GPR109a. The SCFAs activate the inflammasome via GPR43 and GPR109a binding, stimulating tissue repair and contributing to the maintenance of the intestinal barrier [98]. SCFAs bind to GPCR43 receptor present on colonic regulatory T cells (T_{reg}) which then inhibit the pro-inflammatory activity of other immune cells [99].

3.3 Mechanisms of colonization resistance

In the early 1990s, the term "colonization resistance" was coined to define the ability of intestinal microbiota to contain the expansion of enterococci and Enterobacteriaceae pathogens in the gut [100, 101]. The advancements in molecular biology research have allowed to decipher many mechanisms through which bacterial commensals, prevalently from Bacteroidetes, Firmicutes and Actinobacteria phyla [102], limit the growth of pathogens within the intestinal lumen. We can classify these resistance mechanisms as direct or indirect [102].
Among direct mechanisms, commensal species inhibit pathogens through microbe-to-microbe interactions or compete with them for nutrients within the same ecological niches *e.g.* nutrient consumption.
A curious example of direct mechanisms is given by *Escherichia coli* Nissle 1917 (EcN 1917), which is a commensal species belonging to Enterobacteriaceae. EcN 1917 took the name from Prof. Alfred Nissle from Freiburg (Germany) who first isolated this strain in 1917, from the fecal samples of a soldier who was the only one among his comrades not to develop *Shigella*-induced diarrhea during a military mission in the Balkan Peninsula during the First World War [103]. Almost 100 years after EcN 1917 isolation, small proteins called *microcins*, produced by EcN 1917 were found to have a

selective antibiotic activity against adherent–invasive *E. coli* strains and *Salmonella enterica* [104].

Indirect mechanisms of colonization resistance consist of pathways through which commensal bacteria stimulate the host immune system. An example of indirect mechanisms is given by *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*. In murine cecum epithelial cells, these species were shown to promote the expression of interferon-stimulated gene-15 [105], an ubiquitin-like protein which plays a role in responses against viral infections (reviewed in [106]).

Importantly, there are species which use both direct and indirect mechanisms of colonization resistance [102].

In fact, some *Bifidobacterium* spp. isolated from human stools can inhibit the growth of the enterohaemorrhagic *E. coli* O157:H7 strains likely with the production of broad-spectrum anti-microbial compounds [107]. *Bifidobacterium* species produce acetate and butyrate which once protonated at low pH enter *E. coli* cells, acidifying the cytoplasm and thus inhibiting their growth [108]. Moreover, acetate stimulates the regeneration of intestinal epithelium therefore limiting the penetration of bacterial enterotoxins [109].

A further example of interplay between microbiota and immune system is given by the segmented filamentous bacteria (SFB), which are attached to the ileal epithelium and have genetic similarities to *Clostridium* [110]. SFBs indirectly prevent pathogen translocation and invasion by stimulating the production of immunoglobuline A by B cells [111]. In mice, SFB are indirectly involved in the recruitment of neutrophils by inducing the T helper (Th17) cell differentiation [112, 113], thus preventing the infection from *Citrobacter rodentium*, a mouse model of human enterohaemorrhagic and enteropathogenic *E. coli* [112]. SFB and LPS indirectly control the fucosylation status of intestinal *mucin*, a component of mucus. The fucose residue on mucin allows commensal bacteria to attach and grow locally where they compete with pathogens [114]. SFB prompt the release of cytokine IL-22, which induces the expression of 2-α-L-fucosyltransferase 2 (FUT2)[115] in goblet cells [116]. FUT2 catalyzes the fucosylation reaction of mucin.

Chapter 4: Antibiotics, intestinal microbiota and resistome

Antibiotics are broadly used as treatment against infectious diseases. However, bacteria can adapt and develop genetic resistance against antimicrobials.
What are the mechanisms of antibiotic resistance? Are they naturally present in microbiota or acquired? Furthermore, what happens to microbiota under antibiotic therapy? Let's analyse these points in the following paragraphs.

4.1 Antibiotics and their introduction in clinical practise

As we already seen in the paragraph 3.3, compounds with antimicrobial activity are naturally produced by microorganisms [117, 118] *e.g.* in competing for the same ecological niche but also in *quorum sensing* (reviewed in [119]).
The introduction of antibiotics in clinical practice indeed represented one of the most important milestones in modern medicine to successfully combat numerous infections that had previously caused millions of death worldwide.
The use of antimicrobial compounds is not as recent as we might think. In fact, already ancient Egyptians applied mouldy bread or medicinal soil to heal open wounds as reported in the Eber's papyrus from 1550 BC [120]. Infectious diseases were treated also with heavy metals, for example inorganic mercury salts were used to treat syphilis.
For the creation of the first synthetic drug, we had to wait for Paul Ehrlich. Almost 100 years ago, he produced an atoxyl-derivate, *compound 606* (or also named *salvarsan*), against *Treponema pallidum*, the causative agent of syphilis [121]. Alexander Fleming discovered penicillin in 1928 and in the late 1930s we started the industrial production of sulphonamide antibiotics [122].
Although hundreds of antimicrobial molecules exist nowadays, these drugs target and alter a few molecular processes that are indispensable for the bacterial cell division and growth, that is: cell envelope biogenesis, DNA replication, transcription and protein biosynthesis. Collectively, all these processes consume the major fraction of metabolic output of the cell *e.g.* protein synthesis requires 70% of ATP utilisation [123]. Figure 8 reports the main classes of antibiotics (and relative compounds) with their respective cellular targets.
Antibiotics can have either a bactericidal or a bacteriostatic effect. In the former case, the microbe dies, while in the latter case, the bacterial growth, and consequentially bacterial clonal expansion, is arrested. Importantly, the susceptibility to a given antibiotic depends on the metabolic status of the bacterium, and based on the circumstances, it can enhance or decrease the bactericidal effect of a drug [124, 125].

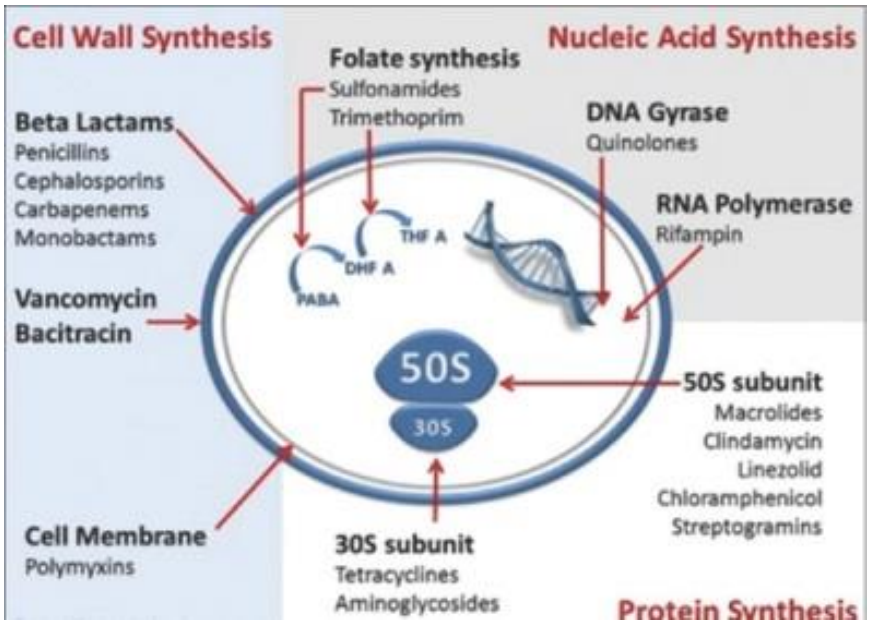

Figure 8. Mechanisms of action of antibiotics.
Antibiotic classes are associated with the targeted biological pathway. The figure was taken from the work of Kapoor and colleagues [126].

4.2 Antibiotic resistance: definition and general mechanisms

Antibiotic resistance (AR) is the ability of a pathogen to survive and grow when exposed to an antimicrobial drug. AR is a very ancient phenomenon occurring long time before the adoption of antibiotics in clinical practise [127]. In fact ARGs encoding resistance against β-lactams and tetracycline antibiotics have been found present in permafrost sediments dating back 30,000 years ago [127]. A possible reason of this phenomenon lies in the fact that, as mentioned above, antibiotics are naturally produced by microbial species.
In medicine, cultures of the isolated pathogens are usually exposed to different doses of a given antibiotic to define the minimum inhibitory concentration (MIC) that is the minimal amount of the drug for which bacterial growth is not observed *in vitro*. Concentrations of antibiotics, called *breakpoints*, allow the classification of the pathogen in antibiotic susceptibility categories such as resistant, intermediate and susceptible. Standardized guidelines, like those published by EUCAST, are implemented in clinical routine laboratories to help clinicians in selecting the most appropriate antibiotics to optimally target the drug regimen.
Antibiotic resistance occurs at different levels depending on the biology of the pathogen. Species forming biofilms, for example, make the penetration of the antibiotic more difficult and thereby reduce its efficiency [128]. From a genetic point of view, we can distinguish two main mechanisms of AR: intrinsic or acquired [129]. Intrinsic resistance solely depends on the physiology of the microorganism – for

instance, vancomycin is ineffective against Gram-negative bacteria as the molecule cannot penetrate the outer membrane.
Acquired resistance defines an ability that is developed by the microbe following a mutation in its genome or acquisition of new DNA though bacteriophages, plasmids or transposons [129]. Therefore, acquired resistance can be encoded by chromosomal and/or plasmid-borne genes.

4.3 The occurrence of multidrug resistance

Resistance to different types of antibiotics is due to the accumulation of several ARGs starting from a so-called founder element *e.g.* chromosomal mutations leading to the overexpression of resistance-encoding genes [128].
The constitution of multiple resistance regions reflects the continuous exchange between chromosomal material and the environmental ARG gene pool [128]. The presence of common sequences between chromosome and plasmid, for example, favor homologous recombination or gene mobilization events (*i.e.* gene capture in a plasmid or another mobile element). Multidrug resistant encoding genes present on the same chromosome or plasmid can be co-selected even when the pathogen is exposed to only one drug. For example, the treatment with the aminoglycoside antibiotic gentamicin can induce a co-selection in *E. coli* populations of those strains with plasmid harbouring extended-spectrum-β-lactamase- and gentamicin-resistance-encoding genes [128]. Importantly the ecological fixation of multidrug resistant genes, either chromosomal or plasmid-borne, in natural population will depend on the overall improvement in bacterial fitness that these genetic elements can offer under selective pressure [128].

4.4 General characteristics of human gut resistome

With the term intestinal *resistome*, we define all antibiotic resistance encoding genes (ARGs; also called antibiotic resistance determinants, ARDs) present in the gut and belonging to pathogenic or non-pathogenic species [130]. ARDs are mostly carried by microbiota commensal species, and apparently their transmission from a species to another within the intestine seems to be rare [131, 132].
Human intestinal resistome is set up soon after birth. Metagenomic studies highlighted the presence of ARGs in healthy neonates before antibiotic exposure (reviewed in [133]). A study conducted on twins revealed that newborns have a similar resistome that it is distinct from that of their mothers. This would mean that maternal microbiota is not a primary factor determining the composition of infant gut resistome which instead is affected also by environmental factors [134]. Resistome composition reflects the microbiota composition which we saw to change with aging. Thus, the presence of some ARGs in the gut seems to be transitory, for example drug

resistance mechanisms based on efflux pump are lost during the first year of life as result of microbiota maturation [134].
According to two metagenomic studies [135, 136], the human intestinal resistome is rich in genes giving resistance to tetracyclines, macrolides, lincosamides, streptogramins and β-lactams. Tetracycline ARGs are often considered as relevant endemic properties of the human intestinal resistome.

4.5 Changes induced by antibiotic treatment in gut microbiota and resistome

Antibiotics can have drastic effects on general microbiota composition, leading to dysbiosis and favoring infection. This phenomenon has been well-known since the introduction of antibiotics in 1940s. The clinical observations that patients treated with penicillin or streptomycin were more prone to infections [137, 138], led scientists to conduct further investigations. In 1960s Miller, Bohnhoff, and Freter showed for the first time that mice treated with streptomycin were more susceptible to *Salmonella enteritidis* [139, 140], *Shigella flexneri*, and *Vibrio cholerae* [141] infections. Thus, based on the results obtained from these studies, we understood that antibiotic treatment can deplete important components of intestinal microbiota and decrease colonization resistance.
The effect of an antibiotic on intestinal microbiota depends on several factors *e.g.* the way of administration, the degree and types of modifications of the molecule before reaching the gut or in the gut itself, its final concentration in the intestinal tract. Based on these principles, antibiotics belonging to the same drug class can impact differently the microbial ecology [70]. For example the carbapenem antibiotic imipenem is prevalently filtered by the kidneys and excreted in urine, explaining that its concentration in stools is <1% of the original administered dose [142]. However, ertapenem from the same carbapenem class is present in feces at higher concentrations [143]. Thus, compared to other carbapenems, imipenem has a limited effect on the intestinal microbiota composition [70].
Metagenomic studies have highlighted that antibiotics promptly decrease microbial diversity and species richness and can have an effect on taxa beyond their normal and known spectrum of action. A 5-day treatment with ciprofloxacin was reported to change the relative abundance of about a third of the intestinal bacterial taxa [144]. Vancomycin which is known to kill Gram-positive bacteria reduces also the abundance of certain Gram-negative Bacteroidetes [145, 146].
In paediatric patients, the utilisation of antibiotics is particularly detrimental as it affects the colonization of *Bifidobacterium* and *Lactobacillus* [15, 147]. Moreover, it was shown that the formation of microbiota might be disturbed by anti-group-B-streptococcal antibiotics administered as prophylactic regime to delivering mothers [148].
Importantly, Forslund and colleagues [136] showed that the composition of human resistome can depend on the usage of antibiotics in animal rearing as growth

promoters. Sublethal concentrations of antibiotics administered to animals would exert a selection pressure making ARGs emerge. Those ARGs would then be transferred to humans via alimentary chain (*farm-to-fork effect*). The resilience of the microbiota, or better its capacity to rescue from the effects of antibiotic consumption, can take months after the termination of an antibiotic treatment course and varies from individual to individual depending on factors like the health status and age (reviewed in [149]).

Chapter 5: Multidrug resistant Enterobacteriaceae, resistance to β-lactam antibiotics and to colistin and epidemiology

In this chapter, I am going to talk about the bacterial family of Enterobacteriaceae, which include enteric pathogens of particular clinic and scientific interest as they have developed resistance to multiple antibiotics, especially β-lactams, thus leading to difficult-to-treat diseases.
I will first describe the ecological characteristics of the group, and then I will focus on the mechanisms of genetic resistance to β-lactams and colistin. I will also provide information on the epidemiology of multidrug resistant species from Enterobacteriaceae.

5.1 Characteristics of Enterobacteriaceae family

Enterobacteriaceae is a family from Proteobacteria phylum which include 28 genera and more than 75 known species [150]. Phylogenomic analyses has allowed to differentiate 6 distinct sub-family clades, named as the "*Escherichia* clade", "*Klebsiella* clade", "*Enterobacter* clade", "*Kosakonia* clade", "*Cronobacter* clade" and "*Cedecea* clade" [150]. These facultative anaerobes can metabolize carbohydrates by fermentation in the intestinal tract. *E. coli* and enterococci represent some of the earliest colonizers of microbiota in newborns [15].
The relative abundance of Enterobacteriaceae is usually <1% of intestinal microbiota in healthy individuals [151]. They are classified in coliforms or non coliforms based on the capacity to ferment completely lactose [152].
Importantly, Enterobacteriaceae include a long list of pathogen species belonging to *Escherichia*, *Klebsiella* and *Enterobacter* genera. Entobacteriaceae pathogens are often associated with diarrhea [153, 154]. One among the most famous groups of bacteria causing diarrhea is enteropathogenic *E. coli* (EPEC), which uses a type III secretion machinery to hijack enterocytes and cause rearrangements of their cytoskeleton. Eventually, EPEC adhesion to the intestinal cell layer results in the loss of microvilli and the formation of actin-rich niches, called *pedestals*, where EPEC hides [155].
Enterobacteriaceae members are classified as Gram-negative and their cell envelope is composed by an inner and an outer membrane between which a thin peptidoglycan (murein) layer is located. The peptidoglycan of Gram-negative bacteria is a network constituted by a polysaccharide, formed by the repetition of β-(1,4)-GlcNAc-β-(1,4)-MurNAc disaccharides, interconnected by oligopeptides [156]. During the peptidoglycan synthesis and before cell division, crosslinking of peptides from different chains is performed by transpeptidase enzyme, also known as a penicillin-binding protein (PBP), anchored on the inner membrane.

5.2 β-lactam antibiotics, mechanisms of action and drug resistance

β-lactams (or beta-lactams) are the most prescribed antibiotics in human medicine: almost a decade ago, they corresponded to the 65% of the total antibiotics market [157].
PBP is the target of β-lactam antibiotics. Despite the diverse chemical nature (discussed below), all β-lactams operate via the same inhibitory mechanism on PBPs. In Gram-negative bacteria, they cross the outer membrane through a porin and gain the periplasmatic space. Here, they function as surrogate substrates for PBP proteins and acylate irreversibly a serine in the active site of the PBPs [158]. Hence, β-lactams prevent the further crosslinking and block the peptidoglycan synthesis.
We can distinguish four groups of β-lactams, which are penicillins, cephalosporins, monobactams and carbapenems. All β-lactams are characterized by a central β-lactam ring and radicals which affect the antibiotic efficiency, its spectrum of action and pharmacokinetics (half-life, distribution and elimination). For example, aminopenicillins (*i.e.* ampicillin and amoxicillin) are penicillin-deriving compounds that present an amino-group as radical and can kill a larger number of Gram-negative bacteria than penicillins [159, 160].
Compared to penicillins whose β-lactam ring is fused to a 5-atom ring, in cephalosporins the β-lactam ring is merged to a six-member ring which is resistant to β-lactamase enzymes [161]. In total, five generations of cephalosporins with increasing spectrum activity have been implemented.
The only clinically used monobactam (no fusion to another ring) is aztreonam and it is effective only against Gram-negative bacteria. Finally carbapenems include numerous semisynthetic compounds (*i.e.* imipenem, meropenem, ertapenem and doripenem) used for very severe infections [162].
The extensive use of β-lactam since their implementation has played a crucial role in rising drug resistance by acting as a strong selection pressure on natural populations [163]. We can distinguish four main mechanisms of resistance to β-lactams [164, 165]:

1. Decreased permeability of outer membranes. This mechanism includes mutations in the regulation pathways of outer membrane proteins (*i.e.* porins) production as it is the case for OprD porin in *Pseudomonas aeruginosa* [166]. Generally mutations in porin-encoding genes cannot *per se* confer resistance and they are typically associated with other resistance mechanisms *e.g.* against β-lactam [167].

2. Upregulation of efflux pumps, *i.e.* the AcrAB-TolC system. AcrB is a resistance-nodulation-cell division protein that binds the membrane fusion protein AcrA and the outer-membrane channel TolC in the periplasm. The formed AcrAB-TolC complex expels β-lactams out of the cell (reviewed in [164]).

3. PBP with decreased affinity to β-lactam. Although this phenomenon is rare in Gram-negative compared to Gram-positive bacteria [165], mutations affecting the

binding affinity of PBPs to penicillin have been reported in Enterobacteriaceae pathogens (reviewed in [165]).

4. β-lactamase enzymes, which we discuss more in details in the next paragraph.

5.3 β-lactamases, mechanisms of resistance and classification

The production of β-lactamases represents the main mechanism through which Gram-negative bacteria can resist to β-lactam treatments [165]. In fact these enzymes hydrolyze the amino-bond of the β-lactam ring, therefore inhibiting its binding to PBP. We distinguish two main mechanism of hydrolysis mediated by β-lactamases: in the former case, an active-site serine is required for the reaction, whereas in the latter one hydrolysis can be achieved only in presence of at least one bivalent Zn atom.
β-lactamase-encoding genes can be located on chromosome and plasmids. The most important outbreaks world-wide have been caused by plasmid-borne β-lactamases [168].
According to several epidemiological studies (reviewed in [165]), 3,000 unique β-lactamase genes exist.
β-lactamases are classified according to two different nomenclatures based on the structure or the enzymatic functionality. The Ambler nomenclature divides β-lactamases in four categories, named from A to D, based on the nature of the active site. Briefly, classes A, C and D have a serine on the active site, whereas class B includes those β-lactamases that require Zn in the active site.
The nomenclature based on the biochemical characteristics of the enzymes, including substrate affinity, hydrolysis rates and inhibitors, was proposed by Bush for the first time in 1989 [169] and revised several times [165, 170].

5.4 Most important β-lactamases

While β-lactamases are produced by both Gram-positive and Gram-negative, we will only focus on Gram-negative organisms producing β-lactamases as these ones were the objective of the metagenomic studies presented in this work.
In particular, I will talk about four types of β-lactamases that are important from a clinical and epidemiological point of view: extended spectrum β-lactamases (ESBLs), ampicillin class C beta-lactamases (AmpC-bls), carbapenemases (CPs) and metallo-β-lactamases. Except for metallo-β-lactamases, all these enzymes are serine β-lactamases [171-174].

5.4.1. Extended spectrum β-lactamases

The definition of ESBL is a bit controversial. According to a designation proposed by Bush and Jacoby [175], ESBLs include enzymes of class A, class C, and class D. Others have restricted the ESBL definition to plasmid-encoded enzymes that have emerged from class A and that belong to Bush's functional group 2b and that degrade penicillins as well as narrow- and expanded-spectrum cephalosporins [170, 175]. ESBLs include three more common enzyme types: TEM, SHV, and CTX-M [165].
183 nonduplicative variants of TEM and 178 variants of SHV are known but not all of them display an ESBL phenotype. Molecular characterization of TEM- and SHV-type ESBLs occurred between the late 1980s and 1990s when they were particularly diffused in the United States and Europe (reviewed in [165]). CTX-M have likely originated from an environmental Enterobacteriaceae species, *Kluyvera* spp. [176].

5.4.2 Carbapenemases

According to a revised classification [165], we can distinguish two clinically important groups of carbapenemases (CPs): species-specific and acquired carbapenemases.
Species-specific CPs are serine β-lactamases whose encoding genes are chromosomally located. SME serine carbapenemases are found only in isolates of *Serratia marcescens* while the IMI/NMC carbapenemase families are only found in isolates from *Enterobacter* spp. [165].
Acquired serine carbapenemases include KPC and OXA carbapenemases which are present in many Enterobacteriaceae genera. KPC was first isolated in *K. pneumoniae* [177] and its spread due to *K. pneumoniae* clonal complex 258 (CC258) [178].
Importantly, some OXA-type lactamase variants are reported to be associated with an ESBL-like resistance phenotype. An important variant of OXA family is encoded by *bla*$_{OXA-48}$. According to epidemiological studies performed on isolates collected from 20 countries between 2012 and 2015 [179], *bla*$_{OXA-48}$ and *bla*$_{OXA-48}$-like genes were reported in 14 different Enterobacteriaceae species. Mobility of KPC and OXA genes are given by the fact there are found within transposons [165].

5.4.3 AmpC β-lactamases

AmpC β-lactamases belong to the Ambler class C. The genes encoding for AmpC is naturally located on the chromosome in many Enterobacteriaceae but it can also be plasmid-borne (reviewed in [165]). AmpC expression can be induced by the accumulation of peptidoglycan oligopeptides following disruption of the peptidoglycan synthesis [180]. Mutations on the regulatory mechanisms of AmpC can lead to constitutive expression and/or overexpression of the enzyme [180].
The first plasmid-borne AmpC was reported in a clinical isolate of *K. pneumoniae* in 1989 from South Korea and such variant was named CMY-1 based on cephamycinase-

like phenotype [181]. Plasmid-borne enzymes originate from different organisms. In particular we distinguish nine families: CMY-1, FOX- and MOX-type enzymes which arose from *Aeromonas* spp.; CMY-2 and cephalosporinase LAT families from *C. freundii*; the MIR and ACT families came from *Enterobacter* spp.; the DHA family from *M. morganii*; the ACC family found in *H. alvei* (reviewed in [165]). Importantly, plasmid-borne AmpC β-lactamases are characterized by a constitutive expression because of high gene numbers and strong promoters.

5.4.4 Metallo-β-lactamases

Metallo-β-lactamases (MBLs) belong to Ambler class B which has evolved independently from serine β-lactamases [182, 183]. The most relevant metallo-β-lactamases belong to the VIM (Verona integron-encoded metallo-β-lactamase) and NDM (New Delhi metallo-β-lactamase) families. Accordingly, VIM MBLs are found mostly in *K. pneumoniae* and *Enterobacter cloacae* complex. The gene *bla*$_{VIM}$ is located within an integron (reviewed in [165]). NDM-type enzymes are found in almost 45% of all MBL-producing Enterobacteriaceae (reviewed in [165]).

5.5 Resistance to colistin

Colistin, also known as polymyxin E, is a cationic polypeptide that is used as last resort antibiotic against Gram-negative infections. Colistin has a bactericidal effect: it leads to cell disruption by interacting with lipid A of the outer membrane [184].
Until 2016, the only mechanisms of colistin resistance were related to chromosomally located genes whose mutations induced a lower affinity of lipid A for colistin [185, 186].
However, a plasmid-mediated resistance to colistin, known as MCR-1, was first reported in *E. coli* strain SHP45 from China [187].
MCR-1 has likely originated from animals and then spread to humans [187], an hypothesis strengthened by finding MCR-1 in commensal *E. coli* isolates from livestock [188].
MCR-1 is a membrane-bound phosphoethanolamine transferase which modifies the lipid A by reducing its binding affinity to colistin [189].

5.6 Origin and epidemiology of multidrug resistant Enterobacteriaceae

Because of the spread of mobile elements carrying antibiotic resistance genes, Gram-negative bacteria, in particular Enterobacteriaceae, can produce multiple β-lactamases.
With the term multidrug resistant Enterobacteriaceae (MRE), we group ESBL-producing and CP-producing Enterobacteriaceae (ESBL-E and CP-E, respectively) and

Enterobacteriaceae producing plasmid-encoded AmpC type cephalosporinases (pAmpC).

In 2018 the World Health Organization included ESBL-E and CP-E among critical multidrug resistant pathogens against which new drugs are urgently required [190].

Among MRE, *E. coli* (EC) and *K. pneumoniae* (KP) have attracted particular attention as they represent the major causative agents of sepsis and septic shock. Before 1990s, ESBL-E was prevalently represented by *K. pneumoniae* and *E. coli* isolates producing only SHV and TEM β-lactamases. Moreover, such isolates were found only in nosocomial infections and very rarely in community-acquired infections (reviewed in [191]). Since 2010 an increased spread and incidence of CTX-M ESBL genes has been reported worldwide [192, 193]. Importantly, CTX-M-15 diffusion is due to the expansion of *E. coli* ST131 [176] which represents the 40-80% of ESBL-producing isolates [194, 195].

The distribution of ESBL-E KP and EC varies from region to region (Figure 9). In Europe, the estimated fecal carriage rate of ESBL-E per country is around 5%. This percentage is almost 14-fold higher in Thailand (data from 2010) [196]. In Africa the carriage is estimated between 10% and 30.9% and almost 12.4% (data from 2011) in Latin America [196].

CR-E incidence also varies across regions. In Europe they are reported to be endemic in Greece and Italy but rare in Scandinavian countries [164]. Finally, the fecal carriage of ESBL/AmpC β-lactamases has been reported to occur in households at a rate of 4% [197].

MRE spreading is of a rural and agricultural origin. In fact, animal and human feces containing MRE usually contaminate environmental and drinking water which then become an important source of MRE transmission to humans [128].

MRE has become a serious public health concern as they are increasing also in countries where antibiotic usage is under control [128]. Besides, MRE usually have more than one resistance mechanism, making the clinical management of such isolates more difficult. For instance according to an epidemiological study performed in California, almost 80% of the CP-E isolates were also carrying an ESBL- or plasmid-encoded AmpC gene [198]. KPC and OXA-48 were also found in combinations with other carbapenemases or metallo-β-lactamases in isolates originated from Americas, Greece and Spain (reviewed in [165]).

Importantly there are lineages that are resistant to other classes of antibiotics beyond β-lactams. An example is given by the fact that high rates of fluoroquinolone resistance correlates with high rates of ESBL EC and KP (Figure 9). Furthermore, CTX-M-type β-lactamase-producing *E. coli* ST131 clade C, which is largely distributed worldwide is also fluoroquinolone-resistant (reviewed in [165]).

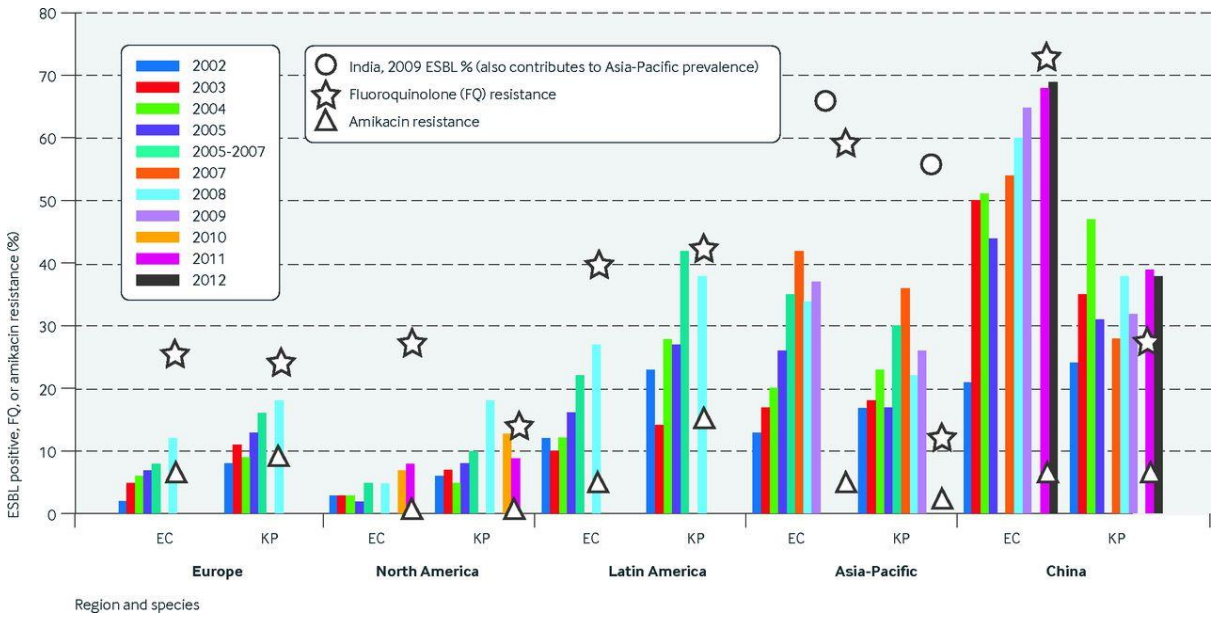

Figure 9. Rates of ESBL *E. coli* (EC) and *K. pneumoniae* (KP) world–wide between 2002 and 2012.

The plot reports also resistance to fluoroquinolones and amikacin (aminoglycoside). The plot was taken from Iredell *et al.* [128]. Permission obtained by BMJ Publishing Group Ltd. (License number 5001270101386 released on Feb. 03, 2021).

Chapter 6: Unwanted 'souvenirs' of tropical vacation: multidrug resistance Enterobacteriaceae acquisition and traveler's diarrhea

The spread of infectious diseases is phenomenon connected to fluxes of human migrations but also to food and goods distribution. The number of people traveling for touristic purposes has increased enormously in the last 60 years: we passed from 25 million arrivals in 1959 to 1.14 billion in 2014 [199] and the number of touristic arrivals in 2018 is reported to be almost 3-fold higher than that registered in 1995 [200].
Traveling to MRE endemic regions is considered as a potential risk for MRE intestinal colonisation [201] and experience of traveler's diarrhea (TD). Therefore, I will discuss more in details what TD is and describe VOYAG-R trial and some related studies that investigated the epidemiological relationship between travel and MRE acquisition.

6.1 Traveler's diarrhea: definition and epidemiology

Traveler's diarrhea is defined as the production of unformed stools for a minimum of 24 hours accompanied by at least one symptom (either mild symptoms like nausea, vomiting, or severe symptoms like strong abdominal cramps and blood in the stools [202, 203]). According to a recently published review [202], the percentage of travelers affected by diarrhea is estimated between 10% and 40%, depending on the destination, and with a higher risk (>20%) in those regions where sanitary conditions are poor such as Africa, Latin America and South Asia [202, 204-206]. The modality of the journey also plays a role as a stay in hotels is less risky than backpacking [205, 207].
In 90% of cases TD is due to bacterial pathogens [208]. In particular, enterotoxigenic *E. coli* (ETEC) is identified as the TD causative agent in 30 to 60% of all cases [203]. However, pathogens causing diarrhea also include *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica* (reviewed in [202]). In ~10% of cases, TD is caused by viruses (*e.g.* noroviruses, rotavirus, astrovirus, and enteric adenovirus) and more rarely by protozoan parasites like *Giardia duodenalis* [202].

6.2 MRE acquisition and TD after traveling to tropics: the VOYAG-R trail and related studies

The VOYAG-R study is a clinical trial conducted in France between February 2012 and April 2013 [209]. The study analyzed the presence of MRE in fecal samples obtained from 574 individuals traveling to 52 tropical countries. For simplicity, countries of destination were divided into three large continental regions: Asia, Africa and Latin America. Moreover, the number of travelers for each region was comparable (~190 individuals per region). The median duration of the travel was close to 3 weeks. Fresh stools were taken and analyzed before the travel to exclude MRE carriage. For subjects

with pre-travel MRE-negative stools, fecal samples were taken several time points after return. MRE-positivity was tested by culturing stool samples in ESBL- or CP-E-enriching media and by disk diffusion antibiotic susceptibility tests.
The resistance mechanism was determined by analyzing the presence of ESBL genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{VEB}$), pAmpC, carbapenemases (*bla*$_{KPC}$ and *bla*$_{OXA-48}$) and metallo-β-lactamase genes (*bla*$_{VIM}$, *bla*$_{NDM}$ and *bla*$_{IMP}$) in stool cultures. In 93.3% of cases *E. coli* was identified as MRE. ESBLs and pAmpC were the most common resistance mechanisms with 91.8% and 8.4% of cases, respectively, while metallo-β-lactamases was detected in <1% of cases. Among ESBLs, *bla*$_{CTX-M}$ was found in the 95.4% of cases. Asian countries were those reporting the highest percentage of pAmpC compared to Sub-Saharan Africa and Latin America.
Half the travelers (292/574) were found to be positive at least for 1 MRE ~1 week following the return to France. Eighty-three individuals were still positive one month after the return. Traveling to Asia was associated with a high risk of MRE acquisition and carriage was also reported by other studies (reviewed in [210]). In particular, the incidence of MRE acquisition among travelers to Asian southern regions was estimated to be between 29-88%.
In the COMBAT study [211] including a cohort of 1,847 Dutch travelers to MRE endemic regions, acquisition rate was reported of 34.3% and traveling in India was associated with the highest risk of MRE acquisition (88.6%).
Besides Asia, the VOYAG-R trial reported also traveling to Peru (Latin America) and Togo (Sub-Saharan Africa) to be associated with remarkable risk of MRE acquisition: 84.4% and 75.0%, respectively.
Beyond geographic destination, antibiotic consumption, especially quinolones [211] and β-lactams [209], and type of travel popped up to be risk factors of MRE acquisition in several studies (reviewed in [210]). In the VOYAG-R study, people who stayed in hotel were less prone to MRE acquisition than other types of traveling (*e.g.* backpacking etc.). TD has been also associated to MRE acquisition and the incidence of diarrhea per tropical region in the VOYAG-R study was comparable (39.7% in Sub-Saharan Africa, 34.8% in Latin America and 45.6% in Asia). This tight association between TD and MRE acquisition [210] is due to the fact that MRE include diarrheagenic species [153, 154].
The VOYAG-R study showed that the duration of travel did not have a significant impact on MRE colonization rate, but an MRE screening study performed on 170 Swiss travelers going to southern Indian regions reported the length of stay as one of MRE acquisition risk factors [212]. Longer stay in endemic regions increases the likelihood of MRE acquisition as a result of a longer exposure of travelers to possible sources of contaminations [209].
The VOYAG-R trial also investigated the carriage of MRE at return and showed that 85% and 95% of the travelers were MRE negative one month and three month after coming back to France, respectively. Thus MRE acquired from tropics does not represent a long lasting reservoir of ESBL-E/CP-E in the community. Moreover, only

1% of travelers who acquired ESBL-E were still colonised 12 months after the return. Of note, this percentage is reported to be higher (11%) in the COMBAT study [211].
The COMBAT study also investigated the transmission of ESBL-E from travelers at return with their householders. The authors found out that the probability of transmission between ESBL-E positive travelers at return and their households was 12%, which is lower than the rate of ESBL-E acquisition in tertiary care hospitals (22.7%) [213].
Vento *et al.* [214] showed that MRE acquisition also occurs among military personnel during missions to endemic regions. The authors reported that US soldiers based in Afghanistan had 5.5-fold higher proportions of multidrug resistant *E. coli* positive individuals than the US-based personnel (11% *versus* 2%).

Chapter 7: Gram-negative infections: therapy duration and emerging alternatives to antibiotics

Gram-negative pathogens, mostly *E. coli*, *K. pneumoniae* and *P. aeruginosa* species [215] enter the human body following ingestion or tissue injuries, gain blood stream so causing *bacteremia*. More in general, bacteremia is defined as the presence of living bacteria in the blood [215] and it can be a transient and mild event but under certain circumstances (*e.g.* weakened immune system) it can lead to life-threatening conditions such as sepsis and septic shock [216]. Bacteremia is treated by administrating antibiotics with a duration that typically varies between 10 and 14 days. Therapy duration is based on opinions of experts rather than on scientific evidence on the benefits of short-term over long-term antibiotic administration [217]. An understanding of advantages to adopt a short or individualized therapy could limit the detrimental effects which can rise with prolonged exposure to antibiotics. This question is addressed by the PIRATE trial, which is presented in this chapter.
Furthermore, although antibiotics represent the gold standard to combat infectious diseases, fecal microbiota transplantation (FMT) has attracted particular attention as potential alternative to treat severe infections as recurrent *C. difficile* [128]. Therefore, I am going to talk about FMT and I will present the R-GNOSIS WP3 that investigated the effects of FMT to decolonize the gut from the ESBL-E and CP-E.

7.1 Short *versus* long antibiotic treatment against Gram-negative bacteremia: the PIRATE study

We have seen that antibiotics can have dramatic effects on the overall composition of intestinal microbiota: antibiotics will also kill commensal bacterial species on top of the bacterial pathogens motivating such therapy. The use of antibiotics can lead to the intestinal colonization of difficult-to-treat pathogens such as *C. difficile* or to the emergence of multidrug resistance [218, 219].
The PIRATE project was a multicentre clinical trial that aimed to investigate the clinical significance of the duration of antibiotic therapy in the context of Gram-negative uncomplicated bacteremia [217].
The PIRATE trial was run between April 2017 and August 2019 and included 509 individuals from three Swiss tertiary care hospitals (Geneva, Lausanne and Sankt-Gallen). The patients were randomized to three different groups. The first included patients with individualized treatments, whereas the remaining two groups were treated for a fixed duration of 7 or 14 days. Sources of bacteremia were prevalently urinary infections (~70%) and the main detected pathogen was *E. coli* in 73-75% of the cases while 5 to 8% of patients had ESBL *E. coli*.
The PIRATE study showed that individualized treatment and 7-day treatment brought comparable clinical failure rates as compared to 14-day therapy for uncomplicated bacteremia, as assessed 30 days after the starting of antibiotic therapy. In other words,

duration of therapy, either individualized or fixed, did not affect the successful treatment of bacteremia.

7.2 Bacteriotherapy: fecal microbiota transplantation and administration of bacterial consortia

FMT and bacterial consortia are strategies aiming at restoring microbial species balance in the intestine after an event affecting microbiota composition, *e.g.* antibiotic treatment.

FMT is a very ancient practice that was already used in China centuries ago when individuals suffering from intestinal infections were offered a diluted preparation from human stools, the "*yellow soup*" [220].

The use of FMT is reported also in Europe in the 18th and 19th centuries and during the Second World War when preparations from human stools were offered to German soldiers experiencing dysentery during military mission in North Africa [220].

More recently, in 1958 Eiseman *et al.* reported the successful use of fecal enemas to treat four patients with *pseudomembranous enterocolitis* [221]. This was really a milestone as in the 1950s pseudomembranous enterocolitis, likely caused by *C. difficile*, was lethal in 75% of cases [222].

Nowadays FMT is a treatment largely used and efficacious for the eradication of recurrent *C. difficile* infection (rCDI). A recently published systematic review by Drekonja *et al.* [223] reported an overall success rate of FMT against rCDI of 85%.

FMT is generally administered with four different strategies [224]: via endoscopy delivery [225], with naso-gastric tube [226], enemas [227] and capsules [228]. Serious adverse events associated to FMT are reported to be rare and to occur more frequently after FMT administration *via* lower gastrointestinal routes than *via* upper gastrointestinal routes [229].

Besides rCDI, FMT has been used also in other contexts. Autologous FMT (that is the patient is administered with a preparation from his/her own feces) was successfully applied in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT) where antibiotics are frequently required to treat infections from opportunistic pathogens [230]. FMT was also reported to reduce incidence of diarrhea following anti-cancer drug therapy [231].

Despite its utilisation, FMT brings some concerns related to the safety of using heterologous donor fecal preparation. Fecal samples and the clinical history of donor individuals are generally carefully analyzed before FMT and clinical-practice guidelines on donor selection have already been published [232]. However, it can happen to accidentally transmit bacteria through donor microbiota administration that would be deleterious for the patient and could even lead in extreme to severe or lethal infections [220].

For these reasons, in some cases, autologous FMT might be applied instead of heterologous FMT. Although autologous and heterologous FMT preparations were

shown to be equivalent concerning the composition of beneficial species, a direct comparison of the efficacy of the two methods is missing [230].
Importantly, the species diversity of donor fecal preparations seems to be an essential characteristic for the clinical success of the FMT procedure [233, 234].
More recently, the concept of a super-donor, that is a donor microbiota more efficient in rescuing microbial composition of recipient than others, made its way in the scientific community [224], opening up the seducing opportunity of use one donor for multiple patients ("*One Stool Fits All*"). However, several clinical trials and case reports argued against this possibility (reviewed in [224]). Thus a better characterization of donor microbiota and donor-patient compatibility is warranted. Unlike FMT, microbe consortia contain a limited number of bacterial species and would eliminate the need of performing large-scale screenings on donor fecal samples. Although bacterial consortia have been used to combat *C. difficile* infections [102] infused bacteria presented short-term intestinal engraftment and their abundance in the gut decreased already 6 months after administration [235]. Machine learning algorithms will likely help in selecting possible important species associated to a successful engraftment [50].

7.2.1 The R-GNOSIS study

"Resistance in Gram-Negative Organisms: Studying Intervention Strategies" (R-GNOSIS) is an international project conducted between 2011 and 2017 aiming to find new strategies for controlling and treating infections from multidrug-resistant Gram-negative bacteria in health-care facilities and community [236]. It is composed by several working packages (WPs), however, for simplicity, I am going to discuss only WP3.
The objective of R-GNOSIS WP3 was to provide a successful intestinal decolonization of ESBL-E and CP-E [237].
A study conducted at the Geneva University Hospitals showed that the treatment with oral neomycin and colistin for 10 days led to a transient decrease of ESBL-E carriage during the treatment and shortly after. Therefore, it was proposed to combine the colistin/neomycin treatment with transplantation of healthy gut microbiota aiming at restoring colonization resistance against ESBL-E/CP-E [238].
The idea of using FMT derived from the experimental evidence that intestinal microbiota opposes to colonization by multidrug resistant bacteria. Caballero *et al.* [239], for example, showed that FMT was able to clear the intestine from carbapenem-resistant *K. pneumoniae* in mice. Therefore, FMT was suggested as procedure likely working also in humans to decolonize intestine from Gram-negative pathogens [240, 241].
R-GNOSIS WP3 was the first multicentre randomized clinical trial assessing the efficacy of the combinations of antibiotics and FMT to eradicate ESBL-E/CP-E from the intestine.

The study included 39 patients from four tertiary-care centres (Geneva, n= 14; Paris n=16; Utrecht, n=7; Tel Aviv, n=2).
The MRE detected during the trial were: *Citrobacter freundii* NDM, *Klebsiella aerogenes* ESBL, *Enterobacter cloacae* ESBL, *E. coli* (ESBL, NDM, OXA), *K. pneumoniae* (OXA, ESBL). *Morganella morganii* ESBL from Morganellaceae family was also detected. Of the 39 patients, 22 received a 5-day treatment with oral neomycin and colistin followed by FMT which was administered either by capsule or by nasogastric tube. In the R-GNOSIS trial oral doses of colistin were adjusted to increase its concentration in the intestine. The remaining 17 out of 39 participants did not receive any treatment planned for the study.
Decolonization was more frequent in treated than untreated group: 9/22 (41%) and 5/17 (29%), respectively. The R-GNOSIS WP3 was the first multi-center trial with a large number of individuals to provide first evidence, although not conclusive because planned sample size was not reached, that antibiotic/FMT could be effective on the intestinal decolonization from ESBL-E/CP-E.

Chapter 8: Aims of the PhD thesis

I took advantage of fecal samples collected in the three clinical trials VOYAG-R, PIRATE and R-GNOSIS, to investigate by metagenomics intestinal microbiota composition: before and after traveling to tropical regions; during and after antibiotic therapy against Gram-negative bacteremia; before and after combined colistin/neomycin-FMT treatment administered to MRE carriers.

For all the three projects, the aims can be summarized by the following points:

1. Describing the dynamics of changes in microbiota composition with time-course analyses.

2. *In silico* identification of bacterial taxa positively or negatively associated with MRE acquisition, MRE carriage and TD diarrhea (VOYAG-R) and short and long antibiotic treatments (PIRATE). I aimed at identify species that could play a role in the MRE intestinal decolonization through FMT (R-GNOSIS).

3. When feasible, I performed functional analyses by characterizing the content of the microbiota-associated antibiotic resistance genes.

Results

Chapter 1: The intestinal microbiota predisposes to traveler's diarrhea and to the carriage of multidrug-resistant Enterobacteriaceae after traveling to tropical regions

Stefano Leo, Vladimir Lazarevic, Nadia Gaïa, Candice Estellat, Myriam Girard, Sophie Matheron, Laurence Armand-Lefèvre, Antoine Andremont, The VOYAG-R study group, Jacques Schrenzel and Etienne Ruppé

1.1. Introduction

The ingestion of contaminated food or contaminated water is the principal mean by which MRE colonize human gut. Poor hygienic conditions, together with uncontrolled usage of antibiotics, make tropics endemic regions for MRE acquisition. Therefore the traveling to tropical regions represents a risk factor for MRE colonization and the development of traveler's diarrhea.
The gut microbiota composition in respect to MRE carriage and occurrence of diarrhea had not yet been analyzed. Therefore, we investigated the changes of metabolically-active microbiota before and after the travel to tropics on a sub-cohort of subjects from the VOYAG-R study. Besides, we linked the microbiota composition to the long-term carriage of MRE.

1.2. Article Status

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1.3. Contributions

I performed RNA extraction, designed the bioinformatics and statistical analysis methods to run, I analyzed and interpreted the data. I contributed to the writing and reviewing of the manuscript. I assembled all the figures and tables for publication.

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RESEARCH PAPER/REPORT

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The intestinal microbiota predisposes to traveler's diarrhea and to the carriage of multidrug-resistant Enterobacteriaceae after traveling to tropical regions

Stefano Leo[a], Vladimir Lazarevic[a], Nadia Gaïa[a], Candice Estellat[b,c,d], Myriam Girard[a], Sophie Matheron [e,f,g], Laurence Armand-Lefèvre[f,g], Antoine Andremont The VOYAG-R study group[f,h], Jacques Schrenzel[a], and Etienne Ruppé[a]*

[a]Genomic Research Laboratory, Service des Maladies Infectieuses, Hôpitaux Universitaires de Genève, Genève, Suisse; [b]AP-HP, Hôpital Bichat, Département d'Epidémiologie et Recherche Clinique, URC Paris-Nord, F-75018 Paris, France; [c]INSERM, CIC 1425-EC, UMR1123, F-75018 Paris, France; [d]Université Paris Diderot, UMR 1123, Sorbonne Paris Cité, F-75018 Paris, France; [e]AP-HP, Hôpital Bichat, Service des Maladies Infectieuses et Tropicales, F-75018 Paris, France; [f]INSERM and Université Paris Diderot, UMR 1137 IAME, F-75018 Paris, France; [g]Laboratoire de Bactériologie, AP-HP, Hôpital Bichat; [h]AP-HP, Hôpital Bichat, Laboratoire de Bactèriologie, F-75018 Paris, France

ABSTRACT

The risk of acquisition of multidrug-resistant Enterobacteriaceae (MRE) and of occurrence of diarrhea is high when traveling to tropical regions. The relationships between these phenomena and the composition of human gut microbiota have not yet been assessed. Here, we investigated the dynamics of changes of metabolically active microbiota by sequencing total RNA from fecal samples taken before and after travel to tropical regions. We included 43 subjects who could provide fecal samples before and after a travel to tropical regions. When found positive by culturing for any MRE after travel, the subjects sent an additional sample 1 month later. In all, 104 fecal samples were considered (43 before travel, 43 at return, 18 one month after travel). We extracted the whole RNA, performed retrotranscription and sequenced the cDNA (MiSeq 2x300bp). The reads were mapped to the reference operational taxonomic units (OTUs) and species/strains using the 16S Greengenes and 23S SILVA databases. We found that the occurrence of diarrhea during the travel was associated with a higher relative abundance of *Prevotella copri* before departure and after return. The composition of microbiota, before travel as well as at return, was not correlated with the acquisition of MRE. However, the clearance of MRE one month after return was linked to a specific pattern of bacterial species that was also found before and after return. In conclusion, we found specific OTUs associated to a higher risk of diarrhea during a stay in tropical regions and to a faster clearance of MRE after their acquisition.

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Introduction

Multidrug-resistant Enterobacteriaceae (MRE) that produce extended-spectrum beta-lactamases (ESBLs), plasmid-encoded AmpC-type cephalosporinases (pAmpC), and/or carbapenemases (CP) have been spreading in the community over the last two decades.[1] MRE represent a major public health issue, as a limited number of antibiotics remains active against these bacteria while very innovative antibiotics are expected to reach the market in the near future.[2] The spread of MRE has been particularly intense in tropical regions, likely owing to poor hygiene conditions and uncontrolled antibiotic usage.[3] Consequently, between 14% and 69% of travelers to tropical regions are reported to acquire MRE, depending on the cohort and specific destination.[4,5] Besides, a high proportion of travelers to these destinations also report the occurrence of diarrhea (traveler's diarrhea) during their trip.[4–8] Among travelers who acquire an MRE during a travel to tropical regions, the observed median carriage is short (≤1 month).[5,6] Still, some of them experience long-

CONTACT Etienne Ruppé etienne.ruppe@aphp.fr IAME, EVRest team, Université Paris Diderot, Sorbonne Paris Cité, Hôpital Bichat - Claude Bernard, HUPNVS, Assistance Publique - Hôpitaux de Paris, 46 rue Henri Huchard, Paris 75877-Cedex 18

*Current affiliation: Laboratoire de Bactériologie, Hôpital Bichat-Claude Bernard, Paris, France

The VOYAG-R study group: Antoine Andremont, Laurence Armand-Lefèvre, Olivier Bouchaud, Yacine Boussadia, Pauline Campa, Bruno Coignard, Paul-Henri Consigny, Assiya El Mniai, Marina Esposito-Farèse, Candice Estellat, Pierre-Marie Girard, Catherine Goujon, Isabelle Hoffmann, Guillaume Le Loup, Jean-Christophe Lucet, Sophie Matheron, Nabila Moussa, Marion Perrier, Gilles Pialoux, Pascal Ralaimazava, Etienne Ruppé, Daniel Vittecoq, Ingrid Wieder, and Benjamin Wyplosz.

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term carriage of MRE, extending up to 1 year in 2.2–11% cases.[5,6]

The capacity of the intestinal microbiota to resist long-term settlement of exogenous bacteria (including MRE) is referred to as colonization resistance[9–11] which is mainly exerted by anaerobes.[9,10] Antibiotics with high activity against anaerobes strongly affect the capacity of the microbiota to prevent colonization by exogenous microorganisms, and thus favor the acquisition and expansion of resistant bacteria.[12] Hence, the restoration of the intestinal microbiota through fecal material transplantation (FMT) has been shown to lower the intestinal concentrations of vancomycin-resistant enterococci (VRE),[13] MRE[14] and more globally, of antibiotic resistance genes.[15] Moreover, the administration of a limited set of intestinal bacteria to mice colonized with VRE reduced the intestinal densities of VRE, suggesting that specific bacteria are involved in colonization resistance.[16]

To date, the link between the composition of the intestinal microbiota and the acquisition of MRE during travel and their clearance after return has not been assessed.

Here, we questioned whether the composition of the intestinal microbiota could be associated with the occurrence of diarrhea and the acquisition of MRE during travel and to the clearance of MRE after return. In particular, we analyzed the pre- and post-travel composition of metabolically active microbiota which has been previously shown to provide a better differentiation between ESBL-carriers and non-carriers.[17]

Methods

Population

The travelers' cohort originates from the VOYAG-R study (ClinicalTrials.gov n°NCT01526187) funded by the French Ministry of Health.[6,18] From February 2012 to April 2013, subjects attending six international vaccination centers in the Paris area, prior to traveling to tropical regions, were asked to provide fecal samples before and after their trip. Only volunteers who had no detectable MRE in the feces taken before their departure, were asked to send a further stool sample within a week after their return. Travelers who revealed positive for MRE after their return were asked to provide additional stool samples 1, 2, 3, 6 and 12 months after their return until MRE was no longer detected. Among the 574 included subjects, 292 (51%) acquired at least one MRE.[6] From November 2012 to March 2013, travelers were provided a supplementary 50 mL RNALater® (Applied Biosystems, Villebon-sur-Yvette, France) containing vial before and after travel and one month after return when found positive for the acquisition of MRE. A total of 43 travelers were provided additional RNALater® containing pots, among whom 18 (41.8%) acquired an MRE (mostly ESBL-producing *Escherichia coli*) during their trip. The comparison between travelers providing additional samples stored in RNALater® (n = 43) and those who did not (n = 534) is described in the Supplementary Table 1. Of note, they experienced less diarrhea (9/43 [20.9%] vs 219/531 [41.7%], p-value <0.01), likely due to different travel destinations (e.g., none of RNALater® travelers went to Peru at that time, Supplementary Figure 8). All of them provided a stool sample one month after they returned, among whom six were still carrying an MRE. Thus, 104 samples were selected (43 before travel, 43 at return, and 18 one month after travel).

Since the number of individuals per visited country was too small to perform meaningful statistical analyses (Supplementary Figure 8), we grouped travelers according to the corresponding continental area of destination: Asia, Sub-Saharan Africa, and Latin America. In particular, of these 43 travelers 15 went to Asia, 15 to Sub-Saharan Africa, and 13 to Latin America.

RNA extraction from stool samples and sequencing

Total RNA (mostly made of ribosomal RNA, e.g., 16S, 23S, and 5S) was extracted for 104 stool samples using the RNeasy Plus Mini Kit (Qiagen, Gaithersburg, USA). The concentration of RNA obtained was measured by Qubit RNA BR Assay Kit or Qubit RNA HS Assay Kit (Life Technologies, Reinach, Switzerland). For simplicity, the terms 16S and 23S refer to 16S and 23S ribosomal RNAs, respectively. The integrity of RNA (ratio 16S/23S) was assessed by the RNA 6000 Nano Kit and RNA 6000 Pico Kit (Agilent Technologies, Plan-les-Ouates, Switzerland) on the Bioanalyzer 2100 system (Agilent Technologies,

Waldbronn, Germany). The detailed protocol can be found in the Supplementary Methods. One hundred nanograms of purified RNA in 10 µL total volume were sent to LGC (Berlin, Germany) for (i) cDNA synthesis using NEBNext mRNA First/Second Strand Synthesis Module (New England Biolabs, Ipswich, USA), (ii) shotgun library preparation using NuGEN Ovation Rapid Library System (NuGEN, San Carlos, USA), and (iii) sequencing (2 × 300 bp) of size-selected fragments (about 300–400 bp) using v3 Illumina chemistry and half the capacity of a MiSeq (Illumina, San Diego, USA) flow cell.

Bioinformatics and statistical analyses

Detailed bioinformatics and statistical methods are reported in Supplementary Materials. Briefly, paired reads were merged and quality-filtered using BBMerge 35.43 (http://bbmap.sourceforge.net) and Mothur v1.35.1,[19] respectively. Operational taxonomic units (OTUs) and bacterial strains species were identified by mapping reads to 16S Greengenes[20] and 23S SILVA[21] databases by USEARCH.[22] The mean mapping rates of quality-filtered merged reads against the Greengenes 16S and SILVA 23S databases were 31.0% (median = 31.0%) and 42.8% (median = 43.2%), respectively. The fraction of non-ribosomal mapping reads was on average 26% (median = 25%). Statistical analyses were performed in PRIMER v6 (PRIMER-E Ltd, Plymouth, UK) and in the R software v3.2.3.

Results

Effect of traveling on microbiota composition

To test the hypothesis whether the modalities and the duration of traveling to tropical regions has an impact on microbiota composition, we analyzed the microbiota profiles at return of 39 travelers that were not administered antibiotics during their trip. Four subjects who took amoxicillin (n = 1), ciprofloxacin (n = 1) and nifuroxazide (n = 2) were not considered. Six subjects who took doxycycline were considered though as we did not detect any effect of doxycycline on the composition of the intestinal microbiota (Table 1). Likewise, we did not observe any significant influence on microbiota composition in relation to the visited region, the type of travel, duration of travel, to the use and the type of malaria prophylaxis (Table 1). However, the proportions of Enterobacteriaceae increased during the travel in all the 39 individuals (Supplementary Figure 3A).

Moreover, we analyzed microbiota profiles before departure, at return, and 1 month after the return of 17 travelers who acquired MRE during their journey and were not treated with antibiotics at return. We observed that the fecal samples taken at different time points (before departure, at return and one month after return) clustered by subject (global PERMANOVA test, p-value <0.0001; Supplementary Figure 5F-G), while travel did not have a significant impact on microbiota profiles (pairwise PERMANOVA tests, p-values ranged between 0.3 and 0.9).

Table 1. Summary of global PERMANOVA analyses performed on the cohort of 39 travelers at return.

Variables	Conditions	rRNA subunit	Pseudo-F	p-value
Region	Sub-Saharan Africa/Latin America/Asia	16S	0.89	0.75
		23S	0.81	0.82
Type of travel	All-inclusive resort/Organized tour/visiting family/mix of all-inclusive resorts and organized tours/backpacking	16S	1.08	0.25
		23S	1.11	0.23
Duration of travel (*)	From 1 to 9 weeks	16S	1.04	0.35
		23S	0.82	0.71
Malaria prophylaxis	No/Yes	16S	0.79	0.86
		23S	0.76	0.80
Type of malaria prophylaxis	Atovaquone-proguanil/Chloroquine/Doxycycline/None	16S	0.95	0.57
		23S	0.92	0.63

(*) The test done was distance-based linear models (DistML; see Supplementary Methods).

Occurrence of diarrhea during the travel

In pre-travel microbiota of people who suffered from diarrhea during the travel (n = 9) the relative abundance of *Prevotella copri* species was >2-fold higher than in subjects who did not experience this condition (n = 34) (Wilcoxon rank sum test, p-value <0.05; Supplementary Figure 1G-H). Nevertheless, the overall composition of pre-travel microbiota was not significantly associated with the occurrence of diarrhea during travel (Table 2; Supplementary Figure 1E).

We, then, analyzed the intestinal microbiota profiles at return by excluding those four travelers who had taken antibiotics during their trip. Microbiota profiles from subjects who had diarrhea significantly differed from those who had not (Table 2; Figure 1(a)). The occurrence of diarrhea was associated with a microbiota significantly less rich and diverse at return as compared to the microbiota of individuals who did not experience this condition during the travel (Wilcoxon rank sum test, p-value <0.05; Figure 1(b)).

At return, people having experienced diarrhea during their travel presented increased proportions of Bacteroidetes and Proteobacteria phyla and decreased proportions of Firmicutes phylum compared to travelers without diarrhea (Figure 1(c)). The great majority of OTUs found to be differentially abundant after travel between people with and without diarrhea mapped to Enterobacteriaceae family, Clostridiales order, and the *P. copri* species (LEfSe and Wilcoxon rank sum test, p-value <0.05; Figure 1(d); Supplementary Figure 2A). In particular, the fecal samples taken at return from travelers who reported diarrhea showed a >2-fold higher proportion of Enterobacteriaceae and *Prevotella copri* and a <2-fold lower proportion of Clostridiales as compared to travelers without diarrhea. The relative abundance of *P. copri* was increased at return only in a fraction of travelers reporting diarrhea (Supplementary Figure 3B).

The results obtained for the samples collected at return from travel were also confirmed by 23S analyses (Supplementary Figure 2B-E). Most Enterobacteriaceae members associated with diarrhea mapped to 23S genes of *Escherichia-Shigella*, while in the 16S analysis the discriminating OTUs from Enterobacteriaceae were not classified at the genus level. Clostridiales included genera belonging to Ruminococcaceae (*Ruminococcus*), Bacteroidaceae (*Bacteroides*) and Lachnospiraceae (*Roseburia*; only for 16S dataset: *Lachnospira and Blautia*).

We then investigated whether diarrhea had protracted effects on gut bacteria composition one month after the return. Therefore, we analyzed samples from 17 travelers who did not take antibiotics during the first month following the return. We did not detect significant differences in the overall microbiota composition in relation to diarrhea (Table 2; Supplementary Figure 5A). Enterobacteriaceae relative abundance returned close to basal level in all 17 travelers but with statistical significance only when the analyses included the individuals who did not suffer from diarrhea (Wilcoxon signed rank test, p-value <0.05; Supplementary Figure 5C-D). One month after return, *Prevotella copri* abundance was still increased in travelers who had diarrhea (Supplementary Figure 3B) whereas Clostridiales members were more abundant in individuals without diarrhea (Supplementary Figure 4).

Table 2. Summary of pairwise PERMANOVA tests performed at each travel time point (*).

Travel time point	Number of travelers	Variable	rRNA subunit	t	p-value
Before travel	43	Diarrhea	16S	1.05	0.25
		during travel	23S	1.1	0.18
		MRE	16S	1.09	0.15
		acquisition	23S	1.05	0.29
At return	39	Diarrhea	16S	1.44	0.003
		during travel	23S	1.8	0.0007
		MRE	16S	1.12	0.12
		acquisition	23S	1.13	0.15
One month after the return	17	Diarrhea	16S	1.04	0.3
		during travel	23S	0.97	0.6
		MRE carriage	16S	1.21	0.01
		at return	23S	1.16	0.07

(*) The number of samples analyzed varied among travel time points according to the use of antibiotics and MRE acquisition. Before travel, we have considered all the 43 travelers. At return, we analyzed only subjects who did not take antibiotics during the travel. One month after the return, we considered travelers who acquired MRE during travel and were not treated with antibiotics after the trip.

Acquisition and carriage of MRE

The acquisition of MRE was not associated with a specific microbiota profile neither before travel nor at return (Table 2; Supplementary Figures 1F and 5E).

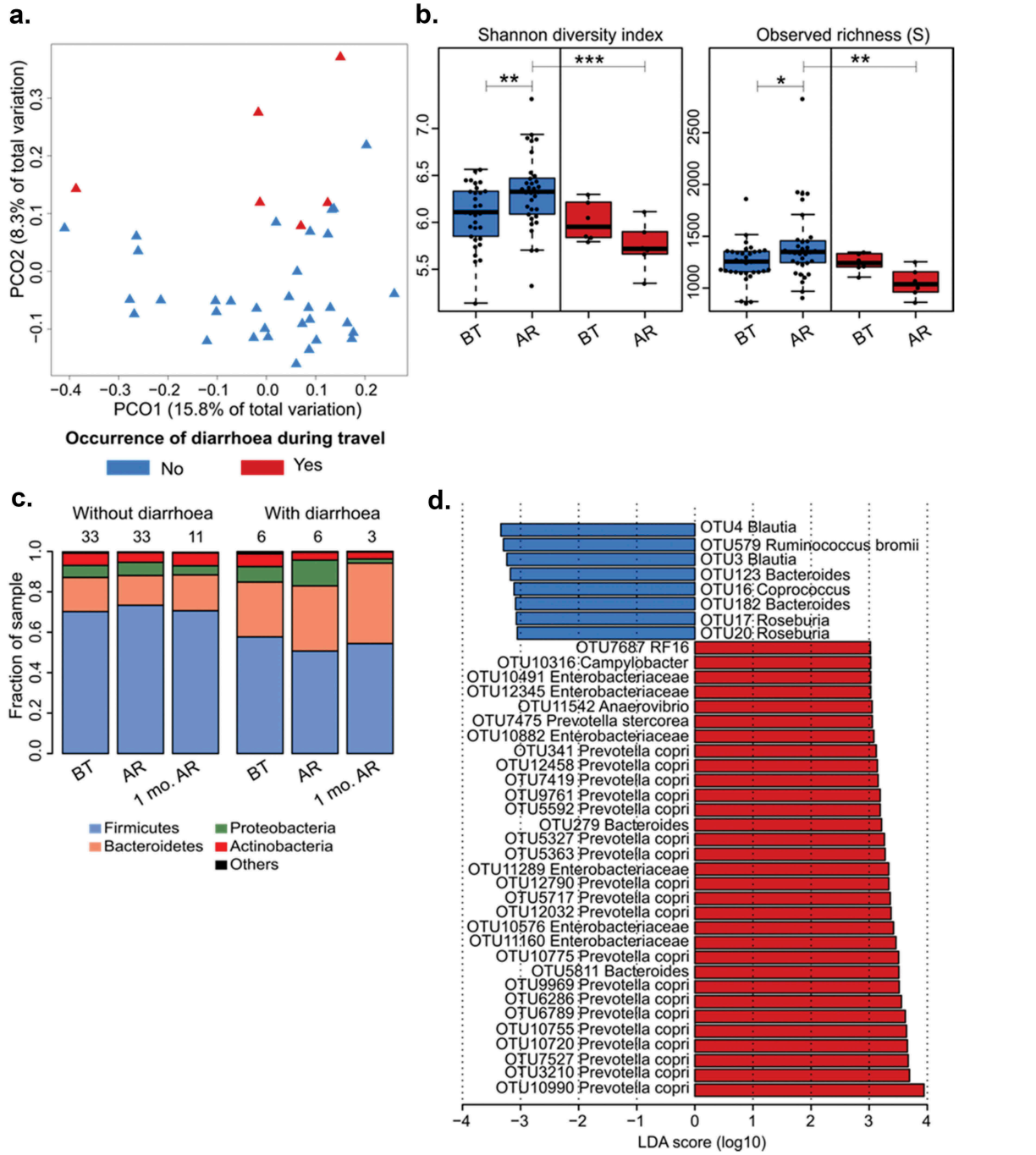

Figure 1. Microbiota changes with respect to the occurrence of diarrhea during the travel. (a) PCoA plot showing the distribution of samples taken at the return, from travelers who had (red triangles) or not (blue triangles) diarrhea during the travel. Of the 6 individuals who suffered from diarrhea 3 went to Asia, 2 to Sub-Saharan Africa and 1 to Latin America. Percentage of variation explained by the first two axes is indicated. (b) Boxplots and dot plots depicting the values and their corresponding distributions of ecological indexes, computed at the 16S OTU level, before travel (BT) and at return (AR), between travelers experiencing or not diarrhea. Stars correspond to the following p-values: * = <0.05; ** = <0.01; *** = <0.001. (c) Bar plots reporting the averaged relative abundance (/100) of phyla detected by mapping to 16S Greengenes database in travelers without diarrhea (n = 33 of which 11 were still MRE positive one month after the return) and those with (n = 6 of which 3 were still MRE positive one month after the return). Numbers at the top of the bars indicate the amount of samples for each travel time point/condition. (D) Bar plot reporting log10-transformed LDA scores of OTUs selected by LEfSe analyses (p-value <0.05). Cohorts of samples and colour label are the same as explained in (a). Only OTUs with the absolute value of LDA score (scaled in log10) of at least 3 are represented. Greengenes taxonomy identifiers for all OTUs are reported in Supplementary Table 4.

We also investigated the association of microbiota composition and the MRE carriage one month after return in the 17 travelers found MRE-positive at return and not treated with antibiotics (one subject took amoxicillin after return). In this case, the composition of the intestinal microbiota of the subjects whose samples became MRE-negative (n = 11) was significantly different from those whose samples remained MRE-positive (n = 6) (Table 2).

OTUs assigned to *Bifidobacterium adolescentis* (OTU6129) and to some Clostridiales (OTU61, OTU7384, OTU405, OTU8089) as well as *Bifidobacterium* strains detected by 23S analyses, were enriched in individuals who had cleared MRE one month after the return (LEfSe and Wilcoxon rank sum test, p-value <0.05; Figure 2(a-b); Supplementary Figure 6A-B). Already before departure, the proportions of these bacteria were higher in MRE carriers who cleared their carriage one month after return than in those who were still positive (Figure 2(a); Supplementary Figure 6A). For most OTUs associated with MRE clearance, the species level taxonomy of *de novo* assembled 16S gene was not available (Supplementary Table 2).

Remarkably, within the subcohorts of 17 travelers, we found that the microbiota profiles of MRE cleared individuals were significantly different from persistent MRE carriers before departure and one month after return (Supplementary Table 3; Figure 2(c); Supplementary Figure 6C).

The observed species richness measured before travel was significantly lower in individuals who acquired MRE during the travel and remained MRE carriers one month after return than in those who cleared their carriage (Supplementary Figure 5B). We found that the relative abundance of several OTUs and strains/species mapping to Enterobacteriaceae significantly decreased more than 2 fold one month after the return, independently of MRE carriage (Supplementary Figure 7). Despite that, the abundance of Proteobacteria phylum, to which Enterobacteriaceae belong, did not change over time or in relation to MRE carriage measured one month after the return (Figure 2(d); Supplementary Figure 6D).

Discussion and conclusions

One of the main results of this study is that travelers who experienced diarrhea (regardless of the etiology) had a higher intestinal abundance of *P. copri*, before the travel, at return, and 1 month after, than those who did not. This suggests that subjects with higher relative abundance of *P. copri* could be at higher risk for diarrhea during travel. Interestingly, *P. copri* has popped up in various metagenomic studies as either a beneficial or pathogenic bacterium: it has been associated to rheumatoid arthritis,[23,24] insulin resistance[25] but also to good health status[26,27] and to an improved glucose homeostasis.[28] In relation to diarrhea, contradictory findings on the role of *P. copri* were documented. This species has been positively associated with parasitic diarrhea[29]; however, it has also been reported to have a potential protective effect against diarrhea caused by enterotoxigenic *E. coli*.[30] Furthermore, *Prevotella* species are involved in the recovery from choleric diarrhea[31] and have lower relative abundance in malnutrition-associated diarrhea.[32] These contradictory findings could be explained by the high inter-strain and inter-individual genetic variations of this species,[33] suggesting that different strains have various functions, antigens and/or metabolites being either beneficial or deleterious for the host. Another characteristic of microbiota profiles of people suffering diarrhea is decreased species richness at return. Diminution of species richness upon diarrhea occurrence is also described in one study by Youmans and colleagues addressing by 16S profiling the intestinal microbiota of travelers returning to USA from Central America or India.[34] Oppositely, subjects suffering from a Norovirus-caused diarrhea had a surprisingly higher intestinal richness and diversity than diarrhea-free travelers. However, comparison of the results from Youmans *et al.* and this study are compromised by the low numbers of reads analyzed per sample (<3000), the absence of pre-travel samples and the collection of samples from symptomatic subjects (while the subjects from VOYAG-R were sampled at distance from diarrheal symptoms).[34]

We observed that the travelers who did not experience diarrhea during travel had a microbiota profile

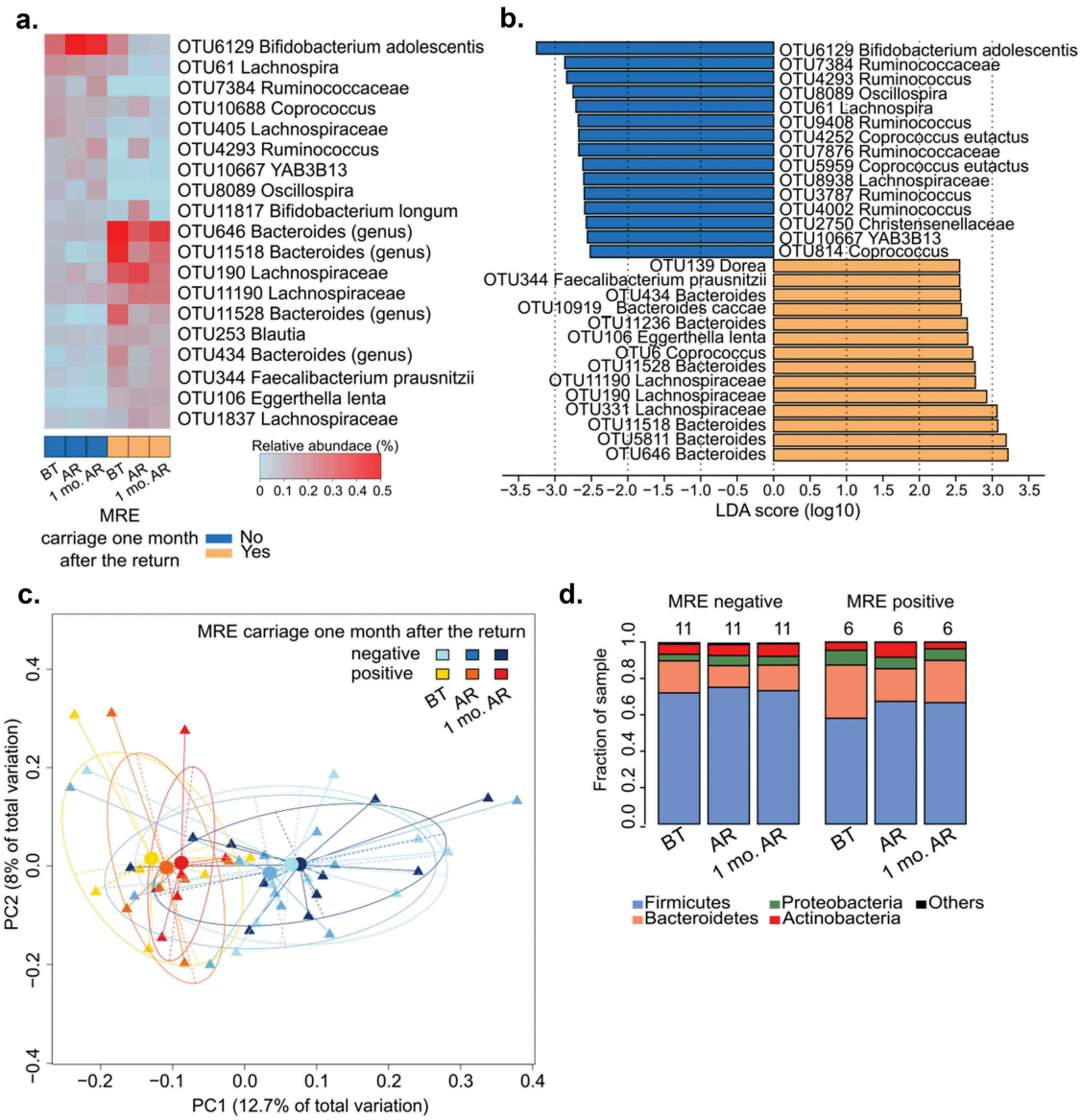

Figure 2. Comparison of travelers who acquired MRE and remained positive (n = 11) or became negative (n = 6) one month after return. (a) Heat map illustrating the mean relative abundance (expressed as percentage of total reads) of 19 OTUs in the three sampling points (BT, AR and 1 mo. AR = before travel, at return and one month after the return, respectively) of travelers MRE positive (yellow bottom bar) and MRE negative (blue bottom bar) one month after the return. OTUs were selected if the p-value was < 0.05 (Wilcoxon rank sum test), the fold change was of at least 2, and when the mean relative abundance was of at least 0.1% in one of the six represented conditions represented (i.e. BT, AR and 1 mo. AR of MRE-positives and of MRE-negatives one month after the return). Greengenes taxonomy identifiers for all OTUs are reported in Supplementary Table 4. (b) Barplot reporting log10-transformed LDA scores from each OTUs resulting significant (p-value < 0.05) from LEfSe analyses on samples from travelers MRE positive (yellow) and MRE negative (blue) one month after the return. Only OTUs with an absolute value of LDA score (scaled in log10) of at least 2.5 were retained for graphic representation. (c) PCoA plot showing the distribution of samples from time points of travelers MRE negative and MRE positive one month after return. PCO1 (12.7%) and PCO2 (8%) represent the degrees of variance of each axis. Samples are grouped in clusters delimited by ellipses. Represented centroids (spheres) capture the origin of each ellipsis. (d) Bar plots reporting the averaged relative abundance (/100) of phyla detected by mapping to 16S Greengenes database in travelers MRE negative (n = 11) and MRE positive travelers (n = 6) one month after return.

enriched in members of *Ruminococcus, Coprococcus*, and *Dorea*. Consistently with our findings, these genera were depleted in 16S microbiota profiles of individuals suffering from post-transplantation[35] and nosocomial diarrhea, including *Clostridium difficile* infection.[36] Moreover, *Roseburia* and *Ruminococcus* species have been shown to prevent gut inflammation by strengthening gut barrier function in mice[37] and by

enhancing starch fermentation in humans,[38] respectively. In addition to an anti-inflammatory role, *Ruminococcus* species were reported to oppose *Vibrio cholerae* colonization in a mouse model by deregulating the expression of virulence factors via quorum sensing.[39] Subjects who did not experience diarrhea during the travel had a richer and more diverse microbiota after travel than before. However, 1 month after return, the diversity and richness for those individuals tended to decrease to a baseline (pre-travel) level. Increase in richness and diversity at return when diarrhea is not experienced could reflect the ingestion of new bacteria that are not met in France, and/or the consumption of food that could act as prebiotics for bacteria in the pre-travel sample.

Besides, we observed an increase of Enterobacteriaceae in all travelers, which was more significant in those subjects who experienced diarrhea during their trip. This could be expected as several diarrhea agents – *E. coli, Shigella* and *Salmonella* – belong to the Enterobacteriaceae family.[40] This increase was transient and the relative abundance of Enterobacteriaceae returned close to baseline one month after travel.

Another significant result of this study is the association between intestinal microbiota composition and clearance of MRE in healthy travelers. Indeed, we observed that a set of bacteria from the Clostridiales order was more abundant in travelers who had no detectable MRE one month after return than in those who remained MRE carriers. Moreover, this pattern was already observed before travel. In addition, among the subjects who acquired MRE during travel, bacterial richness and diversity at baseline (before travel) were lower in individuals who remained MRE carriers one month after travel than in those who did not.

Altogether, our findings support the concept that the intestinal microbiota affects MRE clearance. These results are in line with the observations performed in mice in which some specific OTUs were shown to be associated with the clearance of vancomycin-resistant enterococci[16] – *Listeria monocytogenes*[41] and *Clostridium difficile*[42] – but also with the MRE clearance after fecal material transplantation.[14] Nonetheless, these phenomena were observed after an alteration of the microbiota by antibiotics, whereas our observations were obtained from healthy, antibiotic-free travelers.

The main limitation of this study is that the number of samples is relatively low with regards to some variables such as MRE carriage after the return and the occurrence of diarrhea. We do not exclude that some travelers might have cleaned their gut already before return and therefore this has lowered the number of MRE positive travelers detected after the travel. To overcome the limitation concerning the low number of samples, we combined several biostatistical and bioinformatics approaches which produced consistent results. Of note, we took advantage of using total RNA (rich in ribosomal RNA) to bypass the need for amplification of a specific region of the 16S gene (that leads to biases) and to allow a separate analysis on 16S and 23S taxonomic markers. On the other hand, with our approach, we did not provide insights into bacterial functions since mRNA enrichment steps were not carried out. Another limitation is that we could not precisely identify and characterize the bacterial species associated with the intestinal clearance of MRE despite our attempts to assemble the full 16S genes, hindering the realization of in vitro and in vivo experiments to demonstrate the precise role of these bacteria. Metagenomic sequencing that allows functional analysis could now be used on a similar setting to identify the genes associated with the clearance of MRE irrespectively of the taxonomy of their host. Besides, the etiological agents of traveler's diarrhea were not sought as it was outside of the VOYAG-R study's scope. Consequently, we could not link the composition of the microbiota to the presence of a specific pathogen. Still, the intestinal alteration due to traveler's diarrhea seems to be pathogen-independent.[34] Also, the loperamide intake (potentially involved in the acquisition of MRE[43]) was not recorded in the VOYAG-R study. Finally, we did not consider patients taking antibiotics (other than doxycycline taken for malaria prophylaxis) during the travel and after they returned because we aimed at assessing the link between the composition of the intestinal microbiota and the acquisition/clearance of MRE and the occurrence of diarrhea. Nonetheless, excluding these travelers may prevent from extrapolating our observations to travelers who took antibiotics during the travel

or after they returned. Likewise, our subjects were French citizens traveling to tropical regions and our findings should be confirmed in subjects from other origins.

In conclusion, we showed that the composition of intestinal microbiota is associated with the risk of occurrence of diarrhea in healthy travelers and of carriage of MRE in those who acquired MRE during the travel. Our results call for further functional explorations of the interplay between the intestinal microbiota, traveler's diarrhea, and MRE carriage.

Data availability

Mothur-quality-filtered sequences were deposited as fastq files at the European Nucleotide Archive (ENA) under the project PRJEB24843. Prior to sequences' submission, reads assigned to human genome (vGRCh38.p10) by CLARK (v1.2.3.2)[44] were removed. CLARK taxonomic classification was performed at the phylum level (Chordata).

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Author contributions

ER, LAL, SM, CE, and AA conceived and designed the study. ER, JS and VL supervised the study. The VOYAG-R study group performed the princeps's study. MG and SL performed RNA extraction. ER, VL, SL designed the statistical analyses. SL, VL, and ER analyzed the data. NG helped with read pre-processing. SL, VL, and ER wrote the manuscript. SL assembled all the Figures. JS, CE, and SM revised the manuscript. All authors read and approved the final manuscript.

Disclosure of Potential Conflicts of Interest

All authors have no potential conflicts of interest.

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ORCID

Sophie Matheron http://orcid.org/0000-0001-7879-6553

References

1. Pitout JDD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis. 2008;8:159–166. doi:10.1016/S1473-3099(08)70041-0.
2. Boucher HW, Talbot GH, Benjamin DK, Bradley J, Guidos RJ, Jones RN, Murray BE, Bonomo RA, Gilbert D, America IDSo. 10 x '20 Progress–development of new drugs active against gram-negative bacilli: an update from the infectious diseases society of America. Clin Infect Dis. 2013;56:1685–1694. doi:10.1093/cid/cit152.
3. Woerther P-L, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev. 2013;26:744–758. doi:10.1128/CMR.00023-13.
4. Tängdén T, Cars O, Melhus A, Löwdin E. Foreign travel is a major risk factor for colonization with Escherichia coli producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. Antimicrob Agents Chemother. 2010;54:3564–3568. doi:10.1128/AAC.00220-10.
5. Arcilla MS, van Hattem JM, Haverkate MR, Bootsma MCJ, van Genderen PJJ, Goorhuis A, Grobusch MP, Lashof AMO, Molhoek N, Schultsz C, et al. Import and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study. Lancet Infect Dis. 2017;17:78–85. doi:10.1016/S1473-3099(16)30319-X.
6. Ruppé E, Armand-Lefèvre L, Estellat C, Consigny P-H, El Mniai A, Boussadia Y, Goujon C, Ralaimazava P, Campa P, Girard P-M, et al. High Rate of Acquisition but Short Duration of Carriage of Multidrug-Resistant Enterobacteriaceae After Travel to the Tropics. Clin Infect Dis. 2015;61:593–600. doi:10.1093/cid/civ333.
7. Kantele A, Lääveri T, Mero S, Vilkman K, Pakkanen SH, Ollgren J, Antikainen J, Kirveskari J. Antimicrobials increase travelers' risk of colonization by extended-spectrum betalactamase-producing Enterobacteriaceae. Clin Infect Dis. 2015;60:837–846. doi:10.1093/cid/ciu957.

8. Steffen R, Hill DR, DuPont HL. Traveler's diarrhea: A clinical review. JAMA. 2015;313:71–80. doi:10.1001/jama.2014.17006.
9. Ducluzeau R, Salomon JC, Huppert J. [Implantation of a lysogenic strain and a sensitive strain of Escherichia coli K 12 in the digestive tube of axenic mice: establishment of a balance]. Annales De l'Institut Pasteur. 1967;112:153–161.
10. Vollaard EJ, Clasener HA. Colonization resistance. Antimicrob Agents Chemother. 1994;38:409–414.
11. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 2013;13:790–801. doi:10.1038/nri3535.
12. van der Waaij D, Berghuis-de Vries JM, Lekkerkerk Lekkerkerk-V N JEC. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond). 1971;69:405–411.
13. Stripling J, Kumar R, Baddley JW, Nellore A, Dixon P, Howard D, Ptacek T, Lefkowitz EJ, Tallaj JA, Benjamin WH, et al. Loss of Vancomycin-Resistant Enterococcus Fecal Dominance in an Organ Transplant Patient With Clostridium difficile Colitis After Fecal Microbiota Transplant. Open Forum Infecti Dis. 2015;2:ofv078. doi:10.1093/ofid/ofv078.
14. Singh R, van Nood E, Nieuwdorp M, van Dam B, Ten Berge IJM, Geerlings SE, Bemelman FJ. Donor feces infusion for eradication of extended spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. Clin Microbiol Infect. 2014. doi:10.1111/1469-0691.12683.
15. Millan B, Park H, Hotte N, Mathieu O, Burguiere P, Tompkins TA, Kao D, Madsen KL. Fecal microbial transplants reduce antibiotic-resistant genes in patients with recurrent Clostridium difficile infection. Clin Infect Dis. 2016;62:1479–1486. doi:10.1093/cid/ciw185.
16. Ubeda C, Bucci V, Caballero S, Djukovic A, Toussaint NC, Equinda M, Lipuma L, Ling L, Gobourne A, No D, et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. Infect Immun. 2013;81:965–973. doi:10.1128/IAI.01197-12.
17. Gosalbes MJ, Vázquez-Castellanos JF, Angebault C, Woerther P-L, Ruppé E, Ferrús ML, Latorre A, Andremont A, Moya A. Carriage of enterobacteria producing extended-spectrum β-Lactamases and composition of the gut microbiota in an Amerindian Community. Antimicrob Agents Chemother. 2016;60:507–514. doi:10.1128/AAC.01528-15.
18. Ruppé E, Armand-Lefèvre L, Estellat C, El-Mniai A, Boussadia Y, Consigny PH, Girard PM, Vittecoq D, Bouchaud O, Pialoux G, et al. Acquisition of carbapenemase-producing Enterobacteriaceae by healthy travellers to India, France, February 2012 to March 2013. Euro Surveill. 2014;19(14):pii: 20768..
19. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75. doi:10.1128/AEM.01541-09.
20. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–5072. doi:10.1128/AEM.03006-05.
21. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–D596. doi:10.1093/nar/gks1219.
22. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–2461. doi:10.1093/bioinformatics/btq461.
23. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, Hirota K, Matsushita M, Furuta Y, Narazaki M, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T Cells in the intestine. Arthritis Rheumatol. 2016;68:2646–2661. doi:10.1002/art.39783.
24. Pianta A, Arvikar S, Strle K, Drouin EE, Wang Q, Costello CE, Steere AC. Evidence for immune relevance of prevotella copri, a gut microbe, in patients with rheumatoid arthritis. Arthritis Rheumatol. 2016. doi:10.1002/art.40003.
25. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BAH, Forslund K, Hildebrand F, Prifti E, Falony G, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016;535:376–381. doi:10.1038/nature18646.
26. Glick-Bauer M, Yeh M-C. The health advantage of a vegan diet: exploring the gut microbiota connection. Nutrients. 2014;6:4822–4838. doi:10.3390/nu6114822.
27. Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, Hallen A, Martens E, Björck I, Bäckhed F. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of prevotella. Cell Metab. 2015;22:971–982. doi:10.1016/j.cmet.2015.10.001.
28. De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis. Cell Metab. 2016;24:151–157. doi:10.1016/j.cmet.2016.06.013.
29. Gilchrist CA, Petri SE, Schneider BN, Reichman DJ, Jiang N, Begum S, Watanabe K, Jansen CS, Elliott KP, Burgess SL, et al. Role of the gut microbiota of children in diarrhea due to the protozoan parasite entamoeba histolytica. J Infect Dis. 2016;213:1579–1585. doi:10.1093/infdis/jiv772.
30. Pop M, Paulson JN, Chakraborty S, Astrovskaya I, Lindsay BR, Li S, Bravo HC, Harro C, Parkhill J,

Walker AW, et al. Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic Escherichia coli and subsequent ciprofloxacin treatment. BMC Genomics. 2016;17:440. doi:10.1186/s12864-016-3328-4.

31. David LA, Weil A, Ryan ET, Calderwood SB, Harris JB, Chowdhury F, Begum Y, Qadri F, LaRocque RC, Turnbaugh PJ. Gut microbial succession follows acute secretory diarrhea in humans. mBio. 2015;6. doi:10.1128/mBio.00381-15.
32. Kieser S, Sarker SA, Sakwinska O, Foata F, Sultana S, Khan Z, Islam S, Porta N, Combremont S, Betrisey B, et al. Bangladeshi children with acute diarrhoea show faecal microbiomes with increased Streptococcus abundance, irrespective of diarrhoea aetiology. Environ Microbiol. 2018;20:2256–2269. doi:10.1111/emi.2018.20.issue-6.
33. Zhu A, Sunagawa S, Mende DR, Bork P. Inter-individual differences in the gene content of human gut bacterial species. Genome Biol. 2015;16:82. doi:10.1186/s13059-015-0667-4.
34. Youmans BP, Ajami NJ, Jiang Z-D, Campbell F, Wadsworth WD, Petrosino JF, DuPont HL, Highlander SK. Characterization of the human gut microbiome during travelers' diarrhea. Gut Microbes. 2015;6:110–119. doi:10.1080/19490976.2015.1019693.
35. Lee JR, Muthukumar T, Dadhania D, Toussaint NC, Ling L, Pamer E, Suthanthiran M. Gut microbial community structure and complications following kidney transplantation: A pilot study. Transplantation. 2014;98:697–705. doi:10.1097/TP.0000000000000370.
36. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, Wang GP. Intestinal dysbiosis and depletion of butyrogenic bacteria in clostridium difficile infection and nosocomial diarrhea. J Clin Microbiol. 2013;51:2884–2892. doi:10.1128/JCM.00845-13.
37. Patterson AM, Mulder IE, Travis AJ, Lan A, Cerf-Bensussan N, Gaboriau-Routhiau V, Garden K, Logan E, Delday MI, Coutts AGP, et al. Human gut symbiont roseburia hominis promotes and regulates innate immunity. Front Immunol. 2017;8:1166. doi:10.3389/fimmu.2017.01166.
38. Ze X, Duncan SH, Louis P, Flint HJ. Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. ISME J. 2012;6:1535–1543. doi:10.1038/ismej.2012.4.
39. Hsiao A, Ahmed AMS, Subramanian S, Griffin NW, Drewry LL, Petri WA Jr., Haque R, Ahmed T, Gordon JI. Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. Nature. 2014;515:423–426. doi:10.1038/nature13738.
40. Shah N, DuPont HL, Ramsey DJ. Global etiology of travelers' diarrhea: systematic review from 1973 to the present. Am J Trop Med Hyg. 2009. doi:10.1089/cmb.2012.0021.
41. Becattini S, Littmann ER, Carter RA, Kim SG, Morjaria SM, Ling L, Gyaltshen Y, Fontana E, Taur Y, Leiner IM, et al. Commensal microbes provide first line defense against Listeria monocytogenes infection. J Exp Med. 2017;214:1973–1989. doi:10.1084/jem.20170495.
42. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew M, Viale A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature. 2014. doi:10.1038/nature13828.
43. Kantele A, Mero S, Kirveskari J, Lääveri T. Increased risk for ESBL-producing bacteria from co-administration of loperamide and antimicrobial drugs for travelers' diarrhea. Emerg Infect Dis. 2016;22:117–120. doi:10.3201/eid2201.151272.
44. Ounit R, Wanamaker S, Close TJ, Lonardi S. CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers. BMC Genomics. 2015;16:236. doi:10.1186/s12864-015-1419-2.

Chapter 2: Effects of antibiotic duration on the intestinal microbiota and resistome: the PIRATE RESISTANCE project, a cohort study nested within a randomized trial

Stefano Leo, Vladimir Lazarevic, Elodie von Dach, Laurent Kaiser, Virginie Prendki, Jacques Schrenzel, Benedikt D. Huttner and Angela Huttner

2.1. Introduction

The onset of antibiotic resistance is also driven by antibiotic usage. Whether to shorten the duration of antibiotic administration has a positive effect on the microbiota composition and diversity has not yet been investigated. Therefore, we analyzed the fecal microbiota of adult patients enrolled in the PIRATE trial and treated with short *versus* long antibiotic courses for Gram-negative bacteremia. We characterize the temporal changes of microbiota and resistome during and after treatments. Eventually, we compared those changes with a cohort of patients that were not treated with antibiotics.

2.2. Article Status

The article has been submitted and it is currently under review.
Pre-print is available at: https://papers.ssrn.com/sol3/papers.cfm?abstract_id=3761111
Supplementary Materials are in the Appendix.

2.3. Contributions

I contributed to the curation of clinical metadata and I performed all the bioinformatics and statistical analysis. I contributed to the results' interpretation and to writing and reviewing of the manuscript. I generated all the figures and tables for publication. As corresponding author, I will manage the process of submitting, editing and publishing the manuscript.

Effects of antibiotic duration on the intestinal microbiota and resistome: the PIRATE RESISTANCE project, a cohort study nested within a randomized trial

Stefano Leo*[,1,§], Vladimir Lazarevic[1,§], Elodie von Dach[2], Laurent Kaiser[3,4], Virginie Prendki[3,4,5], Jacques Schrenzel[1,3,4], Benedikt D. Huttner[3,4], Angela Huttner[2,3,4]

1 Genomic Research Laboratory, Division of Infectious Diseases, University Hospitals and University of Geneva, Geneva, Switzerland

2 Clinical Research Center, Geneva University Hospitals and University of Geneva, Geneva, Switzerland

3 Division of Infectious Diseases, Geneva University Hospitals and Faculty of Medicine, Geneva, Switzerland

4 Faculty of Medicine, University of Geneva, Geneva, Switzerland

5 Division of Internal Medicine for the Aged, Hôpital des Trois-Chêne, Thônex-Genève, Geneva, Switzerland

§Equal contribution

* Stefano Leo, MSc

Genomic Research Laboratory

Division of Infectious Diseases

Geneva University Hospitals and University of Geneva

4, Rue Gabrielle Perret-Gentil CH-1211 Geneva

stefano.leo@genomic.ch

T. +41 22 379 47 53

Key words: resistome; antibiotics; faecal microbiota; whole-metagenome shotgun sequencing; treatment duration

Word count: 2534 (max 3500)

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ABSTRACT

Abstract word count: 299

Background: Shortening antibiotic-treatment durations is a key recommendation of antibiotic-stewardship programmes, yet it is based on weak evidence. We investigated whether halving antibiotic courses would reduce antibiotic-resistance genes (ARG) in the intestinal microbiomes of patients treated for gram-negative bacteraemia.

Methods: This nested prospective cohort study included adult patients hospitalized at Geneva University Hospitals (Switzerland) participating in the PIRATE randomized trial assessing non-inferiority of shorter antibiotic courses for gram-negative bacteraemia ('cases') and, simultaneously, hospitalized patients with similar demography and comorbidity yet no antibiotic therapy ('controls'). Stool was collected from case and control patients on days 7, 14, 30 and 90 after antibiotic initiation (day 1) and days 7 and 14 after admission, respectively, and analysed by whole-metagenome shotgun sequencing. The primary outcome was ARG abundance at day 30; secondary outcomes included microbiota-species composition and clustering over time.

Findings: Forty-five patients and 11 controls were included and evaluable; ARG analyses were conducted on the 29 per-protocol patients receiving 7 (±2) days or 14 (±3) days of antibiotic therapy. At day 30, ARGs were detected at similar abundance in patients receiving 7 and 14 days (median counts/million [mCPM]: 96 versus [vs] 71; p=.38). By D30, total ARG content in both groups was similar to that of controls at D7 (362 and 370 mCPM vs 314 mCPM, p=.24 and .19). There were no significant differences among antibiotic-treated patients at any timepoint in bacterial diversity or clustering, but Shannon species diversity was significantly reduced compared to controls through D14 (median 3.12 and 3.24 in the 7-day and 14-day groups vs 3.61 [controls]; p=.04 and .012). Fourteen-day antibiotic courses prompted a persistent trend toward reduced faecal phage content.

Interpretation: Reducing antibiotic durations by half did not result in decreased abundance of ARGs in patients treated for gram-negative bacteraemia, nor did it improve microbiota species diversity.

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RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed with the terms “antibiotics” AND “duration” AND “microbiota” AND “resistome” in ‘All Fields’ for previously published reviews and articles from database creation to December 15, 2020. Ten studies on the effects of large- and narrow-spectrum antibiotics administration on infant or adult microbiota have been documented in a systematic review. Antibiotic course is known to have dramatic effect on microbiota composition as well as in the selection of antibiotic-resistance genes (ARG). In particular, microbiota species diversity can be heavily decreased also after short-term therapy with narrow-spectrum antibiotics. However, whether shorter antibiotic treatments result in a reduction of genetic resistance in the intestinal microbiota as well as to an earlier recovery of microbiota diversity remains an open question. No study has examined the comparative effects of antibiotic therapy on the intestinal microbiota of patients randomized to different antibiotic durations.

Added value of this study

Our metagenomic study shows that short-term (≤7-day) antibiotic therapy for gram-negative bacteraemia did not prompt reduced ARG abundance nor improved microbiota diversity compared to longer (≥14-day) therapy. We provide insights on how microbiota and resistome change upon short-term versus long-term antibiotic courses. In particular, we showed that one week of antibiotic therapy alone is already sufficient to drastically impact faecal microbiota and resistome composition, and does not result in faster recovery than that seen with two weeks of antibiotics.

Implications of all the available evidence

Antibiotic resistance has increased and antimicrobial drug development is slowing. One major factor driving resistance is antibiotic usage in clinical practice. Here we documented that antibiotic therapy increases ARG abundance and compromises microbiota species diversity irrespective of its duration. Our findings highlight the importance of reinforcing surveillance on antibiotic administration.

Preprint not peer reviewed

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INTRODUCTION

Antibiotic overuse is one of the key drivers of antibiotic resistance (1). Epidemiologic data demonstrate that long antibiotic courses select for collateral resistance in the microbiota (2), putting patients at increased risk for later infections by antibiotic-resistant pathogens (3, 4). Metagenomic data are beginning to capture the immediate changes in gut microbiota composition and antibiotic-resistance genes (ARG) after administration of commonly used antibiotics such as ciprofloxacin (5) and imipenem (6). Yet data are lacking as to whether patients randomized to significantly shorter antibiotic courses will see a proportionate reduction in detectable ARG in the intestinal microbiome. It is also as yet unknown whether those patients retain more microbiota diversity and experience earlier recovery.

To answer these questions, we analysed faecal samples collected from hospitalized patients at the time of their inclusion in the 'PIRATE' study (7, 8), a multicentre, point-of-care-randomized trial assessing clinical non-inferiority of 7-day and individualized (C-reactive-protein [CRP]-guided) antibiotic courses to 14-day courses for gram-negative bacteraemia, and in the three months thereafter. The PIRATE study confirmed non-inferiority of shorter antibiotic durations, with similar clinical cure rates in all arms in the three months following randomization.

MATERIALS AND METHODS

The 'PIRATE RESISTANCE Project': study design and participants

Between July 2018 and April 2019, this nested prospective observational cohort study included adult (≥18 years) patients hospitalized at the Geneva University Hospitals and enrolled in the PIRATE trial ('cases') and, simultaneously, hospitalized patients with similar demography and comorbidity but without antibiotic therapy ('controls'). Entry criteria for the PIRATE trial are described elsewhere (7, 8); briefly, immunocompetent adults with gram-negative bacteraemia without complications (*e.g.*, abscess) or source requiring long-term therapy (*e.g.*, endocarditis) were eligible. Stool samples were collected from case patients on days 7 (±2), 14 (±3), 30 (±7) and 90 (±14) after antibiotic initiation and from control patients on days 7 (±2) and 14 (±3) after hospital admission. Characteristics of the study cohort are reported in Table S1.

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Laboratory, bioinformatics and statistical methods

Stool sampling

Stools were collected from a Commode Specimen Container (Covidien Medtronic, Dublin, Ireland) placed over the toilet-seat opening. A few nut-sized sections of faecal material were added to Sarstedt Feces Tube 76x20mm (Nümbrecht, Germany) and then refrigerated at 4 °C. Samples were delivered within 24 h to the Genomic Research Laboratory for storage at -80°C until DNA extraction.

Whole-metagenome shotgun sequencing and bioinformatic analyses

Detailed information is provided in the supplement. DNA extracted from stool together with four no-sample controls (DNA obtained using the same extraction procedure but omitting the addition of stool sample) were sequenced (2 x 150) on a NovaSeq 6000 System (Illumina) at Fasteris (Plan-les-Ouates, Switzerland). Reads were quality filtered with Trimmomatic v0.36 and dereplicated with in-house Perl script. Reads which were classified by CLARK v1.2.5 [11] to the species *Homo sapiens* were removed. Dereplicated reads classified as non-human were then assigned by CLARK to bacterial, viral and fungal species. The relative abundance of bacterial species was expressed as percentage of total number of reads mapping to bacteria.

For each individual, we investigated resistome composition according to the type of antibiotic administered to case patients (beta-lactam, quinolone, folate-pathway antagonist [FPA], aminoglycoside, macrolide and metronidazole). Dereplicated and quality-filtered forward non-human reads were mapped against Resfinder database (9) by USEARCH v11.0.667 (10). The relative abundance of detected ARGs was expressed as counts per million of bacterial reads (CPM).

Patient group assignment, statistical analyses and data visualization

Given the lack of data allowing for hypothesized effect sizes, the sample size was not calculated; a convenience sample of 50 cases and roughly 10 controls were chosen, with its size-guided chiefly by budgetary and logistic feasibility.

For each patient, antibiotic duration was tabulated in days; a gap of ≤24 hours mid-course (*e.g.*, missed dose) was accepted as one day of therapy. Patients were classified by treatment duration and analysis population (Figure S1, Table S2).

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Three analysis populations were created. All enrolled case patients but one were included in the 'baseline population': one patient (number 159) was excluded from all analyses because he had received vancomycin for 22 days prior to enrolment. Patients in the baseline population were subclassified into 'group S' (for "short"; antibiotic duration ≤10 days) or 'group L' (for "long"; duration >10 days).

The 'per-protocol' population consisted of case patients with the following criteria:

(1) ≥2 faecal samples, one being a baseline sample (D7 or D14) and the other a D30 sample (primary outcome),

(2) no further receipt of antibiotics after D14, and

(3) treatment duration of either 7 (±2) or 14 (±3) days.

The third population consisted of control patients. Results of the per-protocol analyses are presented here; results from baseline-population analyses can be found in the supplement.

The four no-sample controls were excluded from statistical analyses and testing. Statistical analyses, including principal coordinate analysis (PCoA), and data visualization were conducted with R v3.2.3 and PRIMER v7 (PRIMER-E Ltd, Plymouth, UK) and are detailed in the supplement. A result was considered significant when its associated p was <.05 (two-sided).

PCoA were performed to visually explore differences between microbiota species profiles (so-called 'beta diversity') among case and control patients at a given time point. PCoA analysis reduces the number of the variables determining the microbiota profile (*e.g.* all microbiota species) to two abstract dimensions that capture the highest variance present in the dataset. These two dimensions are then used to draw a plot where microbiota profile from each sample is represented as a dot. The closer two dots are on the plot, the more similar the corresponding microbiota profiles will be, and vice-versa.

RESULTS

Forty-six case patients and 11 control patients were included; among case patients, 45 and 29 were included in the baseline and per-protocol populations, respectively (Figure S1, Table S2). In the baseline population, 23 patients received ≤10 days of antibiotic therapy (group S) and 22 >10 days (group L). In the per-protocol

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population, 15 patients received 7 days of therapy and 14 received 14 days. Patient demographics and individual antibiotic regimens are described in Tables S1 and S2, respectively.

The resistome

We identified ARGs encoding resistance against 14 unique antibiotic classes and 10 combinations of ≥2 antibiotic classes. In per-protocol patients, ARGs conferring resistance to the antibiotic administered were identified (Figure 1A-B). Yet abundance of ARGs at day 30 did not differ significantly between patients receiving 7 days and those receiving 14 days (median counts/million (mCPM): 96 versus 71, p=.38; Figure 1C). Indeed, when assessing only ARGs conferring resistance to antibiotics given to the patient, the fractions of patients with decreased ARG abundance at D30 were similar between the 7-day and 14-day groups (6/13 [0.46] and 7/13 [0.54], p=1; Table 1).

Further, ARGs conferring resistance to a given antibiotic class were also found in control patients and in case patients not receiving the antibiotic class (Figure 2). When analysing all ARG content at D30 in the 7- and 14-day groups, we found a median relative abundance of 362 mCPM (IQR 270-611) and 370 mCPM (IQR 239-419; p=.425), respectively. Relative abundance of ARGs among controls did not differ significantly from that of case patients (median 314 mCPM [IQR 218-412] at D7 and 380 mCPM [IQR 278-502] at D14).

Median values of ARG normalised counts peaked at the end of treatment for both case-patient groups (D7 and D14, respectively; Figures 1C and 2D). Since all patients except two from the 7-day group (patients 182 and 215) were treated with beta-lactam antibiotics (Table S2), we examined the temporal changes specifically of beta-lactam-resistance-encoding ARG (Figure 1D): medians trended downward in both case-patient groups at the end of therapy (Figure 1D), albeit with no statistical significance.

ARG abundance by treatment duration and distance from last treatment day

Increases in ARG targeting metronidazole were not detected in patients treated with it (Figure S2). Only folate-pathway-antagonist (FPA) use was associated with FPA-targeting ARGs, with their abundance decreasing after FPA use was discontinued. Resistance against antibiotics persisted days after the end of antibiotic treatment

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irrespective of treatment duration (Figure S2). More specifically, we detected ARGs 23 (IQR 10-77) versus 18 (IQR 11-74) days after therapy discontinuation in the 7- and 14-day groups.

Microbiota species composition

Differences in microbiota species composition at D30 between patients receiving 7 and 14 days did not differ significantly (PERMANOVA test, t=1.03, p=0.3).

In PCoA plots, D30 samples tended to cluster by treatment duration and by antibiotic treatment (Figure 3A-B). The sample size did not allow for assessment of clustering at the antibiotic level.

Microbiota species composition among case patients differed significantly from that of control patients at baseline, but differences decreased over time (Table S3). Species richness and diversity were significantly reduced in per-protocol patients receiving 7 days of antibiotic therapy at D7 (p<.001) and D14 (p=.043) compared to controls (Figure S3; Table S4). For patients receiving 14 days, only Shannon diversity was significantly decreased at D7 and at D14 compared to controls (Table S4). In addition, in both treated patient groups these ecological indices increased at D30 and D90 compared to D7 and D14 (Figure S3).

We analysed the temporal changes of median values of the Firmicutes to Bacteroidetes (F:B) ratio over time and observed that medians increased in later time points in both groups (Figure S4). However, the F:B ratio increased in 8/14 and decreased in 8/13 patients from group 7 and 14, respectively, at D30 compared to the end-of-treatment time points (D7, D14 for group 7 and 14, respectively). These differences were not statistically significant.

In per-protocol patients, *Klebsiella pneumoniae* and *Citrobacter freundii* abundance decreased significantly at D14 and D30, respectively, in patients receiving 14 days versus 7 days (Figure 4). Conversely, *Flavonifractor plautii* (Ruminococcaceae) abundance significantly increased at D30 in patients receiving 14 days versus 7 days (Figure 4). Members of the Bifidobacteriaceae (Actinobacteria), Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae and Ruminococcaceae (Firmicutes) families were significantly more abundant at D30 and D90 than they were at D7 in per-protocol patients receiving 7 days of antibiotics (Figure S5). *Alistipes obesi, Anaerobutyricum halli, Coprococcus comes, Romboutsia timonensis, Roseburia intestinalis* and

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Veillonella parvula became more abundant by D30 in comparison to the end-of-treatment time point in all per-protocol patients (Figures S5-6).

Faecal phage composition

On average 0.14% of human-depleted reads mapped to known phage sequences. We detected 875 different phages among which the five most abundant were specific to *Escherichia* (11.7%), *Pseudomonas* and *Salmonella* (7.5%), *Staphylococcus* (7.4%) and *Lactococcus* (6.4%) hosts. Overall phage content was ~4-fold and ~3-fold decreased in the 14-day group (221 mCPM per sample) compared to the 7-day group (809 mCPM per sample, p=.012) and controls (626 mCPM per sample, p=.105), respectively. The difference in phage content was less marked between the 7-day group and control samples (p=.859).

We investigated the temporal dynamics of detected phages in both case and control patients. We observed that the median values of overall phage content in the 7-day group decreased over time and dropped at D30 (344 mCPM) compared to D7 (1021 mCPM, p=1) and D14 (622 mCPM, p= .577; Figure S7A). On the other hand, median values of phage content remained relatively stable over time for the 14-day group and controls (p>.1 and = .049, respectively; Figure S7A).

Observations made at the population level were accompanied by high variability among individual patients of the same group (Figure S7B). Roughly half of the 7-day patients had decreased phage content at D30 compared to D7 (7/14) and D14 (6/11).

The number of ≥2-fold increase in CPM D30 vs D7 and D30 vs D14 were comparable between group 7 and group 14 (D30 vs D7: 5/14 and 3/13; D30 vs D14: 2/11 and 2/13, respectively). For ≥2-fold decrease in CPM, we observed similar trends (D30 vs D7: 5/14 and 3/13; D30 vs D14: 4/11 and 4/13).

DISCUSSION

In this metagenomic study of patients randomized to different durations of antibiotic therapy for gram-negative bacteraemia, we did not find reduced ARG abundance or improved microbiota diversity in patients receiving shorter antibiotic courses: those treated for only seven days had, overall, comparable amounts of ARGs on days

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30 and 90 as those treated for 14 days. These results suggest that one week of antibiotic therapy alone suffices to set in motion changes to the resistome and microbiota that cannot easily be reversed in the short-term.

Despite individual patient variability, we observed differences in phage abundance: patients treated for 14 days had consistently suppressed phage content at all time points, except when treated with anti-FPA drugs. The significance of this finding is difficult to determine. First, the observed differences in phage content were not significant between patient groups; second, the identification of most phage-host pairs in the intestinal microbiota remains uncharacterized, and we are currently able to work only with known phage sequences, leaving much viral 'dark matter' undefined (11).

When comparing antibiotic-treated patients to untreated control patients, significant differences in both microbiota profiles and species diversity were apparent, as observed in other metagenomic studies (12, 13). Once antibiotic therapy was discontinued, case patients saw a gradual recovery in microbiota species composition, with their microbiota profiles approaching those of control patients at later time points. Yet inter-individual variability was high and depended on the antibiotic class received and, likely, other factors. Indeed, when analysing other parameters such as the Firmicutes-to-Bacteroidetes ratio and ecological indices, there was no shared pattern of changes.

Given this variability, we performed pairwise comparisons between groups of treated patients to analyse which species were affected by treatment duration. Longer antibiotic treatment seemed to be accompanied by decreased abundance of *K. pneumoniae* and *C. freundii*, both Enterobacteriaceae. Therefore, a prolonged antibiotic regimen would affect more these species than a short-term treatment, at least in a setting where multidrug-resistant strains of these bacteria are still relatively rare. Yet, in all case patients, antibiotic discontinuation resulted in a significant increase of the butyrate-producing species *A. halli, C. comes,* and *R. intestinalis*; butyrate plays an important role in preserving intestinal epithelium from inflammation (14), in opposing pathogen expansion and in enhancing pathogen clearance from the gut (15).

This study has limitations. Patients were randomized to antibiotic duration only and could receive different antibiotics for their bacteraemia, a variability that reduces the nested study's statistical power given its limited sample size. Detection of ARG was limited to acquired genetic antibiotic resistance; chromosomal mutations, which might lead to similar resistance phenotypes, were not investigated. We were unable to collect samples from patients before their antibiotic therapy began, though the inclusion of several untreated hospitalized

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patients as controls mitigated this limitation. Furthermore, we were unable to study how antibiotic duration impacts transmission of multidrug-resistant organisms (MDRO). Most clinically relevant MDRO such as extended-spectrum beta-lactamase or carbapenemase producing Enterobacterales or methicillin-resistant S*taphylococcus aureus* and vancomycin-resistant *Enterococcus* species are not selected *de novo* through antibiotic exposure but rather acquired through transmission from other patients or the environment. It is likely that the impact of antibiotic duration on transmission of MDRO shows a different dynamic than the impact on the intestinal microbiota.

Bacteraemic patients randomized to shorter antibiotic courses did not have fewer antibiotic-resistance genes or improved intestinal microbiota diversity in the weeks after therapy, suggesting that one week of antibiotic therapy alone is enough to significantly impact the intestinal microbiota and resistome. Larger studies are needed to better characterize these changes and better determine how their impact might be minimized.

Contributors

BH, VL and AH conceptualized and supervised the study. EVD, VP and AH collected the data. SL, VL and AH contributed to the analyses and interpretation of data, and drafting and revision of the manuscript. EVD, LK, VP, JS and BH contributed to the interpretation of data and revision of the manuscript. All authors read and approved the final version of the manuscript.

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Supplementary Material

Supplementary Figures and Tables are available in Supplementary appendix.

Sequencing data availability

Quality-filtered dereplicated non-human reads are available on European Nucleotide Archive Database under the study accession number PRJEB40995.

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Patient and other consents

The present study was approved by the Cantonal Ethics Committee of Geneva (no. 2017-00108); all participants provided written informed consent before enrolment.

Declaration of interests

All authors declare no competing interests.

Source of funding statements

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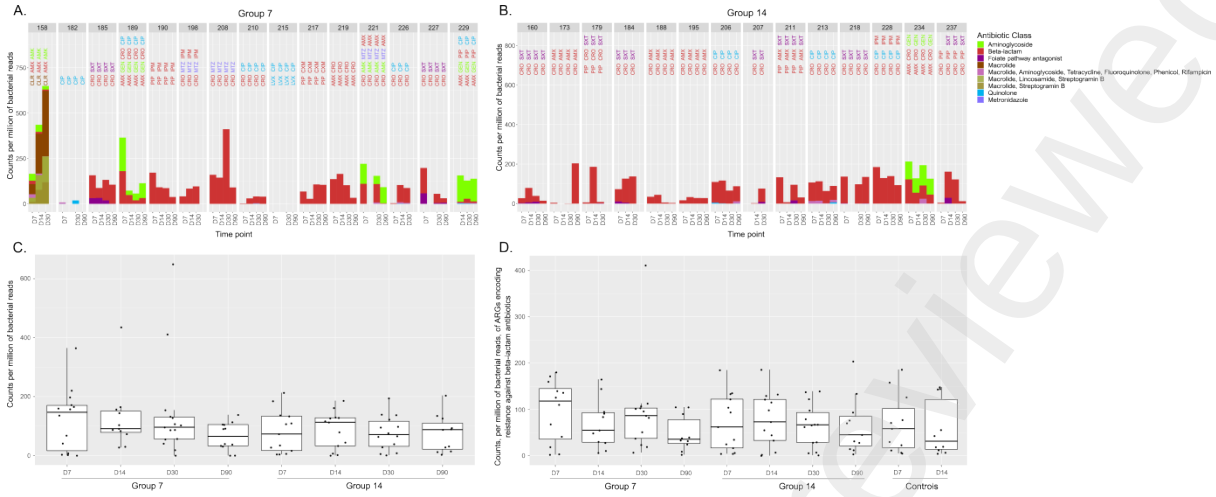

Figure 1. Resistome abundance compared to antibiotic therapy. (A.-B.) Bar plots representing the abundance of antibiotic-resistance-encoding genes in faecal samples at time points (D7, D14, D30 and D90) of patients assigned to 7 days (A.) or 14 days of antibiotic therapy (B.). For each patient, we selected genes encoding resistance against the antibiotics used in treatments till a given time point. When a time point is not included, faeces were not sampled. Antibiotic abbreviations are reported above bars and coloured according to the corresponding antibiotic class. Reads mapping to genes conferring resistance to the same class of antibiotic were summed and normalized to the number of reads mapping to bacteria. The y-axis thus reports the number of reads mapping to a given class per million of bacterial reads. Like antibiotics' abbreviations, bars are coloured according to antibiotic classes (see Abbreviations section). (C.) Boxplots reporting the changes of selected components of the resistome and summarizing data shown in panels A. and B. Dots represent single values. (D.) Boxplots reporting the changes of ARG conferring resistance to beta-lactam antibiotics in patients treated with this class of antibiotic and in controls.

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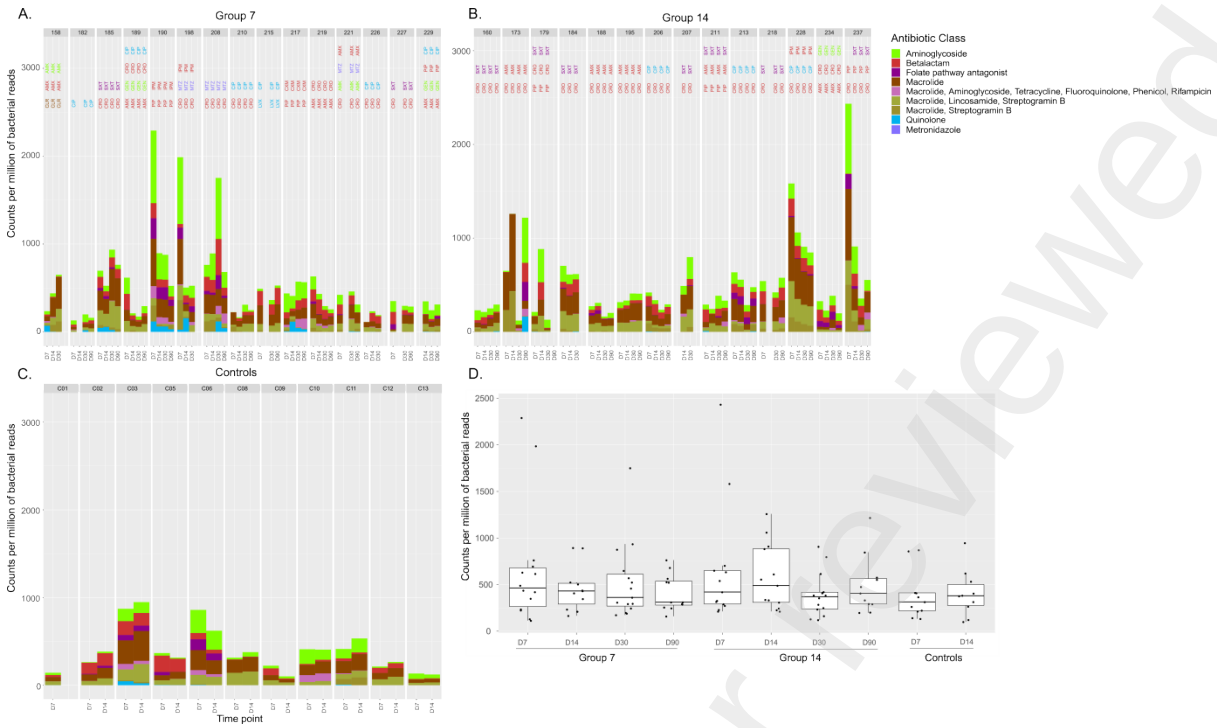

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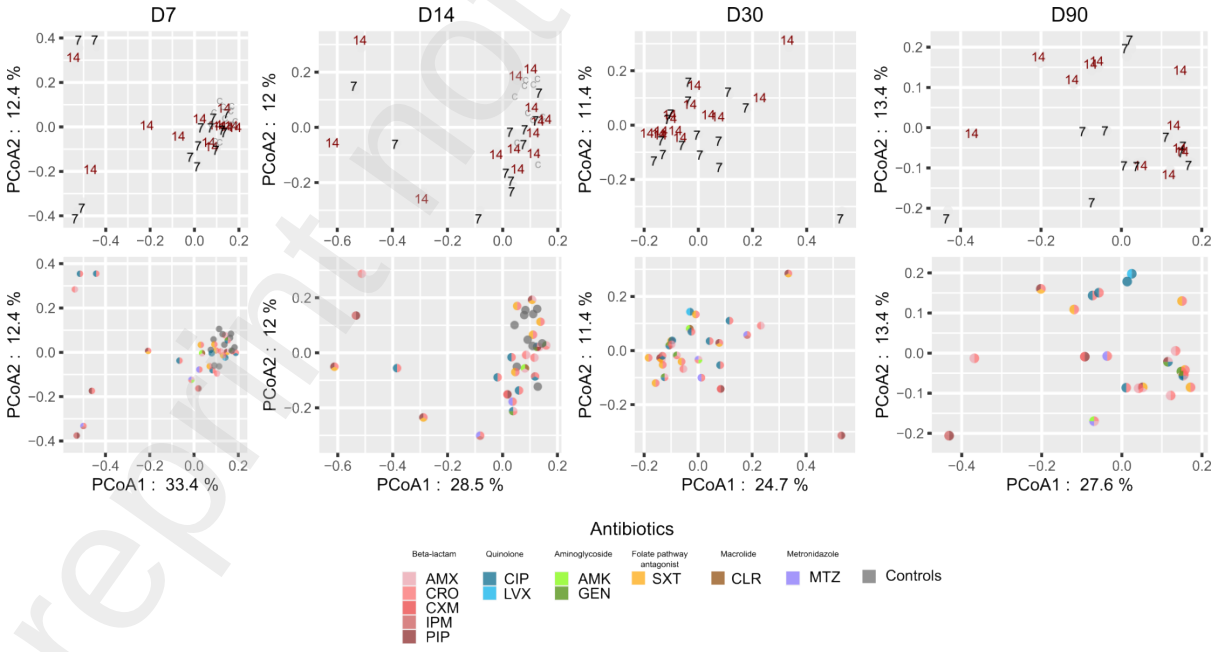
312 **Figure 2. Resistome abundance in treated patients. (A.-B.-C.)** Bar plots as in Figure 2, except that the
313 temporal changes in abundance are reported for genes encoding resistance against all the antibiotics used to treat
314 the cohort of 29 patients (see Abbreviations section). (D) Boxplots reporting the changes of the resistome and
315 summarizing data shown in panels A., B. and C. Dots represent single values.



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317 **Figure 3. Global species microbiota profiles in the 7-day and 14-day groups and in controls.** Principal
318 coordinates analysis (PCoA) plots representing global differences between bacterial species communities of

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faecal samples at a given sampling time point. Top row: PCoA plots where samples are labelled according to treatment duration: 7 = 7-day group; 14= 14-day group. C= control patients. Bottom row: dots are coloured according to antibiotic treatment. Antibiotics belonging to the same class have similar colours. For time points D7 and D14, samples from control patients are also shown (grey dots). The percentage of total data variance is reported for PCoA1 and PCoA2.

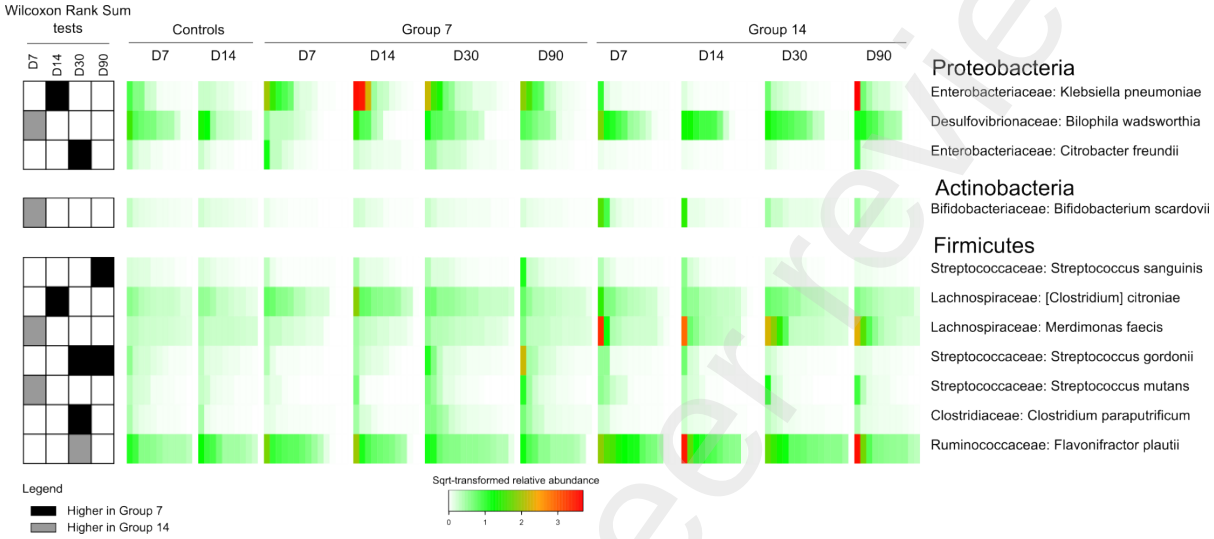

Figure 4. Differentially abundant species between patients treated for 7 days and 14 days. For each of the represented species, we report the significance of differences in the relative abundance between the 7-day and 14-day groups at a given time point (heat map on the left), the relative abundance in the groups (heat map in the middle) and the corresponding phylum and family (on the right). We reported species with differences in relative abundance between the 7- and 14-day groups associated with a significant Wilcoxon rank sum test ($p < .05$), a fold change ≥ 1.5 in the relative abundance and a mean relative abundance $\geq .05\%$ in at least one of the compared groups. Heat map on the right: coloured cells report significant differences ($p < .05$) associated with a ≥ 1.5 fold change in the relative abundance whereas white cells correspond to other cases. In particular, black represents the species significantly more abundant in the 7-day group whereas grey those significantly more abundant in the 14-day group. Heat map in the middle: square-root-transformed relative abundance is colour-scaled as indicated in the legend.

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339 **Table 1. Summary table reporting the number of patients receiving 7- or 14-day antibiotic courses for whom a decrease or increase in resistome abundance was**

340 **observed when comparing time points.** The number of the patients for each comparison refers to Figure 1.

Antibiotic duration	Comparison		Decrease in ARGs abundance at time point 1	Increase in ARGs abundance at time point 1	ARGs not detected	Total no. of samples with detected ARGs	Total no. of samples collected at both time points 1 and 2	Total no. of patients per group
	Time point 1	Time point 2						
7 days	D30	D7	6 /13 [0.46]	7/13[0.54]	1	13	14	15
	D30	D14	5/11 [0.45]	6/11[0.55]		11	11	
	D14	D7	5/10 [0.5]	5/10 [0.5]		10	10	
14 days	D30	D7	7 /13 [0.54]	6/13 [0.46]		13	13	14
	D30	D14	7/13 [0.54]	6/13 [0.46]		13	13	
	D14	D7	4/12 [0.33]	8/12 [0.67]		12	12	

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342 **Abbreviations**

Acronym	Full name	Antibiotic class
AMK	amikacin	Aminoglycoside
GEN	gentamicin	Aminoglycoside
AMX	amoxicillin	Beta-lactam
CRO	ceftriaxone	Beta-lactam
CXM	cefuroxime	Beta-lactam
FEP	cefepime	Beta-lactam
IPM	imipenem	Beta-lactam
PIP	piperacillin	Beta-lactam
CIP	ciprofloxacin	Quinolone
LVX	levofloxacin	Quinolone
SXT	sulfamethoxazole (with trimethoprim)	Folate-pathway antagonist
CLR	clarithromycin	Macrolide
MTZ	metronidazole	Metronidazole

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References

1. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2015.

2. Bell BG, Schellevis F, Stobberingh E, Goossens H, Pringle M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC infectious diseases. 2014;14:13.

3. Zarkotou O, Pournaras S, Tselioti P, Dragoumanos V, Pitiriga V, Ranellou K, et al. Predictors of mortality in patients with bloodstream infections caused by KPC-producing Klebsiella pneumoniae and impact of appropriate antimicrobial treatment. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2011;17(12):1798-803.

4. Chastre J, Wolff M, Fagon JY, Chevret S, Thomas F, Wermert D, et al. Comparison of 8 vs 15 days of antibiotic therapy for ventilator-associated pneumonia in adults: a randomized trial. JAMA. 2003;290(19):2588-98.

5. Stewardson AJ, Gaïa N, François P, Malhotra-Kumar S, Delémont C, Martinez de Tejada B, et al. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients with urinary tract infections: a culture-free analysis of gut microbiota. Clinical Microbiology and Infection. 2015;21(4):344.e1-.e11.

6. Grall N, Lazarevic V, Gaïa N, Couffignal C, Laouénan C, Ilic-Habensus E, et al. Unexpected persistence of extended-spectrum β-lactamase-producing Enterobacteriaceae in the faecal microbiota of hospitalised patients treated with imipenem. International Journal of Antimicrobial Agents. 2017;50(1):81-7.

7. Huttner A, Albrich WC, Bochud P-Y, Gayet-Ageron A, Rossel A, Dach Ev, et al. PIRATE project: point-of-care, informatics-based randomised controlled trial for decreasing overuse of antibiotic therapy in Gram-negative bacteraemia. BMJ Open. 2017;7(7):e017996-e.

8. von Dach E, Albrich WC, Brunel A-S, Prendki V, Cuvelier C, Flury D, et al. Effect of C-reactive Protein–guided antibiotic treatment duration, 7-day treatment, or 14-day treatment on 30-day clinical failure rate in patients with uncomplicated Gram-negative bacteremia: a randomized clinical trial. JAMA. 2020;323(21):2160-9.

9. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. Journal of Antimicrobial Chemotherapy. 2012;67(11):2640-4.

10. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3761111

11. Sutton TDS, Hill C. Gut bacteriophage: current understanding and challenges. Frontiers in Endocrinology. 2019;10(784).

12. Leo S, Lazarevic V, Girard M, Gaïa N, Schrenzel J, de Lastours V, et al. Metagenomic characterization of gut microbiota of carriers of extended-spectrum beta-lactamase or carbapenemase-producing Enterobacteriaceae following treatment with oral antibiotics and fecal microbiota transplantation: results from a multicenter randomized trial. Microorganisms. 2020;8(6).

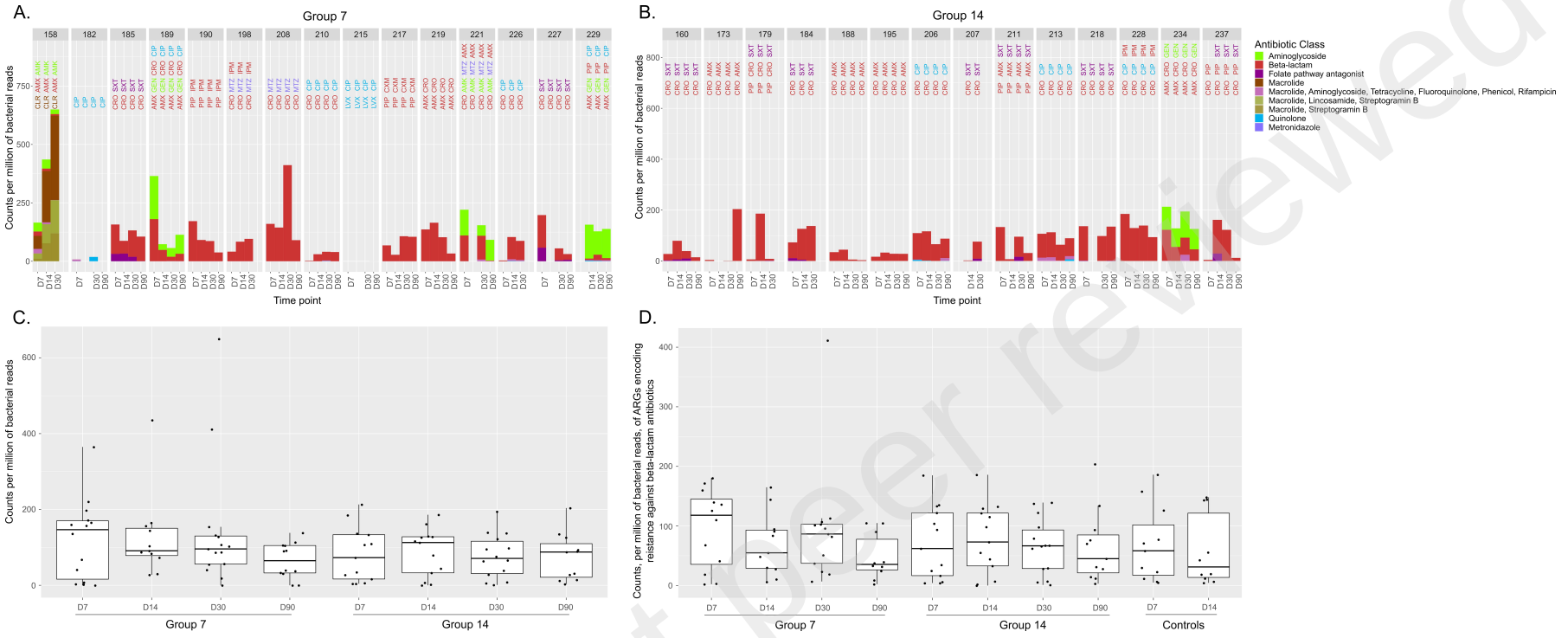
13. Willmann M, Vehreschild MJGT, Biehl LM, Vogel W, Dörfel D, Hamprecht A, et al. Distinct impact of antibiotics on the gut microbiome and resistome: a longitudinal multicenter cohort study. BMC Biology. 2019;17(1):76.

14. Tedelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World journal of gastroenterology. 2007;13(20):2826-32.

15. Sorbara MT, Dubin K, Littmann ER, Moody TU, Fontana E, Seok R, et al. Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification. J Exp Med. 2019;216(1):84-98.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3761111



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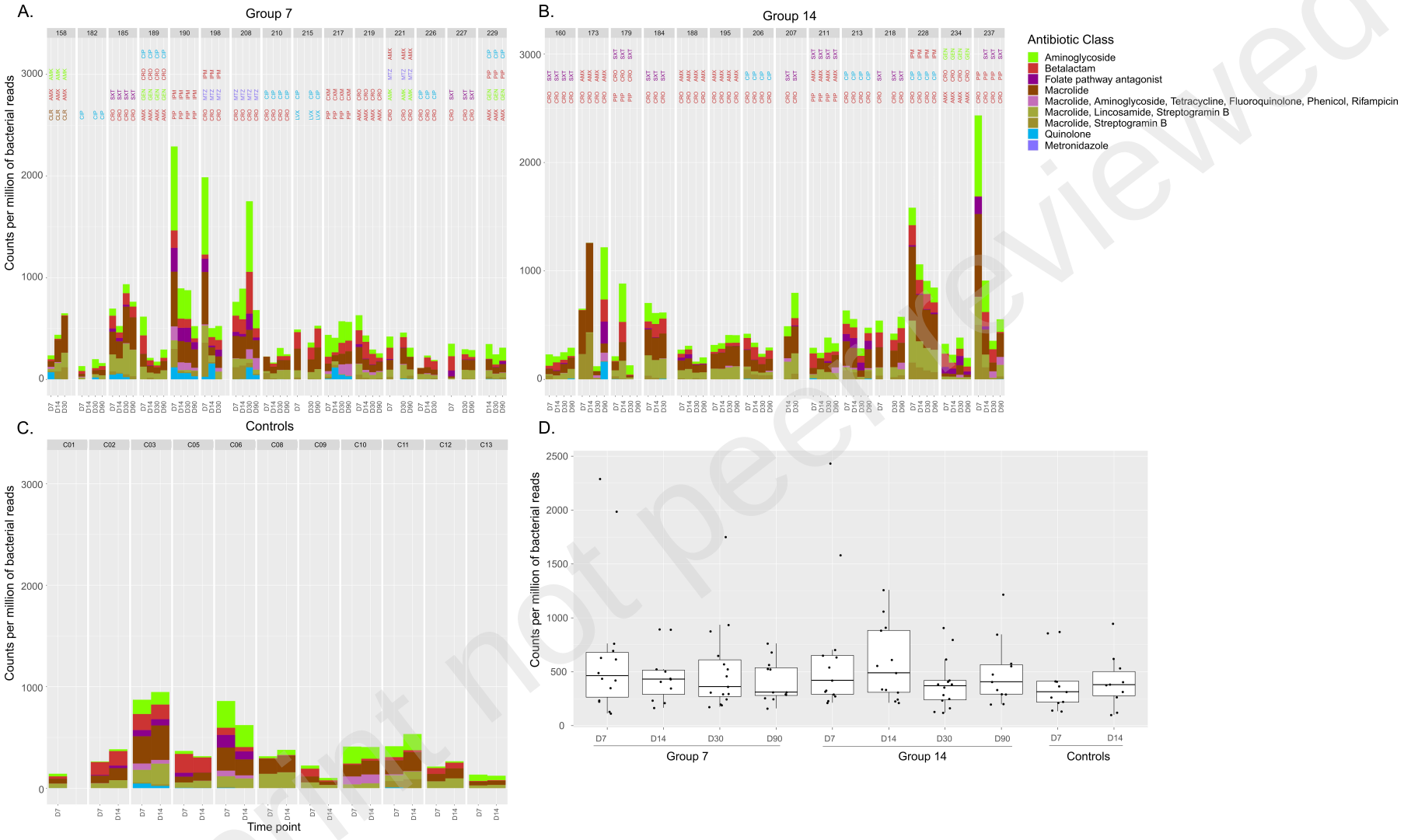

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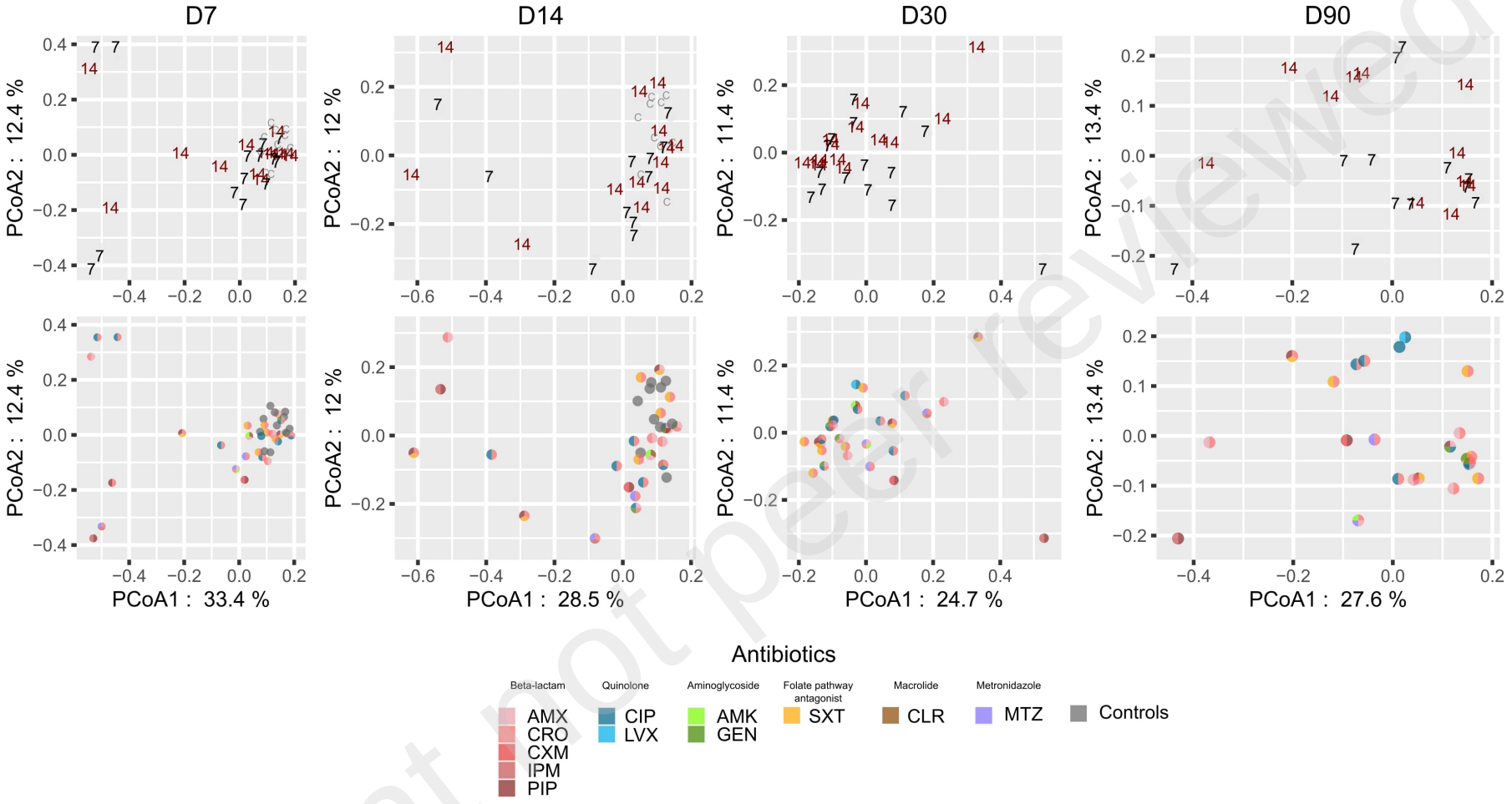

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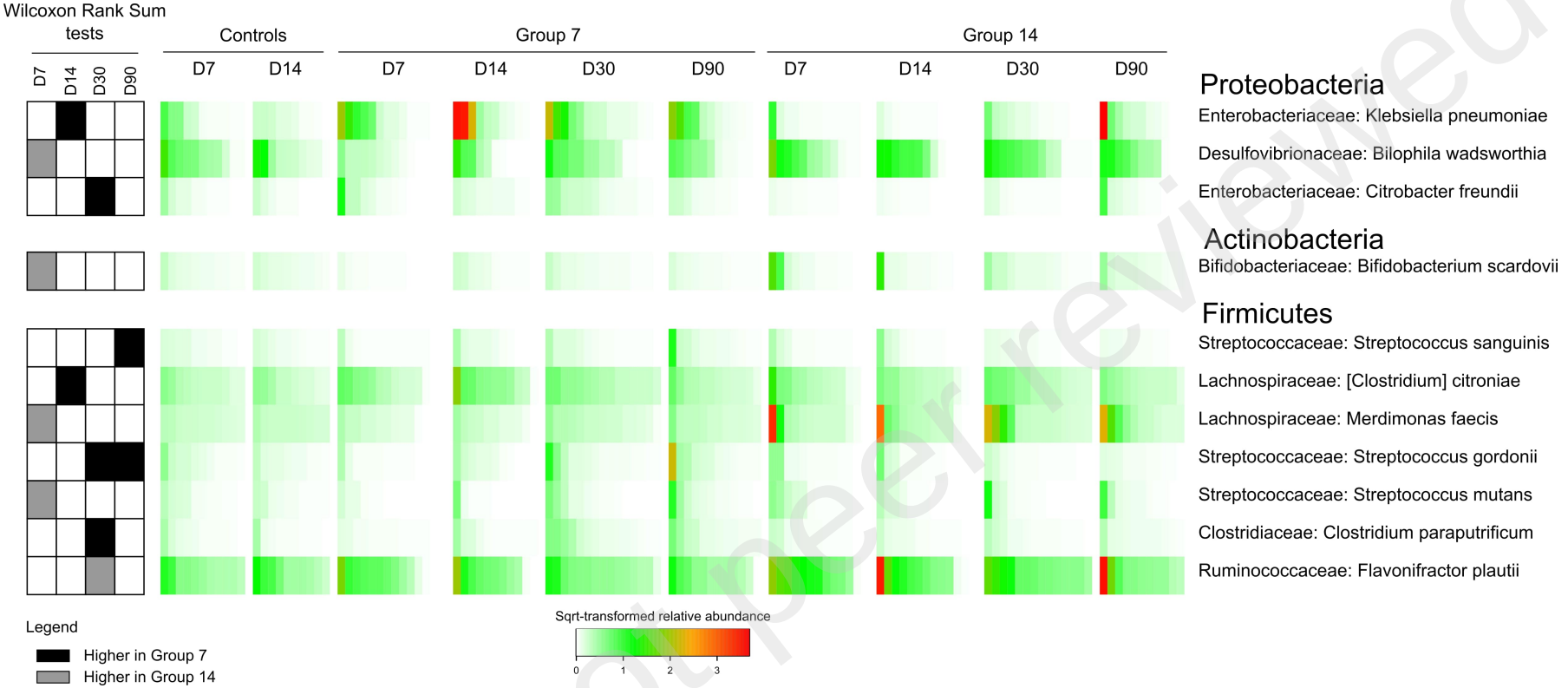

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Chapter 3: Metagenomic characterization of gut microbiota of carriers of extended-spectrum beta-lactamase or carbapenemase-producing Enterobacteriaceae following treatment with oral antibiotics and fecal microbiota transplantation: results from a multicenter randomized trial

Stefano Leo, Vladimir Lazarevic, Myriam Girard, Nadia Gaïa, Jacques Schrenzel, Victoire de Lastours, Bruno Fantin, Marc Bonten, Yehuda Carmeli, Emilie Rondinaud, Stephan Harbarth and Benedikt D. Huttner

3.1. Introduction

Eradication of ESBL-E and CP-E from the gut represents a very hard challenge for clinicians especially when last-resort antibiotics treatments do not work. In this prospective, the R-GNOSIS WP3 study was the first randomized clinical trial aimed at verifying whether or not the combination of antibiotics and fecal microbiota transplantation from healthy donor had an impact in ESBL-E/CP-E gut decolonization. In this work, we characterized the dynamics of microbiota changes before and after antibiotics/FMT treatments from carriers participating to the R-GNOSIS trial. We identified and characterized which components of gut microbiota were affected by treatments. Moreover, we also investigated whether antibiotics/FMT decreased antibiotic-resistance gene content.

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3.3. Contributions

I designed the bioinformatics and statistical analysis methods to mine data. I contributed to the results' interpretation and to the writing and reviewing of the manuscript. I generated all the figures and tables for publication. As corresponding author, I curated the process of submitting, editing and publishing the manuscript.

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Article

Metagenomic Characterization of Gut Microbiota of Carriers of Extended-Spectrum Beta-Lactamase or Carbapenemase-Producing Enterobacteriaceae Following Treatment with Oral Antibiotics and Fecal Microbiota Transplantation: Results from a Multicenter Randomized Trial

Stefano Leo [1,*], Vladimir Lazarevic [1], Myriam Girard [1], Nadia Gaïa [1], Jacques Schrenzel [1,2], Victoire de Lastours [3,4], Bruno Fantin [3,4], Marc Bonten [5,6], Yehuda Carmeli [7], Emilie Rondinaud [8], Stephan Harbarth [2,9] and Benedikt D. Huttner [2,*]

1 Genomic Research Laboratory, Division of Infectious Diseases, University Hospitals and University of Geneva, Rue Michel Servet 1, 1211 Geneva, Switzerland; vladimir.lazarevic@genomic.ch (V.L.); myriam.girard@genomic.ch (M.G.); nadia.gaia@genomic.ch (N.G.); jacques.schrenzel@hcuge.ch (J.S.)
2 Division of Infectious Diseases, Geneva University Hospitals and Faculty of Medicine, Rue Gabrielle-Perret-Gentil 4, 1211 Geneva, Switzerland; stephan.harbarth@hcuge.ch
3 Division of Internal Medicine, Beaujon Hospital, APHP, Boulevard du Général Leclerc 100, 92110 Clichy, France; victoire.de-lastours@aphp.fr (V.d.L.); bruno.fantin@aphp.fr (B.F.)
4 IAME Research Group, UMR 1137, INSERM and University of Paris, Rue Henri Huchard 16, 75870 Paris, France
5 Department of Medical Microbiology, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands; m.j.m.bonten@umcutrecht.nl
6 Julius Center for Health Sciences and Primary Care, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands
7 National Institute for Antibiotic Resistance and Infection Control, Tel Aviv Medical Center, and Sackler Faculty of Medicine, Tel Aviv University, Weizmann Street 6, Tel Aviv 6423906, Israel; yehudac@tlvmc.gov.il
8 Department of Medical Microbiology, APHP, Bichat-Claude-Bernard Hospital, Rue Henri Huchard 46, 75018 Paris, France; emilie.rondinaud@aphp.fr
9 Infection Control Program and WHO Collaborating Center, Geneva University Hospitals, Rue Gabrielle-Perret-Gentil 4, 1211 Geneva, Switzerland
* Correspondence: stefano.leo@genomic.ch (S.L.); benedikt.huttner@hcuge.ch (B.D.H.); Tel.: +41-22-379-41-25 (S.L.); +41-22-372-92-42 (B.D.H.)

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Abstract: Background: The R-GNOSIS (Resistance in Gram-Negative Organisms: Studying Intervention Strategies) WP3 study was the first multicenter randomized clinical trial systematically investigating fecal microbiota transplantation (FMT) for intestinal decolonization of extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) or carbapenemase-producing Enterobacteriaceae (CPE). Here, we characterized the temporal dynamics of fecal microbiota changes in a sub-cohort of the R-GNOSIS WP3 participants before and after antibiotics/FMT using whole metagenome shotgun sequencing. **Methods:** We sequenced fecal DNA obtained from 16 ESBL-E/CPE carriers having received oral colistin/neomycin followed by FMT and their corresponding seven donors. Ten treatment-naïve controls from the same trial were included. Fecal samples were collected at baseline (V0), after antibiotics but before FMT (V2) and three times after FMT (V3, V4 and V5). **Results:** Antibiotic treatment transiently decreased species richness and diversity and increased the abundance of antibiotic resistance determinants (ARDs). *Bifidobacterium* species, together with butyrate- and propionate-producing species from Lachnospiraceae and Ruminococcaceae families were significantly enriched in post-FMT microbiota of treated carriers. After FMT, the proportion of Enterobacteriaceae was lower compared to baseline

but without statistical significance. **Conclusions:** Combined antibiotic and FMT treatment resulted in enrichment of species that are likely to limit the gut colonization by ESBL-E/CPE.

Keywords: fecal microbiota transplantation; extended-spectrum beta-lactamase-producing Enterobacteriaceae; carbapenemase-producing Enterobacteriaceae; microbiome; whole metagenome shotgun sequencing

1. Introduction

Multidrug-resistant Gram-negative bacteria, such as extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) and carbapenemase-producing Enterobacteriaceae (CPE) were classified by the World Health Organization in 2018 as "critical" priority pathogens for research and development of new antibiotics [1]. This classification is justified by the significant burden of infections caused by these pathogens in terms of morbidity and mortality [2,3]. Since colonization precedes infection in most patients, there have been numerous attempts to eradicate carriage, albeit with little lasting success [4,5]. Indeed, the 2019 clinical guidelines of ESCMID-EUCIC (European Society of Clinical Microbiology and Infectious Diseases—European Committee on Infection Control) on decolonization of multidrug-resistant Gram-negative bacteria carriers do not recommend routine use of interventions aimed at achieving decolonization based on a systematic review and appraisal of the published literature [6].

Fecal microbiota transplantation (FMT) has gained increasing interest as intervention for decolonization of ESBL-E/CPE carriers due to its high efficiency and safety for the treatment of recurrent *Clostridioides difficile* infection. Furthermore, some animal experiments have shown promising results and numerous case reports and case series reporting anecdotal "success" of FMT for ESBL-E/CPE decolonization have been published over the last few years [7–10].

The recently published R-GNOSIS (Resistance in Gram-Negative Organisms: Studying Intervention Strategies) WP3 study [11] was the first randomized clinical trial assessing FMT for decolonization of ESBL-E/CPE carriers. While the intervention effect did not reach statistical significance (odd ratios for decolonization was 1.7 [95% CI 0.4–6.4]), possibly because of the failure to achieve the planned sample size, the loss of ESBL-E/CPE carriage detectable by culture was more frequent in FMT-treated participants than in the treatment-naïve control group.

A better understanding of the dynamics of microbiota changes induced by antibiotic treatment and FMT, and of the role of donor microbiota would be crucial in selecting patients and donors for decolonization strategies. In this nested cohort study, we selected carriers and donors from the R-GNOSIS WP3 trial to explore these questions through metagenomic analyses of the collected stool samples.

2. Materials and Methods

2.1. Description of Study Sub-cohort

Criteria for selection of carriers and donors into the R-GNOSIS WP3 study are explained in detail elsewhere [11]. Briefly, immunocompetent adults colonized with ESBL-E were eligible if they had experienced ≥1 episode of symptomatic infection with ESBL-E within ≤180 days before inclusion. Adults colonized with CPE did not have to meet this requirement. Thirty-nine patients in four centers (Geneva [Ge], Switzerland; Paris [Pa], France; Utrecht [Ut], the Netherlands; Tel Aviv [TA], Israel) were randomized to either control (no intervention; "treatment-naïve") or a 5-day course of oral antibiotics (colistin and neomycin sulphate) followed by frozen FMT (named "FMT-treated" hereafter) from unrelated healthy donors. FMT was administered via oral capsules (Ge, Pa) or via nasogastric tube (Ut, TA).

We selected a convenience sub-cohort of 26 ESBL-E/CPE carriers (Ge $n = 12$, Pa $n = 7$, Ut $n = 5$, TA $n = 2$) for the metagenomic analysis. Carriers were selected based on the fact that most of them had completed the last follow-up examination (V5) at the time when the official study/financing period ended (spring 2017); they thus represent the first individuals recruited for the main study. Twenty-four of 26 patients were colonized by ESBL-E and 8 were CPE colonized (of these 6 were also ESBL-E carriers).

Of 26 patients, 16 were FMT-treated and 10 were treatment-naïve. We also selected 7 corresponding stool donors. The study was approved by local institutional review boards and national regulatory agencies in all centers, and all participants (recipients and donors) provided written informed consent.

The sex ratio (female:male) was 1 and the mean age was 63–64 years old in both FMT-treated and treatment-naïve groups (Table S1). The mean body mass index (BMI) of treatment-naïve and FMT-treated individuals was 31.7 and 26.2 kg/m^2, respectively (Table S1); this difference was not associated with statistical significance (*t*-test, $p = 0.054$).

Stools were sampled from all carriers at five time points: baseline (V0), 8–14 days after randomization (V2) (after antibiotics but before FMT in the intervention group), 15–28 days after randomization (V3), 35–48 days after randomization (V4), and 5–7 months after randomization (V5). Details of the antibiotic treatment, FMT procedure and sample storage are reported in Supplementary Materials.

2.2. Whole Metagenome Shotgun Sequencing and Data Analyses

A detailed description of sample processing and metagenomic analysis is provided in Supplementary Materials. In total, we sequenced 21 samples from 7 donors, 76 from the 16 FMT-treated carriers and 47 from the 10 treatment-naïve carriers, with a mean number of raw read pairs of 5.5 M per sample. For donor microbiota, we sequenced DNA from aliquots of the native stools and/or FMT preparation, but for the purpose of this study, we analyzed only 12 stool suspensions used for FMT. Whole metagenome shotgun sequencing (2×150) was performed on a NextSeq 500 system (Illumina, San Diego, CA, USA).

Raw reads were quality-filtered with Trimmomatic v0.36 [12] and then mapped by Kraken2 [13] to a human genome (GRCh38.p7). Human-classified reads were removed and remaining sequences (on average 3.9 M read pairs per sample) were assigned to bacteria, viruses and fungi by Kraken2 using a confidence score of 80%. After the filtering steps, we identified 402 bacterial species belonging to the major gut microbiota phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Moreover, host-depleted R1 reads were mapped against EzBioCloud 16S rRNA gene sequence [14] and ResFinder [15] databases with USEARCH v10 [16]. Statistical analyses were performed in PRIMER v7 (PRIMER-E Ltd., Plymouth, UK) and in the R software v3.2.3.

3. Results

3.1. Donor Microbiota

Since multiple samples from the same donor have been sequenced, we first looked at how the donor samples are related to each other. We found that samples from the same donor (administered to different carriers) clustered together in the principal coordinate analysis (PCoA) plot (Figure 1A). Donor samples also clustered according to the transplantation center and microbiota composition differed between the two FMT preparations (capsule and suspension for nasogastric administration) although not significantly (PERMANOVA, $p = 0.06$).

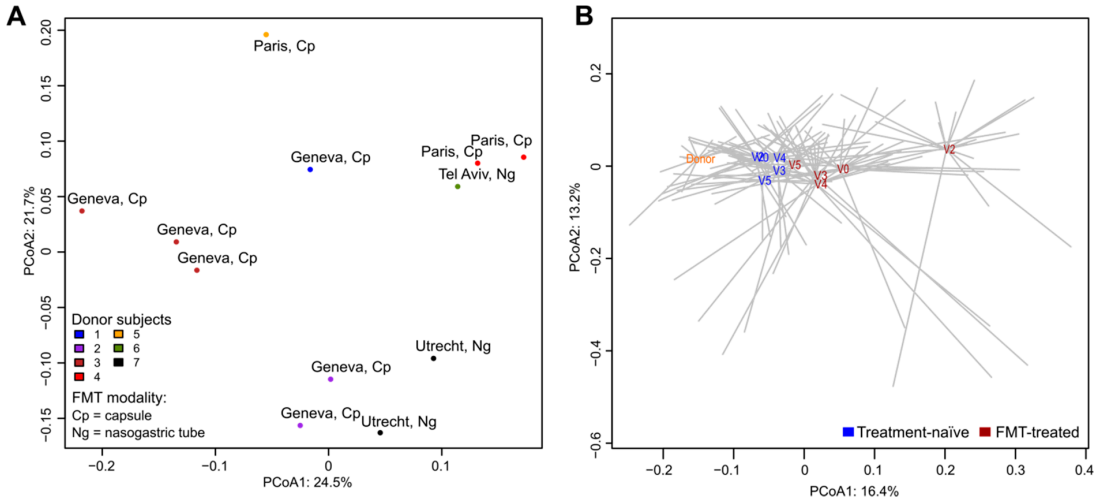

Figure 1. Global differences between microbial communities of fecal samples assessed by principal coordinates analysis (PCoA). The analyses were based on the relative abundance of bacterial species. The percentage of total data variance is reported for PCoA1 and PCoA2. (**A**) Samples from different donors. Data points are color-coded according to the donor. Each sample label reports the center of transplantation and the modality of FMT administration. (**B**) Comparison of samples from donors and FMT-treated and treatment-naïve carriers. Centroids (averaged microbiota profiles) of donors (orange) and of each sampling point (V0, V2, V3, V4 and V5) of treatment-naïve (blue) and FMT-treated (dark red) carriers are represented.

We then compared samples from donors with those collected from all time points of FMT-treated and treatment-naïve carriers. In the PCoA plot, donor samples clustered apart from other samples (Figure 1B) and they were significantly different in microbiota composition from FMT-treated and treatment-naïve microbiota (PERMANOVA, $p < 0.05$).

3.2. Impact of Antibiotic Treatment on Microbiota Profiles and Resistome of FMT-treated Carriers

Colistin/neomycin treatment had a significant effect on global microbiota composition. Microbiota at V2 (i.e., after antibiotic treatment but before FMT) was significantly different from microbiota of baseline (V0) and from later time points (PERMANOVA test, $p < 0.05$; Figure 2A). The administration of the study antibiotics caused a significant decrease of species diversity and richness (Wilcoxon signed rank test, $p < 0.05$; Figure 2B,C).

At V2, we also observed a significantly decreased Firmicutes/Bacteroidetes ratio and a significant increase in the abundance of antibiotics resistance determinants (ARDs) compared to baseline (Wilcoxon signed rank test, $p < 0.05$; Figure 2D,E). In particular, the abundance of genes for tetracycline, aminoglycoside and beta-lactam resistance, which were the most detected ARDs, significantly increased at V2 compared to V0 (Wilcoxon signed rank test, $p < 0.05$, Figure S1). Importantly, we did not detect acquired ARDs to colistin (*mcr-1* to *mcr-5*), by querying the ResFinder database.

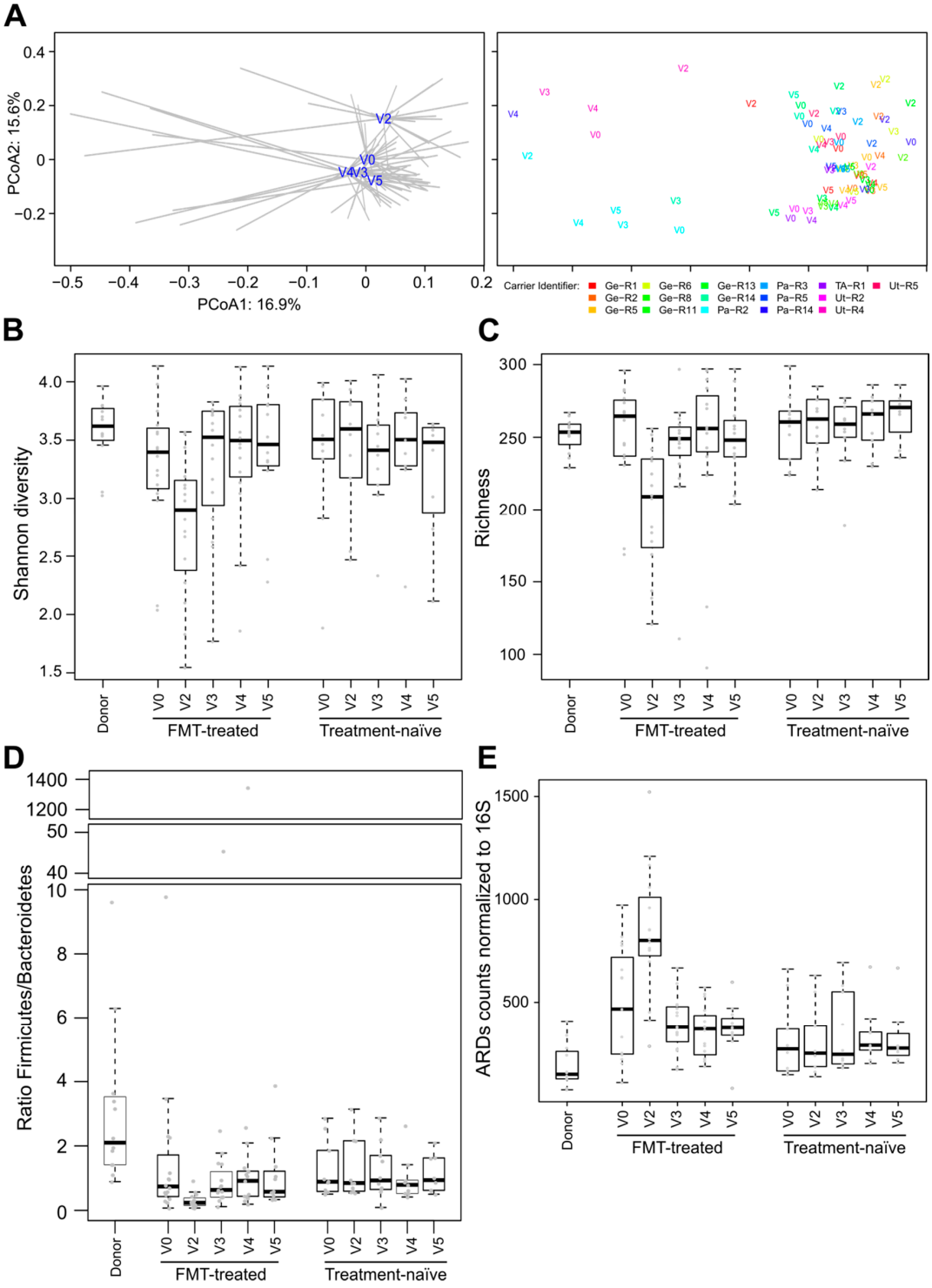

Figure 2. Effect of colistin/neomycin on microbiota composition of FMT-treated carriers. (**A**) PCoA plots of microbiota profiles computed on the species relative abundance of FMT-treated individuals. On the left, centroids (averaged microbiota profiles) from each time point of FMT-treated carriers are represented. Each sample is connected to its corresponding centroid with grey lines. On the right, samples are labeled according to the time point and colored according to the carrier. Shannon diversity (**B**) and species richness (**C**) were computed after rarefying sequencing reads to 40,000. (**D**) Ratio of Firmicutes to Bacteroidetes. (**E**) Number of reads assigned to ARDs and normalized to 1000 16S rRNA gene read counts.

3.3. Post-FMT Microbiota and Its Association with Donor Microbiota

Decrease in species diversity and richness at V2 was followed by a significant increase of those indices at post-FMT time point V3 (Wilcoxon signed rank test, $p < 0.05$; Figure 2B,C). Likewise, after FMT administration, the Firmicutes/Bacteroidetes ratio and the ARDs content significantly increased and decreased, respectively (Wilcoxon signed rank test, $p < 0.05$), reverting to baseline levels (Figure 2D,E).

Post-treatment microbiota was similar to that of the baseline in terms of global species composition, richness and diversity (Figure 2). This was confirmed by the PERMANOVA test that revealed no statistically significant differences ($p > 0.05$) between V0 and post-FMT time points V3 and V4.

To analyze the association between stool microbiota of carriers and donors, we computed the Bray–Curtis index (BCi), where the higher the value, the more similarity there is between the two groups. BCi median values for donor versus carrier microbiota were lower at post-FMT time points V3 and V4 than at V0 and at V2; however, no significant p-values were associated with these differences (Figure 3A).

For each of the 16 FMT-treated carriers, we identified species shared with their corresponding donors. In most cases, the number of these shared species was higher at V3 and V4 than at baseline and V2 (Figure 3B). Of 32 species that were shared with the donor at V3 or V4 in at least half ($n \geq 8$) transplanted carriers (Table 1), 23 were found to be differentially abundant (Wilcoxon signed rank test, $p < 0.05$) in at least one pairwise comparison between the first four time point samples (V0, V2, V3 and V4) of FMT-treated carriers (Figure 4).

Table 1. Thirty-two species most frequently shared between the donors and the transplanted carriers. Species present in at least 8 FMT-treated carriers at V3 or at V4 are shown. For each species, numbers indicate in how many carriers they were present at a given visit. For each visit, we report the number of FMT-treated carriers analyzed ("n"). Species reported in Figure 4 are marked with an "X" in the column "Differentially abundant".

Species	V0 (n = 16)	V2 (n = 16)	V3 (n = 16)	V4 (n = 16)	V5 (n = 12)	Differentially Abundant
Alistipes putredinis	4	3	9	9	7	X
Alistipes shahii	5	6	8	7	6	
Anaerostipes hadrus	6	1	9	9	7	X
Bacteroides caccae	4	4	7	11	9	X
Bacteroides ovatus	11	12	13	13	10	X
Bacteroides sp. 4_1_36	10	11	11	11	8	
Bacteroides sp. D20	12	13	14	13	10	X
Bacteroides stercoris	6	5	10	10	8	
Bacteroides thetaiotaomicron	9	10	11	10	7	X
Bacteroides uniformis	13	14	12	13	11	X
Bacteroides vulgatus	13	14	11	12	10	
Bifidobacterium adolescentis	4	1	8	9	8	X
Bifidobacterium catenulatum	2	0	8	7	5	X
Bifidobacterium longum	13	9	15	14	11	
Blautia obeum	14	9	15	15	11	X
Collinsella aerofaciens	4	2	14	13	10	X
Coprococcus comes	5	2	8	10	8	X
Dorea formicigenerans	7	3	11	11	8	X
Dorea longicatena	8	3	11	12	11	
Escherichia coli	9	3	6	9	8	X
[Eubacterium] rectale	7	4	10	11	10	X
Faecalibacterium prausnitzii	12	4	13	11	11	X
Fusicatenibacter saccharivorans	9	7	12	12	10	X
Gemmiger formicilis	6	2	12	12	11	X
Lachnospiraceae bacterium 7_1_58FAA	8	5	7	8	5	
Odoribacter splanchnicus	9	7	8	10	6	
Roseburia faecis	11	0	9	10	9	X
Roseburia intestinalis	12	1	7	12	9	X
Roseburia inulinivorans	9	0	7	10	8	X
Ruminococcus sp. 5_1_39BFAA	13	1	15	15	11	X
Ruminococcus sp. SR1/5	11	6	12	13	10	
Sutterella wadsworthensis	3	0	11	11	7	X

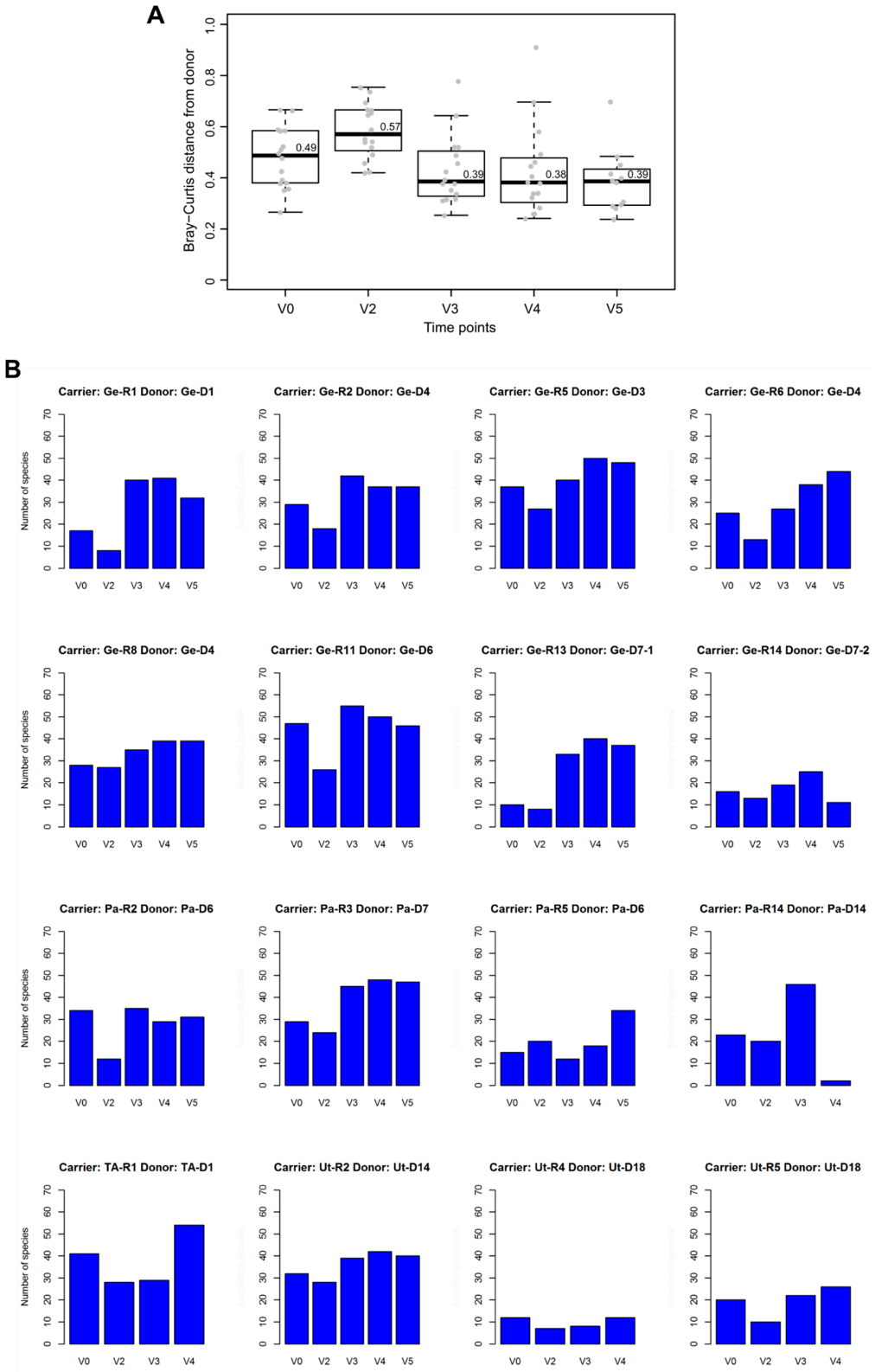

Figure 3. Donor microbiota and their associations with recipient microbiota. (**A**) Boxplot representing the Bray–Curtis distance (see the main text) between donors and FMT-treated carriers at each time point. Median values are reported above the corresponding (thick) line of boxplots. (**B**) Bar plots representing the number of species shared with the donor at each time point for each FMT-treated carrier. Carrier and corresponding donor identifiers are indicated at the top of each bar plot.

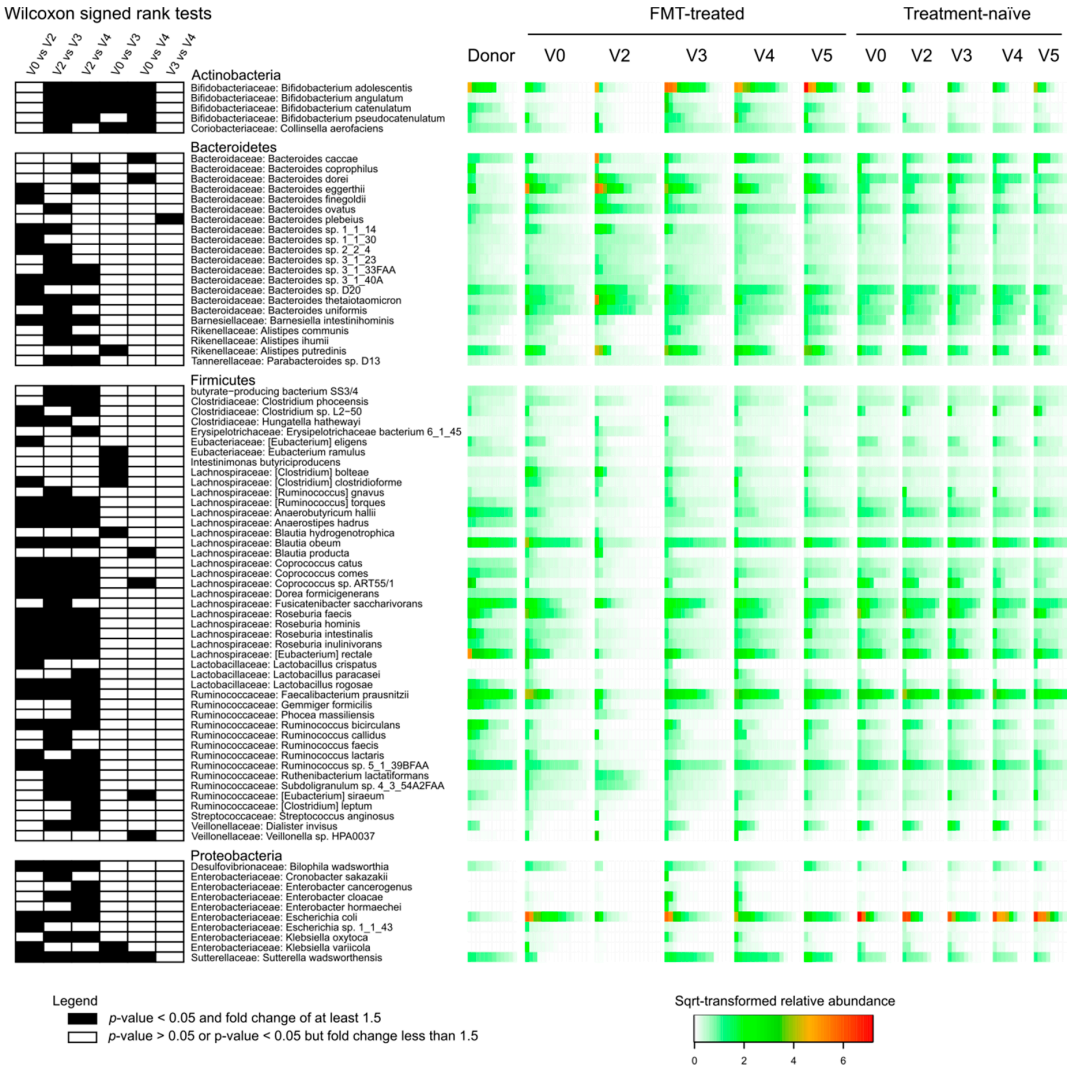

Figure 4. Differentially abundant species in FMT-treated carriers. For each of the 81 represented species, we report the statistical significance (Wilcoxon signed rank test) of changes in the relative abundance between time points (left), phylum and family assignments (middle) and the relative abundance in donor, FMT-treated and treatment-naïve carriers (right). Importantly, we selected species for which at least one of the 6 pairwise comparisons reported (V0 vs. V2, V0 vs. V3, V0 vs. V4, V2 vs. V3, V2 vs. V4, V3 vs. V4) was statistically significant ($p < 0.05$) with a ≥1.5 fold change in the relative abundance and a mean relative abundance ≥0.1% in at least one of the two compared groups. Significant differences ($p < 0.05$) associated with a ≥1.5 fold change in the relative abundance are represented as black shaded cells; white cells denote other cases. Relative abundances are square-root-transformed and color-scaled as indicated in the legend.

We found that four *Bifidobacterium* species and *Collinsella aerofaciens* (Actinobacteria) were significantly more abundant in post-FMT time points than at V0 and V2 (Figure 4). Several other species, belonging to the genera *Bacteroides*, *Alistipes* (Bacteroidetes), *Eubacterium*, *Clostridium*, *Intestinimonas*, *Blautia*, *Coprococcus*, *Veillonella* (Firmicutes), *Klebsiella* and *Sutterella* (Proteobacteria), were also found differentially abundant between baseline and post-FMT time points (Figure 4). The antibiotic treatment was associated with significant changes in the relative abundance between V2 and all three other time points (V0, V3 and V4) for 23 species from the phyla Bacteroidetes, Firmicutes and Proteobacteria (Figure 4).

3.4. Comparisons of Microbiota of FMT-Treated and Treatment-Naïve Individuals

Differences in microbiota composition between the time points in treatment-naïve carriers were not statistically significant (PERMANOVA, $p > 0.05$). Likewise, we did not detect significant differences in global microbiota composition and ecological indexes between FMT-treated and treatment-naïve carriers except at the time point V2. *Bifidobacterium* species, including *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, were significantly more abundant in FMT-treated than in treatment-naïve carriers at V4 and/or V3 (post-FMT) time points (Wilcoxon rank sum test, $p < 0.05$; Figure S2). On the other hand, *Lactobacillus ruminis* had significantly lower relative abundance at V3, V4 and V5 in FMT-treated subjects when compared to treatment-naïve individuals (Wilcoxon rank sum test, $p < 0.05$; Figure S2).

3.5. Effect of FMT/antibiotics on the Abundance of Proteobacteria, Enterobacteriaceae and of Selected Beta-lactam ARDs

Proteobacteria and Enterobacteriaceae levels were higher in carriers as compared to donors (Figure 5). In FMT-treated carriers, colistin/neomycin treatment resulted in a transient and significant decrease in the abundance of Proteobacteria and Enterobacteriaceae (Wilcoxon signed rank test, $p < 0.05$; Figure 5A,B). After FMT administration, the abundance of Enterobacteriaceae remained lower relative to baseline but without statistical significance. *Escherichia coli*, the most abundant Enterobacteriaceae species detected in our analyses, followed a similar temporal pattern (Figure 4).

We assessed the abundance of putative $bla_{\text{CTX-M}}$, bla_{NDM}, bla_{OXA}, bla_{SHV}, bla_{TEM} and bla_{KPC} genes (from the ResFinder database), which may be associated with ESBL-E and CPE [17,18], to determine whether antibiotics/FMT treatment caused a change in the abundance of these resistance genes. This analysis included samples from 14 FMT-treated individuals who had detectable *bla* genes ($bla_{\text{CTX-M}}$, bla_{NDM}, bla_{OXA}, bla_{SHV} and bla_{TEM}) at baseline. In 10 of them, the *bla* gene content was higher at baseline than at later time points (Figure 5C). Importantly, no such temporal pattern was observed in treatment-naïve carriers (Figure 5C).

3.6. Comparison of Metagenomic Results with R-GNOSIS ESBL-E/CPE Decolonization Outcomes

The primary objective of the R-GNOSIS WP3 trial was to evaluate if antibiotics followed by FMT treatment resulted in the absence of detectable carriage of ESBL-E/CPE at V4 by analyzing stool culture at this time point. We therefore subdivided FMT-treated and treatment-naïve carriers in "decolonized" and "persistently colonized" categories depending on whether their fecal samples were found ESBL-E/CPE negative or positive at V4 in the R-GNOSIS study. Accordingly, 9 (out of 16) FMT-treated carriers and three (out of 9) treatment-naïve carriers were considered decolonized (Table S1). The treatment-naïve carrier TA-R2 was excluded from this analysis as no sample for this subject was taken at V4.

Colistin/neomycin administration had a significant impact on microbiota composition irrespective of the V4-decolonization status; in PCoA, fecal microbiota from V2 clustered apart from the other time point samples (Figure 1B and Figure S3A), and these differences were significant (PERMANOVA, $p < 0.05$) in both persistently colonized and decolonized subgroups. Microbial profiles of FMT-treated carriers were significantly different between decolonized and persistently colonized groups (PERMANOVA test, $p < 0.05$) at V3 but not at V0, V2, V4 and V5. For treatment-naïve carriers (Figure S3B), differences in microbiota between decolonized and persistently colonized carriers were not statistically significant (PERMANOVA, $p > 0.05$) at any time point.

Eventually, we compared metagenomic predictions of the presence of detected ESBL and carbapenemase genes ($bla_{\text{CTX-M}}$, bla_{NDM}, bla_{OXA}, bla_{SHV} and bla_{TEM}) with the results from R-GNOSIS WP3 conventional testing. Phenotypic analyses did not consistently match with metagenomic predictions of ESBL-E/CPE carriage (Figure S3C). Decolonization status (ESBL-E/CPE negative or positive) at V4 had concordant outcomes between the two approaches (phenotypic and metagenomic) in 68% (17/25) of cases (77.8% [7/9] for treatment-naïve and 62.5% [10/16] for FMT-treated carriers; see also Table S2).

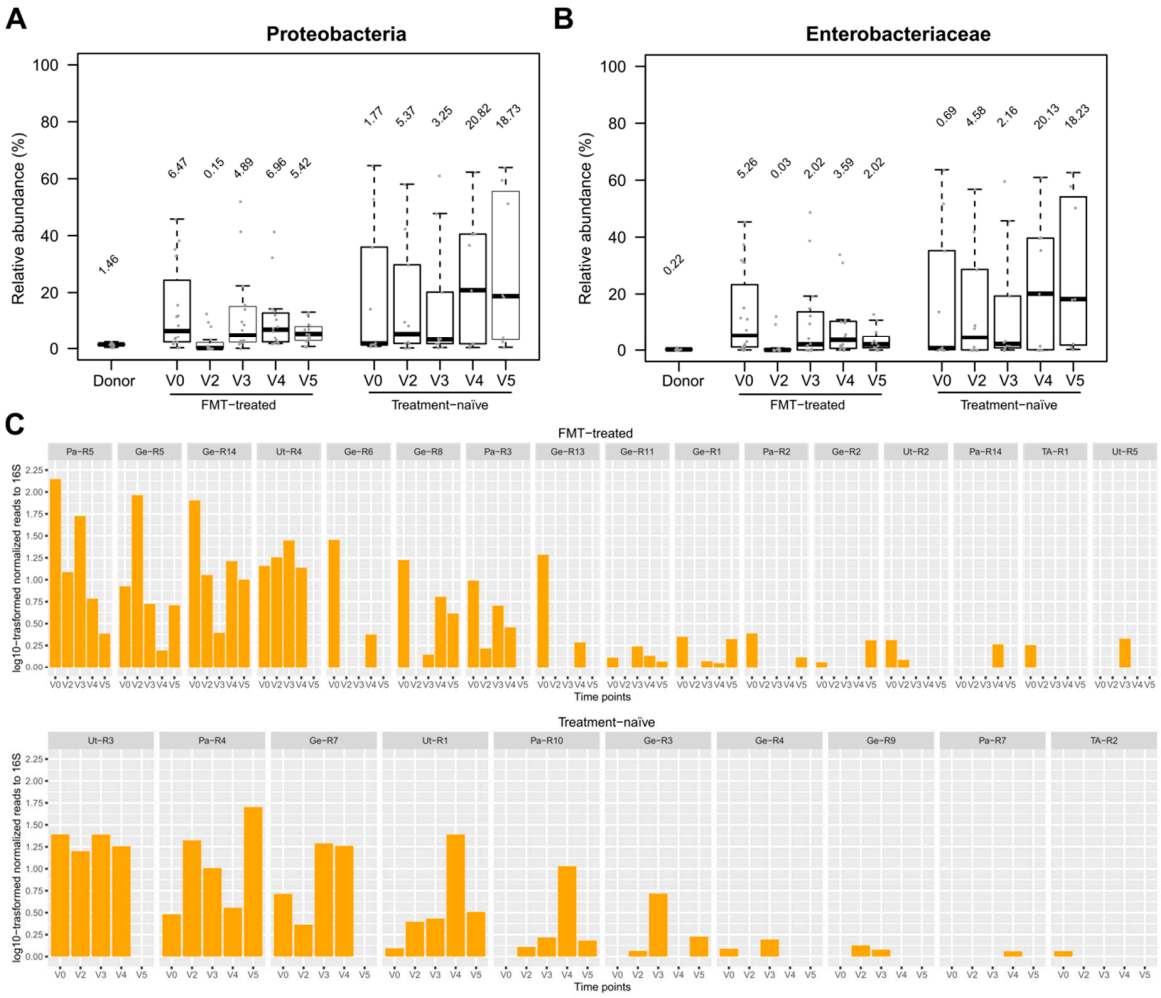

Figure 5. Effect of antibiotics/FMT treatment on the relative abundance of Enterobacteriaceae and the abundance of putative ESBL and carbapenemase genes. Boxplots combined with dotplots represent the relative abundance of Proteobacteria (**A**) and Enterobacteriaceae (**B**) in donors, FMT-treated and treatment-naïve carriers. (**C**) Counts of reads assigned to detected $bla_{\text{CTX-M}}$, bla_{NDM}, bla_{OXA}, bla_{SHV} and bla_{TEM} genes were normalized to read counts of 16S rRNA genes and multiplied by 1000 prior to log10-transformation.

4. Discussion

The R-GNOSIS WP3 study [11] was the first multicenter randomized clinical trial systematically investigating FMT for decolonization of ESBL-E/CPE from the gut. In the present study we characterized the recipient and donor fecal microbiota of a sub-cohort of the R-GNOSIS WP3 participants by whole metagenome shotgun sequencing.

The first main finding of our analyses was that antibiotic treatment resulted in a profound change in microbiota composition with reduced species richness and diversity, lower Firmicutes/Bacteroidetes ratio and decreased proportions of Proteobacteria and Enterobacteriaceae. A decreased Firmicutes/Bacteroidetes ratio has been previously reported in hypertensive rat models following neomycin treatment [19].

Metagenomic predictions for ESBL-E and CPE were 72% concordant with phenotypic screening and both approaches have their own advantages and disadvantages. In line with the results of phenotypic antibiotic susceptibility tests performed in the main study [11], we did not detect acquired colistin resistance genes, although we cannot exclude the presence of resistance mechanisms not detectable by our approach [20]. Determinants encoding resistance to aminoglycosides, a class to

which neomycin belongs, were abundant in our metagenomic data, even in samples collected before treatment. Since phenotypic neomycin susceptibility testing was not performed in the main study, the correlation between metagenomic and culture-based results could not be assessed. The most frequently identified ARDs in our study were tetracycline resistance genes, which have been previously shown to be the most abundant ARDs in human gut commensals but with a marked geographic variation [21,22]. A large use of aminoglycosides, tetracycline and other antibiotics in animal rearing probably promoted transmission of ARDs from food animals to humans [22].

Effects of antibiotic treatment on the species richness and diversity as well as on ARD content were transitory; after FMT administration, their values restored to baseline levels. In particular, the gut microbiota from post-FMT time points, as compared to V2 (the end of antibiotic treatment), was significantly enriched in Lachnospiraceae (Firmicutes), Ruminococcaceae (Firmicutes), and *Alistipes* species (Bacteroidetes). These taxa include producers of propionate and butyrate [23], which are known to reduce inflammation of intestinal mucosa [24].

Relative to the baseline, post-FMT microbiota was significantly enriched in *Bifidobacterium* species and *C. aerofaciens*. No such increase was detected over the same time period in treatment-naïve carriers. *Bifidobacterium* species are known to attenuate inflammation [25] and some strains of *B. pseudocatenulatum* and *B. catenulatum* recovered from human fecal samples have been shown to exert growth inhibition on enterotoxigenic *E. coli* strain cultures [26]. *C. aerofaciens* might have an anti-inflammatory effect on intestinal epithelium since this species also includes butyrate-producer strains [27].

In FMT-treated carriers, we found a decrease in the proportion of Enterobacteriaceae in post-FMT-microbiota compared to baseline, albeit not statistically significant, and we observed that putative ESBL and carbapenemase genes were more abundant at baseline than at any later sampling point in 10 out of 16 cases.

In a nonrandomized prospective single-center cohort study conducted on seven hematologic patients in Poland, higher abundance of *Barnesiella intestini hominis* in donor fecal microbiota was associated with successful decolonization of *Klebsiella pneumoniae* harboring New Delhi metallo-β-lactamase-1 (NDM-1) in FMT recipients [28]. Moreover, the genus *Barnesiella* has been reported to confer resilience to vancomycin-resistant *Enterococcus faecium* intestinal colonization in a mouse model [29]. In our study, the relative abundance of *Barnesiella intestini hominis* significantly increased after FMT-treatment (V3) as compared to V2 (Figure 4).

Although the overall (Bray–Curtis) dissimilarity between donor and carrier microbiota was not significantly different for baseline and post-FMT time points, our results suggest that FMT increases the proportion of species antagonistic to ESBL-E and of species that reinforce the gut epithelium barrier by modulating inflammatory processes.

Microbiota collected at V3 (after FMT), but not at the next visit (V4), at which the decolonization status was defined, were significantly different between colonized and decolonized individuals, which suggests that gut decolonization of ESBL-E/CPE was associated with short-term microbiota changes.

Our study has several limitations: (i) the sample size of our cohort makes it difficult to reach definitive conclusions; (ii) the limited number of donors did not allow us to discriminate possible differences in preservation of donor microbiota components between capsules and suspensions for nasogastric tube administration; (iii) in analyses aimed at identifying differentially abundant species, we excluded V5 samples because several carriers had not been sampled at this time point; (iv) metagenomic reads assigned to ARDs, fungi and viruses had low counts. Nevertheless, in line with previous studies [30–32], DNA bacteriophages (*Myoviridae*, *Podoviridae* and *Siphoviridae* families) were the most abundant viruses and *Saccharomyces* was the most frequently identified fungal genus (Figure S4).

In conclusion, our findings give insights on microbiota composition under combined treatment of antibiotics and FMT. Following this treatment, increased relative abundance of certain species possibly

limits the colonization by ESBL-E/CPE. Further studies with larger sample size and use of FMT without antibiotics are needed to clarify these observations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/6/941/s1, Figure S1: ARD classes, Figure S2: Differentially abundant species between FMT-treated and treatment-naïve carriers, Figure S3: Comparisons of metagenomic analyses with R-GNOSIS ESBL-E/CPE decolonization outcomes, Figure S4: Most abundant viral families and fungal genera detected by metagenomics, Table S1: Summary table describing gender, age and body mass index (BMI) of donors and carriers analyzed in this study, Table S2: Comparison of ESBL-E/CPE detection as performed by phenotypic tests and as predicted by metagenomic analyses for the sub-cohort of ESBL-E/CPE carriers analyzed in the present study.

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References

1. Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D.L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y.; et al. Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* **2018**, *18*, 318–327. [CrossRef]
2. Stewardson, A.J.; Marimuthu, K.; Sengupta, S.; Allignol, A.; El-Bouseary, M.; Carvalho, M.J.; Hassan, B.; Delgado-Ramirez, M.A.; Arora, A.; Bagga, R.; et al. Effect of carbapenem resistance on outcomes of bloodstream infection caused by Enterobacteriaceae in low-income and middle-income countries (PANORAMA): A multinational prospective cohort study. *Lancet Infect. Dis.* **2019**, *19*, 601–610. [CrossRef]
3. Stewardson, A.; Fankhauser, C.; Augelis, G.D.; Rohner, P.; Safran, E.; Schrenzel, J.; Pittet, D.; Harbarth, S. Burden of bloodstream infection caused by extended-spectrum β-lactamase–producing Enterobacteriaceae determined using multistate modeling at a Swiss University Hospital and a nationwide predictive model. *Infect. Control Hosp. Epidemiol.* **2015**, *34*, 133–143. [CrossRef] [PubMed]
4. Bert, F.; Larroque, B.; Paugam-Burtz, C.; Dondero, F.; Durand, F.; Marcon, E.; Belghiti, J.; Moreau, R.; Nicolas-Chanoine, M.-H. Pretransplant fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae and infection after liver transplant, France. *Emerg. Infect. Dis.* **2012**, *18*, 908–916. [CrossRef]
5. Rieg, S.; Küpper, M.F.; de With, K.; Serr, A.; Bohnert, J.A.; Kern, W.V. Intestinal decolonization of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBL): A retrospective observational study in patients at risk for infection and a brief review of the literature. *BMC Infect. Dis.* **2015**, *15*, 475. [CrossRef]
6. Tacconelli, E.; Mazzaferri, F.; de Smet, A.M.; Bragantini, D.; Eggimann, P.; Huttner, B.D.; Kuijper, E.J.; Lucet, J.C.; Mutters, N.T.; Sanguinetti, M.; et al. ESCMID-EUCIC clinical guidelines on decolonization of multidrug-resistant Gram-negative bacteria carriers. *Clin. Microbiol. Infect.* **2019**, *25*, 807–817. [CrossRef]
7. Drekonja, D.; Reich, J.; Gezahegn, S.; Greer, N.; Shaukat, A.; MacDonald, R.; Rutks, I.; Wilt, T.J. Fecal microbiota transplantation for Clostridium difficile infection: A systematic review. *Ann. Intern. Med.* **2015**, *162*, 630–638. [CrossRef]

8. Caballero, S.; Carter, R.; Ke, X.; Sušac, B.; Leiner, I.M.; Kim, G.J.; Miller, L.; Ling, L.; Manova, K.; Pamer, E.G. Distinct but spatially overlapping intestinal niches for vancomycin-resistant Enterococcus faecium and carbapenem-resistant Klebsiella pneumoniae. *PLoS Pathog.* **2015**, *11*, e1005132. [CrossRef]
9. Tavoukjian, V. Faecal microbiota transplantation for the decolonization of antibiotic-resistant bacteria in the gut: A systematic review and meta-analysis. *J. Hosp. Infect.* **2019**, *102*, 174–188. [CrossRef]
10. Catho, G.; Huttner, B.D. Strategies for the eradication of extended-spectrum beta-lactamase or carbapenemase-producing Enterobacteriaceae intestinal carriage. *Expert Rev. Anti-Infect. Ther.* **2019**, *17*, 557–569. [CrossRef]
11. Huttner, B.D.; de Lastours, V.; Wassenberg, M.; Maharshak, N.; Mauris, A.; Galperine, T.; Zanichelli, V.; Kapel, N.; Bellanger, A.; Olearo, F.; et al. A 5-day course of oral antibiotics followed by faecal transplantation to eradicate carriage of multidrug-resistant Enterobacteriaceae: A randomized clinical trial. *Clin. Microbiol. Infect.* **2019**. [CrossRef] [PubMed]
12. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef] [PubMed]
13. Wood, D.E.; Salzberg, S.L. Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **2014**, *15*, R46. [CrossRef] [PubMed]
14. Yoon, S.-H.; Ha, S.-M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617. [CrossRef]
15. Zankari, E.; Hasman, H.; Cosentino, S.; Vestergaard, M.; Rasmussen, S.; Lund, O.; Aarestrup, F.M.; Larsen, M.V. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* **2012**, *67*, 2640–2644. [CrossRef]
16. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef]
17. Wilson, H.; Török, M.E. Extended-spectrum β-lactamase-producing and carbapenemase-producing Enterobacteriaceae. *Microb. Genom.* **2018**, *4*, e000197. [CrossRef]
18. Liakopoulos, A.; Mevius, D.; Ceccarelli, D. A Review of SHV extended-spectrum β-lactamases: Neglected yet ubiquitous. *Front. Microbiol.* **2016**, *7*. [CrossRef]
19. Galla, S.; Chakraborty, S.; Cheng, X.; Yeo, J.; Mell, B.; Zhang, H.; Mathew, A.V.; Vijay-Kumar, M.; Joe, B. Disparate effects of antibiotics on hypertension. *Physiol. Genom.* **2018**, *50*, 837–845. [CrossRef]
20. Falagas, M.E.; Kasiakou, S.K.; Saravolatz, L.D. Colistin: The revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin. Infect. Dis.* **2005**, *40*, 1333–1341. [CrossRef]
21. Hu, Y.; Yang, X.; Qin, J.; Lu, N.; Cheng, G.; Wu, N.; Pan, Y.; Li, J.; Zhu, L.; Wang, X.; et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.* **2013**, *4*, 2151. [CrossRef] [PubMed]
22. Forslund, K.; Sunagawa, S.; Kultima, J.R.; Mende, D.R.; Arumugam, M.; Typas, A.; Bork, P. Country-specific antibiotic use practices impact the human gut resistome. *Genome Res.* **2013**, *23*, 1163–1169. [CrossRef] [PubMed]
23. Vital, M.; Karch, A.; Pieper, D.H. Colonic butyrate-producing communities in humans: An overview using omics data. *mSystems* **2017**, *2*, e00130-17. [CrossRef]
24. Tedelind, S.; Westberg, F.; Kjerrulf, M.; Vidal, A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. *World J. Gastroenterol.* **2007**, *13*, 2826–2832. [CrossRef] [PubMed]
25. Khokhlova, E.V.; Smeianov, V.V.; Efimov, B.A.; Kafarskaia, L.I.; Pavlova, S.I.; Shkoporov, A.N. Anti-inflammatory properties of intestinal Bifidobacterium strains isolated from healthy infants. *Microbiol. Immunol.* **2012**, *56*, 27–39. [CrossRef]
26. Delcaru, C.; Alexandru, I.; Podgoreanu, P.; Cristea, V.C.; Bleotu, C.; Chifiriuc, M.C.; Bezirtzoglou, E.; Lazar, V. Antagonistic activities of some Bifidobacterium sp. strains isolated from resident infant gastrointestinal microbiota on Gram-negative enteric pathogens. *Anaerobe* **2016**, *39*, 39–44. [CrossRef]
27. Qin, P.; Zou, Y.; Dai, Y.; Luo, G.; Zhang, X.; Xiao, L. Characterization a novel butyric acid-producing bacterium *Collinsella aerofaciens* subsp. *Shenzhenensis* subsp. nov. *Microorganisms* **2019**, *7*, 78. [CrossRef]

28. Bilinski, J.; Grzesiowski, P.; Sorensen, N.; Madry, K.; Muszynski, J.; Robak, K.; Wroblewska, M.; Dzieciatkowski, T.; Dulny, G.; Dwilewicz-Trojaczek, J.; et al. Fecal microbiota transplantation in patients with blood disorders inhibits gut colonization with antibiotic-resistant bacteria: Results of a prospective, single-center study. *Clin. Infect. Dis.* **2017**, *65*, 364–370. [CrossRef]
29. Ubeda, C.; Bucci, V.; Caballero, S.; Djukovic, A.; Toussaint, N.C.; Equinda, M.; Lipuma, L.; Ling, L.; Gobourne, A.; No, D.; et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. *Infect. Immun.* **2013**, *81*, 965. [CrossRef]
30. Hoyles, L.; McCartney, A.L.; Neve, H.; Gibson, G.R.; Sanderson, J.D.; Heller, K.J.; van Sinderen, D. Characterization of virus-like particles associated with the human faecal and caecal microbiota. *Res. Microbiol.* **2014**, *165*, 803–812. [CrossRef]
31. Shkoporov, A.N.; Clooney, A.G.; Sutton, T.D.S.; Ryan, F.J.; Daly, K.M.; Nolan, J.A.; McDonnell, S.A.; Khokhlova, E.V.; Draper, L.A.; Forde, A.; et al. The human gut virome is highly diverse, stable, and individual specific. *Cell Host Microbe* **2019**, *26*, 527–541.e5. [CrossRef] [PubMed]
32. Grall, N.; Lazarevic, V.; Gaïa, N.; Couffignal, C.; Laouénan, C.; Ilic-Habensus, E.; Wieder, I.; Plesiat, P.; Angebault, C.; Bougnoux, M.E.; et al. Unexpected persistence of extended-spectrum β-lactamase-producing Enterobacteriaceae in the faecal microbiota of hospitalised patients treated with imipenem. *Int. J. Antimicrob. Agents* **2017**, *50*, 81–87. [CrossRef] [PubMed]

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General Discussion

Facing the increased incidence of multidrug resistant Gram-negative bacteria, there is a need to better understand the risk factors and associated mechanisms leading to drug resistance and carriage of intestinal pathogens.
Intestinal microbiota plays a fundamental role in the maintenance of homeostasis of intestinal epithelium [102]. Besides, microbiota communicates with immune cells to signal the presence of pathogens and can directly oppose pathogen colonization [102].
Changes in microbiota composition can lead to dysbiosis, a condition that can favor intestinal inflammation and subsequent pathogen infection.
Traveling to tropical regions and antibiotic treatment have been associated to ESBL-E and CP-E acquisition, however, few studies have addressed, so far, the role of microbiota with respect to these risk factors [209, 242]. While antibiotics have been shown to perturb the intestinal ecosystem [243], whether short therapy duration limited the detrimental effects of these drugs on human microbiota composition had been poorly investigated. Finally, alternative strategies to antibiotics, like fecal microbiota transplantation, were shown successful in the eradication of *C. difficile* infection [223] and have been applied to treat ESBL-E and CP-E carriage [237]. The analyses of the changes in microbiota composition before and after FMT would lead to an understanding of the effect of FMT on microbiota and possibly on colonization/decolonization dynamics of ESBL-E and CP-E.
During my PhD, I investigated all these important questions by taking advantages of fecal samples collected during three previously published clinical trials: VOYAG-R [209], PIRATE [217] and R-GNOSIS WP3 [237].
Here, I will recapitulate the main findings of the three projects and discuss some aspects that were not addressed (or fully addressed) in the manuscripts presented in the Results section.

1. The VOYAG-R trial and microbiota species involved in susceptibility to traveler's diarrhea

The number of people traveling for touristic purposes incredibly increased during the last years [199, 200].
Travel to tropical regions has been pointed out as particularly risky for colonization with MRE and experiencing diarrhea. Tropical regions are known to be endemic for MRE likely because of absence of appropriate hygiene practices and unrestricted antibiotic administration [196]. Based on experimental evidences that intestinal microbiota can protect from pathogen intestinal invasion and colonization [102], we challenged the hypothesis that microbiota could prevent from MRE acquisition and from traveler's diarrhea. Thus time-series analyses of fecal microbiota changes were peformed before departure, soon after or one month after the return to three continental MRE endemic regions. Importantly, I would like to underline that the

decision to use cDNA sequencing rather than DNA sequencing was supported by the work by Gosalbes *et al.* [244] according to which the analyses of metabolically active microbiota composition provide better segregation between ESBL-E positive and negative individuals. Such an approach brought the advantage to infer microbial taxonomy by considering different regions along the whole length of both 16S and 23S ribosomal RNA encoding genes. Furthermore, different bioinformatics databases and pipelines allowed us to strengthen our observation on microbiota composition changes.

We find out that MRE acquisition does not depend on the initial microbiota composition, which, in turn, confers susceptibility to traveler's diarrhea and affects MRE clearance after the return.

Although the rate of TD occurrence during the travel was reported to be 40.1% in the VOYAG-R trial [209], in the metagenomic sub-study only nine out of the 43 (20.9%) individuals experienced TD.

The aetiology of TD was not investigated but we demonstrated that TD was associated with high proportions of Enterobacteriaceae, thus it is likely that TD had a bacterial origin as Enterobacteriaceae include diarrheagenic species such as *E. coli*.

We also analyzed the composition of travelers experiencing TD (TD+) and compared them to diarrhea-free individuals (TD-) before departure and return. While TD+ microbiota at return from travel was enriched in *Prevotella copri*, Enterobacteriaceae and *Campylobacter* species, it had decreased proportions of Lachnospiraceae (*i.e. Blautia*, *Coprococcus* and *Roseburia* species) and Ruminococcaceae (*i.e. Ruminoccus*) members.

A study conducted on Swedish travelers to TD endemic countries (including Egypt, Tunisia, Curaçao, Turkey, Ecuador, Bangladesh and Tanzania), showed that pre-travel microbiota of individuals who did not acquire the enteropathogen *Campylobacter* was enriched in Lachnospiraceae in particular in *Dorea* and *Coprococcus* [245]. In the same study, species richness and diversity were already lower before departure in individuals who were going to be infected by *Campylobacter* compared to those people who remained uninfected. While Lachnospiraceae were confirmed to be a correlate of protection against TD, in our study we did not find significant differences in global microbiota composition between TD+ and TD- individuals before travel. Pre-travel similarity in microbiota composition between TD+ and TD- individuals was also confirmed in a study performed on a cohort of 11 US soldiers sent to Honduras for a military mission [246]. On the other hand, we showed that microbiota of TD+ individuals was significantly less rich and diverse than microbiota of TD- group collected at return.

Furthermore, we demonstrated that travelers who developed diarrhea during travel had higher loads of *P. copri* before departure. Thus, this observation led us to hypothesize that pre-travel abundance of *P. copri* could induce susceptibility to experiencing diarrhea.

Iljazovic *et al.* [247] demonstrated that mice having *Prevotella intestinalis*, a murine-specific commensal that has colonization rate similar to *P. copri* in humans [247, 248], increased inflammation responses when intestinal barrier was artificially damaged.
P. intestinalis was shown to reduce the concentration of acetate in the gut and to decrease intestinal IL-18 levels. By disturbing homeostatic IL-18 concentration, *P. intestinalis* is believed to exacerbate intestinal inflammation occurring after injury of intestinal epithelium.
P. copri is reported to be present in 39.1% of healthy individuals [249] where it can represent one third of the fecal microbiota population [250]. In particular *Prevotella* genus has also been previously described as a major component of one of the possible enterotypes [56].
A study conducted on more than 1,000 genomes [250], mostly deriving from intestinal metagenomes from 25 different countries of six continents, revealed four large clades of *P. copri*, all with a certain degree of geographical stratification. The study showed that *P. copri* is underrepresented in individuals from Western countries likely because of a diet poor in fibers and complex carbohydrates, which, instead, are abundant in the alimentary regimen of ancient and non-Westernized cultures. None of the four clades was associated to a particular disease and presented different metabolic pathways for carbohydrates utilisation. Strains from different clades were also reported to exist in the same individual.
Walters *et al.* [246], compared our results on pre-travel microbiota of TD+ and TD- groups with those reported from Pop *et al.* [251] and Youmans *et al.* [252].
Pop *et al.* analyzed the microbiota composition changes in individuals that were and were not administered ETEC strain H10407 following treatment with ciprofloxacin. Youmans *et al.* investigated microbiota composition of individuals traveling from US to Central America or India and stratified them in three classes: pathogen-associated TD+ (from Norovirus, ETEC, and ETEC plus pathogenic *E. coli* co-infections), no-identified-pathogen TD+ and TD-.
More generally, there was a very poor overlap between species negatively or positively associated to diarrhea between the three studies.
According to Walters *et al.* [246], *P. copri* was found positively associated to pre-travel TD+ microbiota only in our study, Youmans *et al.* did not provide pre-travel samples and Pop *et al.* reported *P. copri* as protective species against TD.
However, this meta-analysis did not take in account that different study designs, bioinformatics and biostatistics approaches were used in all the three studies.
Whether *P. copri* is beneficial or detrimental for microbiota is a complex question that should be addressed taking in account the overall genomic (and geographical) variability of the species. With our work, we linked for the first time *P. copri* to the occurrence of TD in a cohort of French travelers going to tropical regions.

2. Effect of antibiotic therapy duration on microbiota, resilience and reconstitution of normal flora

Besides favoring pathogen infection [253], antibiotic treatment can select pre-existing resistant strains and thus increases the ARGs content [253].
Whether a long antibiotic therapy is more efficacious than a short one in treating infections is under debate. The duration of antibiotic therapy against Gram-negative bacteremia is conventionally fixed between 10 and 14 days exclusively based on opinions of experts [217]. The PIRATE trial showed that there are no actual differences in the treatment response between therapy durations of 7 and of 14 days [217].
We challenged the hypothesis that short treatment can limit the detrimental effects of antibiotics on microbiota and resistome composition. Thus, in the PIRATE RESISTANCE study, we analyzed the dynamics of changes in microbiota and resistome composition in 15 and 14 patients treated for 7 or 14 days against Gram-negative bacteremia, respectively.
We showed that there were no significant differences in species and ARDs composition between 7-day and 14-day groups and that short-term therapy is already sufficient to prompt changes in microbiota *e.g.* decreased species richness and diversity.
Different durations were not associated to significant changes in the overall phage composition.
ARG content, and in particular abundance of ARGs encoding resistance to the drug used in therapy, was maximal during antibiotic treatment in both patient groups. Importantly, ARGs were also detected in control patients, who were hospitalized and presented co-morbidities and, therefore, are not expected to carry a "healthy" microbiota. The presence of ARGs in control patients remarks the fact that ARGs are normally present in microbiota in absence of antibiotic therapy [131, 132]. The inter-species ARG exchange is reported to occur rarely in intestinal environment [131, 132]. Therefore, the antibiotic treatment is the major driving force explaining increasing the overall ARG content in case patients. Unfortunately fecal samples were not collected in case patients before antibiotic administration, thus allowing reconstructing only a part of the dynamics of resistome changes upon therapy.
Antibiotic treatment, irrespective of therapy duration, decreased the proportions of several species mostly belonging to Lachnospiraceae and Ruminococcaceae families (Firmicutes). Reduction in abundance of Lachnospiraceae and Ruminococcaceae following antibiotic treatment has also been shown in the R-GNOSIS metagenomic analyses (Figure 4; Results, Chapter 3). Thus, it is clear that antibiotics inevitably kill or reduce important components of microbiota beyond the clinical targets.
Microbiota seemed to be resilient to antibiotic therapy because post-treatment bacterial profiles of PIRATE RESISTANCE patients tended to be similar to those of control patients. However, genetic resistance to therapy antibiotics was still detected roughly 20 days after therapy interruption and in extreme cases even 74-77 days later.
Reconstitution of intestinal flora after antibiotic treatment is also achieved with fecal

microbiota transplantation. In fact we showed that FMT increased species diversity and richness but decreased the overall content of ARGs in microbiota in the R-GNOSIS project, so compensating the negative impact of colistin/neomycin therapy. Similar results were achieved in patients undergoing allo-HSCT and treated with antibiotics to reduce the risk of collateral infections following post-transplant immunosuppression. Autologous FMT with fecal material taken before the allo-HSCT intervention was shown to be sufficient to restore the microbiota species diversity after antibiotic administration [230].
Our work provided further evidence that even shorter antibiotic therapy increases the abundance of ARGs in the intestine and kills important components of intestinal microbiota. Thus, an aware use of antibiotics in clinical practise is mandatory. Moreover, strategies to restore microbiota composition after antibiotic course should and could be put in place.

2.1 Doxycycline treatment for malaria prophylaxis and microbiota composition of VOYAG-R travelers

A total of 23 out of 42 (54.7%) VOYAG-R travelers received malaria prophylaxis either with Atovaoquone-proguanil (n=16) or doxycycline (n=6) or chloroquine (n=1).
Of particular concern was the administration of doxycycline which is a tetracycline antibiotic.
Elvers *et al.* [243] have recently reviewed the effect of doxycycline on intestinal microbiota, reporting that changes in bacterial composition are dose-dependent.
In fact, when doxycycline was administered at suboptimal concentration (20 mg for 9 months) to patients suffering of *periodontitis*, it did not alter significantly intestinal microbiota composition nor prompted selection of tetracycline resistance [254].
Treatment with oral 40 mg daily dose of doxycycline for 16 weeks on healthy individuals caused small changes in *E. coli* and enterococci abundance and had no impact on the anaerobes' composition compared to placebo group [255]. Those changes were reported to be transient and returned to pre-treatment composition 28 days after interruption of the therapy. When doxycycline was given 100–150 mg (which is considered as the normal dose [243]) for 7–10 day [256, 257], it caused a significant decrease in *Bifidobacterium* diversity, it increased tetracycline resistance and led to the loss of *Fusobacterium* spp. Long-term effects of normal doxycycline dose on intestinal microbiota have not been investigated yet [243].
Unfortunately, information concerning dose and duration of doxycycline therapy in our cohort of travelers is missing, thus preventing a fair comparison with these previously published studies.
Based on the anti-malaria prophylaxis guidelines by the Société de pathologie infectieuse de langue française (SPILF), doxycycline is administered with a daily dose of 100 mg from the day before the departure to four weeks after the return [258].

It is likely that for our cohort the duration of doxycycline administration was variable given the different length of stay in tropical regions. Of the 6 travelers treated with doxycycline, one acquired MRE, experienced diarrhea and had antibiotics during the travel; another one had no diarrhea symptoms but acquired MRE and thus fecal samples were also analyzed one month after return.
In our work we showed that global microbiota composition, based on 16S and 23S rRNA taxonomic profiles, of travelers administered malaria prophylaxis was not significant different from that of travelers who did not received the therapy (Table 1; Results, Chapter 1). Moreover, we did not find significant differences in microbiota composition in respect to the type of anti-malaria drug used (Atovaoquone-proguanil, doxycycline, chloroquine, no drugs; Table 1; Results, Chapter 1). The inclusion of doxycycline-treated travelers did not influence the conclusions of our analyses.

3. Shifts in microbiota composition after fecal microbiota transplantation

FMT has been recently proposed to eradicate multidrug resistance colonization, *i.e.* ESBL-E and CP-E, from the gut, and the R-GNOSIS WP3 was the first randomized multicentre clinical trial to assess this possibility. The trial failed to provide conclusive results that FMT could help in decolonizing the intestine from ESBL-E/CP-E, likely because of its sample size. However, at 35–48 days after carriers' randomization the proportion of ESBL-E/CP-E decolonized subjects was higher in FMT-treated group than in those individuals receiving no treatments. Thus, we characterized the microbiota profiles of 16 FMT-treated and 10 treatment-naïve carriers from fecal samples that were collected at 5 different time points before and after antibiotics/FMT treatments during the trial. We also characterized the composition of donor fecal matter used for FMT. We observed that ESBL-E/CP-E carriers, irrespective of intervention group, had higher proportions of Enterobacteriaceae than donors with a relative abundance superior to 1% that is considered as average abundance of this group of bacteria in the gut [10].
By performing time-series analyses, we found out that antibiotic and FMT treatments lead to a shift in microbiota and resistome compositions which were not observed in treatment-naïve carriers.
E. coli was found decreased after antibiotics and FMT administration. Moreover, the content of β-lactam-resistance encoding genes was also decreased after treatment in 10 out of 14 carriers ESBL-E/CP-E compared to baseline (Figure 5; Results, Chapter 3). We showed that FMT was able to increase species richness and diversity reduced by antibiotic therapy. Post-FMT microbiota was more similar in bacterial composition to that of donors. Furthermore, a higher number of species was shared between donor and recipient in post-FMT microbiota than in pre-FMT microbiota.
I did not investigate the engraftment of donor strains in recipient, however, donor strains are known to be more likely successfully engrafted when strains of the same

species are already present in the recipient [259]. Donor and recipient strains are found to co-exist more than three months after FMT [259].
We also reported that the same donor administered to different patients can lead to diverse decolonization outcomes. Thus, factors driving successful pathogen clearance are dependent on pre-FMT recipient microbiota, besides donor, and need to be investigated in further details. Some preliminary analyses showed that success of FMT in treating chemotherapy-induced diarrhea could depend on the degree of strain diversity in the recipient after FMT administration [231].

3.1 *Bifidobacterium* species and FMT treatment

Bifidobacterium species were affected differently by antibiotic and FMT treatment in the R-GNOSIS metagenomic study. In fact, they were significantly more abundant in post-FMT time points than at baseline and post-antibiotic time point of treated individual. Post-FMT *Bifidobacterium* loads were also more abundant than treatment-naïve microbiota.
Increased intestinal abundance of *Bifidobacterium bifidum*, not found to be differentially abundant in our analyses, was reported to be associated with successful eradication of CP-E from a single-center study investigating oral administration of capsulized-FMT on a cohort of 15 CP-E carriers [260].
A single-arm, open-label study investigating a cohort of 10 patients suffering from IBD and treated with FMT, reported that *Bifidobacterium*-rich donor microbiota restored intestinal microbial balance in recipient patients [261]. However, the abundance of *Bifidobacterium* in recipient patients was not associated to a relief of the disease. Another study reported that *Bifidobacterium* strains acquired from the donor have been detected in the microbiota of the recipient one year after FMT administration against recurrent *C. difficile* infection [262].
Bifidobacterium species are known to contribute to the sustainment for intestinal epithelium barrier. In fact, *Bifidobacterium longum* has been reported to induce expression of tight-junction proteins, such as *occludin* in human keratinocytes [263] and in murine intestinal epithelial cells [264]. *Bifidobacterium* species are also involved in the regulation of intestinal inflammation by upregulating the expression of anti-inflammatory cytokines (reviewed in [265]).
Moreover, the administration of *B. longum* as probiotic in a mouse model for post-infectious visceral hypersensitivity, demonstrated the same effect as the administration of crude FMT [264]. Thus, in some cases probiotics containing *Bifidobacterium* strains could replace FMT that has disadvantages related to the preparation and safety of administration. Results from the PLACIDE study testing the efficacy of a multistrain preparation of lactobacilli and bifidobacteria on the occurrence of antibiotic-associated diarrhea and *C. difficile* diarrhea in elderly population, showed that this multistrain probiotic did not prompt any improvement of patient conditions [266]. Failure of intestinal engraftment of the probiotic species

could explain this outcome. Thus, like FMT, the understanding of the factors driving probiotics engraftments requires further investigation. The authors from PLACIDE study highlighted that a further exploration of microbiota components involved in antibiotic-associated diarrhea is mandatory as well as to study effects of probiotics administration in a younger population associated with less comorbidities than that of the trial [266]. In addition, the possibility of using probiotics with a more limited number of strains should be also explored [266]. Thus, whether probiotics could substitute FMT preparations is still an open question. Indeed, metagenomic-based studies could bring an important contribution in this field by allowing a deeper characterization of microbiota components.

4. Lachnospiraceae and Ruminococcaceae and their possible role in intestinal microbiome

Inflammation at level of intestinal epithelium is thought to locally increment blood flow thus increasing the concentration of oxygen in the intestinal lumen [80]. Increased oxygen concentrations lead to the death of obligate bacterial anaerobes, including Clostridia to which Lachnospiraceae and Ruminococcaceae belong, and to the expansion of some groups of bacteria such as Enterobacteriaceae [80]. In fact Enterobacteriaceae can switch from anaerobic to aerobic respiration and can metabolize nitrates produced by inflamed gut (reviewed in [80]).
In the context of intestinal microbiota, Lachnospiraceae and Ruminococcaceae species limit inflammation responses through the production of SCFAs, contribute to the renewal of the intestinal epithelium and play a role in the resistance to pathogen colonization [80].
In the VOYAG-R study, we reported that Lachnospiraceae and Ruminococcaceae are associated to the intestinal clearance of MRE acquired after traveling to tropical regions and that they are negatively correlated with the occurrence of TD. However, the function of certain Lachnospiraceae and Ruminococcaceae species should be better clarified as for example members from Ruminococcaceae family are reported to act either as protectors against TD (*Ruminiclostridium* spp.) or promoters of TD (Ruminococcaceae UCG-013) [246].
In the PIRATE RESISTANCE study, post-antibiotic-treatment microbiota has higher loads of Lachnospiraceae and Ruminococcaceae members than on-treatment microbiota. Finally, together with *Bifidobacterium* spp., Lachnospiraceae and Ruminococcaceae increased after FMT. Our analyses do not show whether Lachnospiraceae and Ruminococcaceae have a role in the ESBL-E/CP-E decolonization through FMT, but Bar-Yoseph and colleagues reported these bacteria as key features of post-FMT microbiota in individuals successfully decolonized from CP-E [260]. Moreover, Schubert *et al.* [267] also described *Bacteroides*, Lachnospiraceae and Ruminococcaceae species as fundamental factors of transplantation to restore the "healthy" microbiota in rCDI.

In conclusion, increased abundance of some Lachnospiraceae and Ruminococcaceae members seems to be associated with potential benefits for microbiota.
Given the limitations of the trials and of the metagenomic analyses presented in this thesis, further investigations are needed for a full understanding of the potential ecological impact and of the clinical role of these species in the intestine.

5. Conclusions

With my work, I showed association between microbiota composition, MRE clearance and diarrhea. I reported that antibiotics shift the composition of microbiota towards dysbiosis irrespective of therapy duration, but that this trend can be inverted by therapy interruption and with fecal microbiota transplantation.
Finally, I have identified key species which might play a role in the dynamics of changes of microbiota.
Those *Bifidobacterium*, Lachnospiraceae and Ruminococcaceae species associated with the intestinal loss of MRE might be isolated from fecal samples and co-cultured with MRE strains to better understand which molecular pathways are involved.
The genomes of *Prevotella copri* strains could be recovered from fecal samples of the travelers to tropics and compared to each other to see whether there are genetic traits capable to explain the association with TD.
The use of culturomics, organoids [13, 268] and mouse model could better clarify the functional roles of these species in physiological conditions mimicking those of the human intestine.
Such information will be of interest for translational microbiota research and could lead to more targeted therapies, to prevent intestinal MRE colonization and/or to enhance intestinal MRE clearance.
Release of antibiotics into the environment as consequence of human activities is capable to select for multidrug resistance organisms even at low drug concentrations [102] and the intestinal resistome continuously exchanges genetic material with the environment [128]. Thus, characterization of microbiota and resistome is essential for a better understanding and management of infectious diseases, especially in this moment when rise of antibiotic resistance is increasing worldwide.

References

1. Marchesi JR, Ravel J: **The vocabulary of microbiome research: a proposal**. *Microbiome* 2015, **3**(1):31.
2. **Antony van Leeuwenhoek and his "Little Animals": being some account of the father of Protozoology and Bacteriology and his multifarious discoveries in these disciplines**. *Nature* 1932, **130**(3288):679-680.
3. Leidy J: **Reviews: a flora and fauna within living animals**. *Quarterly Journal of Microscopical Science* 1854, **s1-2**(7):192.
4. Pasteur L: **Observations relatives à la note précédente de M. Duclaux.** *CR Acad Sci* 1885, **100, 68**.
5. Rackaityte E, Lynch SV: **The human microbiome in the 21st century**. *Nature Communications* 2020, **11**(1):5256.
6. Breitschwerdt EB, Linder KL, Day MJ, Maggi RG, Chomel BB, Kempf VAJ: **Koch's postulates and the pathogenesis of comparative infectious disease causation associated with Bartonella species**. *J Comp Pathol* 2013, **148**(2-3):115-125.
7. Podolsky SH: **Metchnikoff and the microbiome**. *The Lancet* 2012, **380**(9856):1810-1811.
8. Escherich T: **Die Darmbakterien des Neugeborenen und Sauglings.** . *Fortschritte der Medizin* 1885, **3, 515-522**.
9. Farré-Maduell E, Casals-Pascual C: **The origins of gut microbiome research in Europe: From Escherich to Nissle**. *Human Microbiome Journal* 2019, **14**:100065.
10. Rajilić-Stojanović M, de Vos WM: **The first 1000 cultured species of the human gastrointestinal microbiota**. *FEMS Microbiol Rev* 2014, **38**(5):996-1047.
11. Lagkouvardos I, Overmann J, Clavel T: **Cultured microbes represent a substantial fraction of the human and mouse gut microbiota**. *Gut Microbes* 2017, **8**(5):493-503.
12. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, Dantas G, Gordon JI: **Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice**. *Proc Natl Acad Sci U S A* 2011, **108**(15):6252-6257.
13. Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, Levasseur A, Rolain J-M, Fournier P-E, Raoult D: **Culturing the human microbiota and culturomics**. *Nature Reviews Microbiology* 2018, **16**(9):540-550.
14. Midtvedt T, Bjørneklett A, Carlstedt-Duke B, Gustafsson B, Høverstad T, Lingaas E, Norin K, Saxerholt H, Steinbakk M: **The influence of antibiotics upon microflora-associated characteristics in man and mammals**. *Prog Clin Biol Res* 1985, **PMID: 4022981**.
15. Fouhy F, Ross RP, Fitzgerald GF, Stanton C, Cotter PD: **Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps**. *Gut Microbes* 2012, **3**(3):203-220.
16. Woese CR, Fox GE: **Phylogenetic structure of the prokaryotic domain: The primary kingdoms**. *Proceedings of the National Academy of Sciences* 1977, **74**(11):5088.
17. Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors**. *Proc Natl Acad Sci U S A* 1977, **74**(12):5463-5467.
18. Janda JM, Abbott SL: **16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls**. *Journal of Clinical Microbiology* 2007, **45**(9):2761.

19. Kitahara K, Miyazaki K: **Revisiting bacterial phylogeny: natural and experimental evidence for horizontal gene transfer of 16S rRNA**. *Mob Genet Elements* 2013, **3**(1):e24210-e24210.
20. Mardis ER: **Next-generation DNA sequencing methods**. *Annual Review of Genomics and Human Genetics* 2008, **9**(1):387-402.
21. Handelsman J: **Metagenomics: application of genomics to uncultured microorganisms**. *Microbiol Mol Biol Rev* 2004, **68**(4):669-685.
22. Brosius J, Palmer ML, Kennedy PJ, Noller HF: **Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli**. *Proc Natl Acad Sci U S A* 1978, **75**(10):4801-4805.
23. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS *et al*: **Structure, function and diversity of the healthy human microbiome**. *Nature* 2012, **486**(7402):207-214.
24. Johnson JS, Spakowicz DJ, Hong B-Y, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M *et al*: **Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis**. *Nature Communications* 2019, **10**(1):5029.
25. Emonet S, Lazarevic V, Leemann Refondini C, Gaïa N, Leo S, Girard M, Nocquet Boyer V, Wozniak H, Després L, Renzi G *et al*: **Identification of respiratory microbiota markers in ventilator-associated pneumonia**. *Intensive Care Medicine* 2019, **45**(8):1082-1092.
26. Leo S, Cherkaoui A, Renzi G, Schrenzel J: **Mini review: clinical routine microbiology in the era of automation and digital health**. *Frontiers in Cellular and Infection Microbiology* 2020, **10**(706).
27. Mardis ER: **The impact of next-generation sequencing technology on genetics**. *Trends in Genetics* 2008, **24**(3):133-141.
28. Needleman SB, Wunsch CD: **A general method applicable to the search for similarities in the amino acid sequence of two proteins**. *Journal of Molecular Biology* 1970, **48**(3):443-453.
29. Smith TF, Waterman MS: **Identification of common molecular subsequences**. *Journal of Molecular Biology* 1981, **147**(1):195-197.
30. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool**. *Journal of Molecular Biology* 1990, **215**(3):403-410.
31. Stackebrandt E, Goebel BM: **Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology**. *International Journal of Systematic and Evolutionary Microbiology* 1994, **44**(4):846-849.
32. Stackebrandt E, Ebers J: **Taxonomic parameters revisited: tarnished gold standards**. *Microbiol Today* 2006, **33:152–155**.
33. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ *et al*: **Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities**. *Applied and Environmental Microbiology* 2009, **75**(23):7537.
34. Edgar RC: **Search and clustering orders of magnitude faster than BLAST**. *Bioinformatics* 2010, **26**(19):2460-2461.
35. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI *et al*: **QIIME allows analysis of high-throughput community sequencing data**. *Nature Methods* 2010, **7**(5):335-336.

36. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: **Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB**. *Applied and Environmental Microbiology* 2006, **72**(7):5069.
37. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO: **The SILVA ribosomal RNA gene database project: improved data processing and web-based tools**. *Nucleic Acids Research* 2013, **41**(D1):D590-D596.
38. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM: **Ribosomal Database Project: data and tools for high throughput rRNA analysis**. *Nucleic acids research* 2014, **42**(Database issue):D633-D642.
39. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A, Huttenhower C, Segata N: **MetaPhlAn2 for enhanced metagenomic taxonomic profiling**. *Nature Methods* 2015, **12**(10):902-903.
40. Ounit R, Wanamaker S, Close TJ, Lonardi S: **CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers**. *BMC Genomics* 2015, **16**(1):236.
41. Wood DE, Salzberg SL: **Kraken: ultrafast metagenomic sequence classification using exact alignments**. *Genome Biology* 2014, **15**(3):R46.
42. Calle ML: **Statistical analysis of metagenomics data**. *Genomics Inform* 2019, **17**(1):e6-e6.
43. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV: **Identification of acquired antimicrobial resistance genes**. *J Antimicrob Chemother* 2012, **67**(11):2640-2644.
44. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen A-LV, Cheng AA, Liu S *et al*: **CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database**. *Nucleic acids research* 2020, **48**(D1):D517-D525.
45. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain J-M: **ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes**. *Antimicrobial agents and chemotherapy* 2014, **58**(1):212-220.
46. Anderson MJ: **A new method for non-parametric multivariate analysis of variance**. *Austral Ecology* 2001, **26**(1):32-46.
47. Love MI, Huber W, Anders S: **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2**. *Genome Biology* 2014, **15**(12):550.
48. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD: **Analysis of composition of microbiomes: a novel method for studying microbial composition**. *Microb Ecol Health Dis* 2015, **26**:27663-27663.
49. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C: **Metagenomic biomarker discovery and explanation**. *Genome Biology* 2011, **12**(6):R60.
50. Smillie CS, Sauk J, Gevers D, Friedman J, Sung J, Youngster I, Hohmann EL, Staley C, Khoruts A, Sadowsky MJ *et al*: **Strain tracking reveals the determinants of bacterial engraftment in the human gut following fecal microbiota transplantation**. *Cell Host & Microbe* 2018, **23**(2):229-240.e225.
51. Kong S, Zhang YH, Zhang W: **Regulation of intestinal epithelial cells properties and functions by amino acids**. *BioMed Research International* 2018, **2018**:2819154.
52. Azzouz L, Sharma S: **Physiology, large intestine. [Updated 2020 Jul 27].** *StatPearls [Internet] Treasure Island (FL): StatPearls Publishing; Available from: https://wwwncbinlmnihgov/books/NBK507857/* 2020.

53. Rose C, Parker A, Jefferson B, Cartmell E: **The characterization of feces and urine: a review of the literature to inform advanced treatment technology**. *Crit Rev Environ Sci Technol* 2015, **45**(17):1827-1879.
54. Sender R, Fuchs S, Milo R: **Revised estimates for the number of human and bacteria cells in the body**. *PLOS Biology* 2016, **14**(8):e1002533.
55. Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, Ley RE, Farnleitner AH: **Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades**. *Nature Communications* 2019, **10**(1):2200.
56. Voreades N, Kozil A, Weir TL: **Diet and the development of the human intestinal microbiome**. *Frontiers in microbiology* 2014, **5**:494-494.
57. Yan W, Sun C, Zheng J, Wen C, Ji C, Zhang D, Chen Y, Hou Z, Yang N: **Efficacy of fecal sampling as a gut proxy in the study of chicken gut microbiota**. *Frontiers in Microbiology* 2019, **10**:2126.
58. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T *et al*: **A human gut microbial gene catalogue established by metagenomic sequencing**. *Nature* 2010, **464**(7285):59-65.
59. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, Mele MC: **What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases**. *Microorganisms* 2019, **7**(1):14.
60. Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P, Balamurugan R: **The Firmicutes/Bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients?** *Nutrients* 2020, **12**(5):1474.
61. Pokusaeva K, Fitzgerald GF, van Sinderen D: **Carbohydrate metabolism in Bifidobacteria**. *Genes Nutr* 2011, **6**(3):285-306.
62. Felis G, Dellaglio F: **Taxonomy of Lactobacilli and Bifidobacteria.** *Curr Issues Intest Microbiol* 2007, **8(2):44-61. PMID: 17542335**.
63. Hoyles L, McCartney AL, Neve H, Gibson GR, Sanderson JD, Heller KJ, van Sinderen D: **Characterization of virus-like particles associated with the human faecal and caecal microbiota**. *Research in Microbiology* 2014, **165**(10):803-812.
64. Shkoporov AN, Clooney AG, Sutton TDS, Ryan FJ, Daly KM, Nolan JA, McDonnell SA, Khokhlova EV, Draper LA, Forde A *et al*: **The Human gut virome is highly diverse, stable, and individual specific**. *Cell Host & Microbe* 2019, **26**(4):527-541.e525.
65. Aggarwala V, Liang G, Bushman FD: **Viral communities of the human gut: metagenomic analysis of composition and dynamics**. *Mobile DNA* 2017, **8**(1):12.
66. Cornuault JK, Petit M-A, Mariadassou M, Benevides L, Moncaut E, Langella P, Sokol H, De Paepe M: **Phages infecting Faecalibacterium prausnitzii belong to novel viral genera that help to decipher intestinal viromes**. *Microbiome* 2018, **6**(1):65.
67. Laforest-Lapointe I, Arrieta M-C: **Microbial eukaryotes: a missing link in gut microbiome studies**. *mSystems* 2018, **3**(2):e00201-00217.
68. Lukeš J, Stensvold CR, Jirků-Pomajbíková K, Wegener Parfrey L: **Are human intestinal Eukaryotes beneficial or commensals?** *PLOS Pathogens* 2015, **11**(8):e1005039.
69. Nieves-Ramírez ME, Partida-Rodríguez O, Laforest-Lapointe I, Reynolds LA, Brown EM, Valdez-Salazar A, Morán-Silva P, Rojas-Velázquez L, Morien E, Parfrey LW *et al*: **Asymptomatic intestinal colonization with protist Blastocystis is strongly associated with distinct microbiome ecological patterns**. *mSystems* 2018, **3**(3):e00007-00018.

70. Woerther P-L, Lepeule R, Burdet C, Decousser J-W, Ruppé É, Barbier F: **Carbapenems and alternative β-lactams for the treatment of infections due to extended-spectrum β-lactamase-producing Enterobacteriaceae: What impact on intestinal colonisation resistance?** *International Journal of Antimicrobial Agents* 2018, **52**(6):762-770.
71. Gilbert SF: **A holobiont birth narrative: the epigenetic transmission of the human microbiome**. *Front Genet* 2014, **5**:282-282.
72. Arboleya S, Suárez M, Fernández N, Mantecón L, Solís G, Gueimonde M, de los Reyes-Gavilán CG: **C-section and the neonatal gut microbiome acquisition: consequences for future health**. *Annals of Nutrition and Metabolism* 2018, **73(suppl 3)**(3):17-23.
73. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, Knight R, Heath AC, Leibel RL *et al*: **The long-term stability of the human gut microbiota**. *Science* 2013, **341**(6141):1237439-1237439.
74. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto J-M *et al*: **Enterotypes of the human gut microbiome**. *Nature* 2011, **473**(7346):174-180.
75. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R *et al*: **Linking long-term dietary patterns with gut microbial enterotypes**. *Science* 2011, **334**(6052):105-108.
76. Huse SM, Ye Y, Zhou Y, Fodor AA: **A core human microbiome as viewed through 16S rRNA sequence clusters**. *PLOS ONE* 2012, **7**(6):e34242.
77. Koren O, Knights D, Gonzalez A, Waldron L, Segata N, Knight R, Huttenhower C, Ley RE: **A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets**. *PLOS Computational Biology* 2013, **9**(1):e1002863.
78. Rojo D, Hevia A, Bargiela R, López P, Cuervo A, González S, Suárez A, Sánchez B, Martínez-Martínez M, Milani C *et al*: **Ranking the impact of human health disorders on gut metabolism: Systemic lupus erythematosus and obesity as study cases**. *Sci Rep* 2015, **5**(1):8310.
79. Moya A, Ferrer M: **Functional redundancy-induced stability of gut microbiota subjected to disturbance**. *Trends in Microbiology* 2016, **24**(5):402-413.
80. Zeng MY, Inohara N, Nuñez G: **Mechanisms of inflammation-driven bacterial dysbiosis in the gut**. *Mucosal immunology* 2017, **10**(1):18-26.
81. Dani A: **Colonization and infection**. *Cent European J Urol* 2014, **67**(1):86-87.
82. Parada Venegas D, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, Harmsen HJM, Faber KN, Hermoso MA: **Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases**. *Frontiers in Immunology* 2019, **10**(277).
83. Louis P, Flint HJ: **Formation of propionate and butyrate by the human colonic microbiota**. *Environmental Microbiology* 2017, **19**(1):29-41.
84. McNeil NI, Cummings JH, James WP: **Short chain fatty acid absorption by the human large intestine**. *Gut* 1978, **19**(9):819-822.
85. Kim S, Kim J-H, Park BO, Kwak YS: **Perspectives on the therapeutic potential of short-chain fatty acid receptors**. *BMB Rep* 2014, **47**(3):173-178.
86. Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P: **Phylogenetic distribution of three pathways for propionate production within the human gut microbiota**. *The ISME Journal* 2014, **8**(6):1323-1335.

87. Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ: **Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon**. *J Bacteriol* 2004, **186**(7):2099-2106.
88. Louis P, Young P, Holtrop G, Flint HJ: **Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene**. *Environmental Microbiology* 2010, **12**(2):304-314.
89. Spahn TW, Kucharzik T: **Modulating the intestinal immune system: the role of lymphotoxin and GALT organs**. *Gut* 2004, **53**(3):456-465.
90. Dohi T, Rennert PD, Fujihashi K, Kiyono H, Shirai Y, Kawamura YI, Browning JL, McGhee JR: **Elimination of colonic patches with lymphotoxin β receptor-Ig prevents Th2 cell-type colitis**. *The Journal of Immunology* 2001, **167**(5):2781.
91. Jacob E, Baker SJ, Swaminathan SP: **'M' cells in the follicle-associated epithelium of the human colon**. *Histopathology* 1987, **11**(9):941-952.
92. Okumura R, Takeda K: **Roles of intestinal epithelial cells in the maintenance of gut homeostasis**. *Experimental & Molecular Medicine* 2017, **49**(5):e338-e338.
93. Günther J, Seyfert H-M: **The first line of defence: insights into mechanisms and relevance of phagocytosis in epithelial cells**. *Seminars in Immunopathology* 2018, **40**(6):555-565.
94. Lei-Leston AC, Murphy AG, Maloy KJ: **Epithelial cell inflammasomes in intestinal immunity and inflammation**. *Frontiers in Immunology* 2017, **8**(1168).
95. Elinav E, Strowig T, Kau Andrew L, Henao-Mejia J, Thaiss Christoph A, Booth Carmen J, Peaper David R, Bertin J, Eisenbarth Stephanie C, Gordon Jeffrey I *et al*: **NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis**. *Cell* 2011, **145**(5):745-757.
96. Winter SE, Winter MG, Xavier MN, Thiennimitr P, Poon V, Keestra AM, Laughlin RC, Gomez G, Wu J, Lawhon SD *et al*: **Host-derived nitrate boosts growth of E. coli in the inflamed gut**. *Science* 2013, **339**(6120):708-711.
97. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J, Blugeon S, Bridonneau C, Furet J-P, Corthier G *et al*: **Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients**. *Proceedings of the National Academy of Sciences* 2008, **105**(43):16731.
98. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C *et al*: **Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome**. *Nature Communications* 2015, **6**(1):6734.
99. Bollrath J, Powrie F: **Feed your T_{regs} more fiber**. *Science* 2013, **341**(6145):463.
100. Clasener HAL, Vollaard EJ, van Saene HKF: **Long-term prophylaxis of infection by selective decontamination in leukopenia and in mechanical ventilation**. *Reviews of Infectious Diseases* 1987, **9**(2):295-328.
101. van der Leur JJJPM, Thunnissen PLM, Clasener HAL, Muller NF, Dofferhoff ASM: **Effects of imipenem, cefotaxime and cotrimoxazole on aerobic microbial colonization of the digestive tract**. *Scandinavian Journal of Infectious Diseases* 1993, **25**(4):473-478.
102. Buffie CG, Pamer EG: **Microbiota-mediated colonization resistance against intestinal pathogens**. *Nature Reviews Immunology* 2013, **13**(11):790-801.

103. Scaldaferri F, Gerardi V, Mangiola F, Lopetuso LR, Pizzoferrato M, Petito V, Papa A, Stojanovic J, Poscia A, Cammarota G *et al*: **Role and mechanisms of action of Escherichia coli Nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update**. *World J Gastroenterol* 2016, **22**(24):5505-5511.
104. Sassone-Corsi M, Nuccio S-P, Liu H, Hernandez D, Vu CT, Takahashi AA, Edwards RA, Raffatellu M: **Microcins mediate competition among Enterobacteriaceae in the inflamed gut**. *Nature* 2016, **540**(7632):280-283.
105. Sonnenburg JL, Chen CTL, Gordon JI: **Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host**. *PLOS Biology* 2006, **4**(12):e413.
106. Perng Y-C, Lenschow DJ: **ISG15 in antiviral immunity and beyond**. *Nature Reviews Microbiology* 2018, **16**(7):423-439.
107. Gagnon M, Kheadr EE, Le Blay G, Fliss I, x: **In vitro inhibition of Escherichia coli O157:H7 by bifidobacterial strains of human origin**. *International Journal of Food Microbiology* 2004, **92**(1):69-78.
108. Booth IR: **Regulation of cytoplasmic pH in bacteria**. *Microbiol Rev* 1985, **49**(4):359-378.
109. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T *et al*: **Bifidobacteria can protect from enteropathogenic infection through production of acetate**. *Nature* 2011, **469**(7331):543-547.
110. Hedblom GA, Reiland HA, Sylte MJ, Johnson TJ, Baumler DJ: **Segmented filamentous bacteria – metabolism meets immunity**. *Frontiers in Microbiology* 2018, **9**(1991).
111. Talham GL, Jiang HQ, Bos NA, Cebra JJ: **Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system**. *Infect Immun* 1999, **67**(4):1992-2000.
112. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV *et al*: **Induction of intestinal Th17 cells by segmented filamentous bacteria**. *Cell* 2009, **139**(3):485-498.
113. Cua DJ, Tato CM: **Innate IL-17-producing cells: the sentinels of the immune system**. *Nature Reviews Immunology* 2010, **10**(7):479-489.
114. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, Bogatyrev SR, Ismagilov RF, Pamer EG, Turnbaugh PJ *et al*: **Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness**. *Nature* 2014, **514**(7524):638-641.
115. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, Takeyama N, Kamioka M, Sakamoto M, Matsuki T *et al*: **Innate lymphoid cells regulate intestinal epithelial cell glycosylation**. *Science* 2014, **345**(6202):1254009.
116. Hurd EA, Holmén JM, Hansson GC, Domino SE: **Gastrointestinal mucins of Fut2-null mice lack terminal fucosylation without affecting colonization by Candida albicans**. *Glycobiology* 2005, **15**(10):1002-1007.
117. Netzker T, Flak M, Krespach MKC, Stroe MC, Weber J, Schroeckh V, Brakhage AA: **Microbial interactions trigger the production of antibiotics**. *Current Opinion in Microbiology* 2018, **45**:117-123.
118. Rea MC, Sit CS, Clayton E, Connor PM, Whittal RM, Zheng J, Vederas JC, Ross RP, Hill C: **Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile**. *Proceedings of the National Academy of Sciences* 2010, **107**(20):9352.
119. Fajardo A, Martínez JL: **Antibiotics as signals that trigger specific bacterial responses**. *Current Opinion in Microbiology* 2008, **11**(2):161-167.

120. Haas LF: **Papyrus of Ebers and Smith**. *J Neurol Neurosurg Psychiatry* 1999, **67**(5):578-578.
121. Gelpi A, Gilbertson A, Tucker JD: **Magic bullet: Paul Ehrlich, Salvarsan and the birth of venereology**. *Sex Transm Infect* 2015, **91**(1):68-69.
122. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J: **Call of the wild: antibiotic resistance genes in natural environments**. *Nature Reviews Microbiology* 2010, **8**(4):251-259.
123. Stouthamer AH: **A theoretical study on the amount of ATP required for synthesis of microbial cell material**. *Antonie van Leeuwenhoek* 1973, **39**(1):545-565.
124. Stokes JM, Lopatkin AJ, Lobritz MA, Collins JJ: **Bacterial metabolism and antibiotic efficacy**. *Cell Metabolism* 2019, **30**(2):251-259.
125. Lobritz MA, Belenky P, Porter CBM, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil AS, Collins JJ: **Antibiotic efficacy is linked to bacterial cellular respiration**. *Proc Natl Acad Sci U S A* 2015, **112**(27):8173-8180.
126. Kapoor G, Saigal S, Elongavan A: **Action and resistance mechanisms of antibiotics: a guide for clinicians**. *J Anaesthesiol Clin Pharmacol* 2017, **33**(3):300-305.
127. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R *et al*: **Antibiotic resistance is ancient**. *Nature* 2011, **477**(7365):457-461.
128. Iredell J, Brown J, Tagg K: **Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications**. *BMJ* 2016, **352**:h6420.
129. Hawkey PM: **The origins and molecular basis of antibiotic resistance**. *BMJ (Clinical research ed)* 1998, **317**(7159):657-660.
130. Costa VM, McGrann KM, Hughes DW, Wright GD: **Sampling the antibiotic resistome**. *Science* 2006, **311**(5759):374.
131. van Schaik W: **The human gut resistome**. *Philos Trans R Soc Lond B Biol Sci* 2015, **370**(1670):20140087-20140087.
132. Ruppé E, Ghozlane A, Tap J, Pons N, Alvarez A-S, Maziers N, Cuesta T, Hernando-Amado S, Clares I, Martínez JL *et al*: **Prediction of the intestinal resistome by a three-dimensional structure-based method**. *Nature Microbiology* 2019, **4**(1):112-123.
133. Schwartz DJ, Langdon AE, Dantas G: **Understanding the impact of antibiotic perturbation on the human microbiome**. *Genome Medicine* 2020, **12**(1):82.
134. Moore AM, Ahmadi S, Patel S, Gibson MK, Wang B, Ndao MI, Deych E, Shannon W, Tarr PI, Warner BB *et al*: **Gut resistome development in healthy twin pairs in the first year of life**. *Microbiome* 2015, **3**:27-27.
135. Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, Pan Y, Li J, Zhu L, Wang X *et al*: **Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota**. *Nature Communications* 2013, **4**(1):2151.
136. Forslund K, Sunagawa S, Kultima JR, Mende DR, Arumugam M, Typas A, Bork P: **Country-specific antibiotic use practices impact the human gut resistome**. *Genome Res* 2013, **23**(7):1163-1169.
137. Lipman MO, Coss JA, Boots RH: **Changes in the bacterial flora of the throat and intestinal tract during prolonged oral administration of penicillin**. *The American Journal of Medicine* 1948, **4**(5):702-709.
138. Keefer CS: **Alterations in normal bacterial flora of man and secondary infections during antibiotic therapy**. *The American Journal of Medicine* 1951, **11**(6):665-666.

139. Miller CP, Bohnhoff M, Rifkind D: **The effect of an antibiotic on the susceptibility of the mouse's intestinal tract to Salmonella infection**. *Trans Am Clin Climatol Assoc* 1956, **68**:51-58.
140. Bohnhoff M, Miller CP, Martin WR: **Resistance of the mouse'intestinal tract to experimental Salmonella infection.** *Journal of Experimental Medicine* 1964, **120**(5):805-816.
141. Freter R, With the Technical Assistance of David H: **Experimental enteric Shigella and Vibrio infections in mice and guinea pigs**. *Journal of Experimental Medicine* 1956, **104**(3):411-418.
142. Drusano GL, Standiford HC: **Pharmacokinetic profile of Imipenem/cilastatin in normal volunteers**. *The American Journal of Medicine* 1985, **78**(6, Supplement 1):47-53.
143. Pletz MWR, Rau M, Bulitta J, De Roux A, Burkhardt O, Kruse G, Kurowski M, Nord CE, Lode H: **Ertapenem pharmacokinetics and impact on intestinal microflora, in comparison to those of ceftriaxone, after multiple dosing in male and female volunteers**. *Antimicrobial agents and chemotherapy* 2004, **48**(10):3765-3772.
144. Dethlefsen L, Huse S, Sogin ML, Relman DA: **The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing**. *PLOS Biology* 2008, **6**(11):e280.
145. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M *et al*: **Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans**. *The Journal of Clinical Investigation* 2010, **120**(12):4332-4341.
146. Isaac S, Scher JU, Djukovic A, Jiménez N, Littman DR, Abramson SB, Pamer EG, Ubeda C: **Short- and long-term effects of oral vancomycin on the human intestinal microbiota**. *Journal of Antimicrobial Chemotherapy* 2017, **72**(1):128-136.
147. Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, Shirakawa T, Sonomoto K, Nakayama J: **Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota**. *FEMS Immunology & Medical Microbiology* 2009, **56**(1):80-87.
148. Aloisio I, Mazzola G, Corvaglia LT, Tonti G, Faldella G, Biavati B, Di Gioia D: **Influence of intrapartum antibiotic prophylaxis against group B Streptococcus on the early newborn gut composition and evaluation of the anti-Streptococcus activity of Bifidobacterium strains**. *Applied Microbiology and Biotechnology* 2014, **98**(13):6051-6060.
149. Thiemann S, Smit N, Strowig T: **Antibiotics and the intestinal microbiome: individual responses, resilience of the ecosystem, and the susceptibility to infections**. In: *How to Overcome the Antibiotic Crisis : Facts, Challenges, Technologies and Future Perspectives.* edn. Edited by Stadler M, Dersch P. Cham: Springer International Publishing; 2016: 123-146.
150. Alnajar S, Gupta RS: **Phylogenomics and comparative genomic studies delineate six main clades within the family Enterobacteriaceae and support the reclassification of several polyphyletic members of the family**. *Infection, Genetics and Evolution* 2017, **54**:108-127.
151. Hopkins MJ, Sharp R, Macfarlane GT: **Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles**. *Gut* 2001, **48**(2):198-205.

152. Parker N, Schneegurt M, Tu AT, Forster BM, Lister P: **Microbiology**. *ASM-Press openstax - https://openstaxorg/details/books/microbiology* 2017.
153. Youssef M, Shurman A, Bougnoux M-E, Rawashdeh M, Bretagne S, Strockbine N: **Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan**. *FEMS Immunology & Medical Microbiology* 2000, **28**(3):257-263.
154. Thielman NM, Guerrant RL: **Acute Infectious Diarrhea**. *New England Journal of Medicine* 2004, **350**(1):38-47.
155. Celli J, Deng W, Finlay BB: **Enteropathogenic Escherichia coli (EPEC) attachment to epithelial cells: exploiting the host cell cytoskeleton from the outside**. *Cellular Microbiology* 2000, **2**(1):1-9.
156. Glauner B, Höltje J, Schwarz U: **The composition of the murein of Escherichia coli**. *J Biol Chem* 1988, **1263(21):10088-95. PMID: 3292521.**
157. Pandey N, Cascella M: **Beta lactam antibiotics. [Updated 2020 Sep 9]**. *In: StatPearls [Internet] Treasure Island (FL): StatPearls Publishing Available from: https://wwwncbinlmnihgov/books/NBK545311/* 2020
158. Yocum RR, Rasmussen JR, Strominger JL: **The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase**. *Journal of Biological Chemistry* 1980, **255**(9):3977-3986.
159. Reeves DS, Bullock DW: **The aminopenicillins: Development and comparative properties**. *Infection* 1979, **7**(5):S425-S433.
160. Bush K, Bradford PA: **β-lactams and β-lactamase inhibitors: an overview**. *Cold Spring Harb Perspect Med* 2016, **6**(8):a025247.
161. Neu HC: **Relation of structural properties of beta-lactam antibiotics to antibacterial activity**. *The American Journal of Medicine* 1985, **79**(2, Supplement 1):2-13.
162. Paterson DL, Bonomo RA: **Extended-spectrum beta-lactamases: a clinical update**. *Clinical microbiology reviews* 2005, **18**(4):657-686.
163. Medeiros AA: **Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics**. *Clinical Infectious Diseases* 1997, **24**(Supplement_1):S19-S45.
164. Eichenberger EM, Thaden JT: **Epidemiology and mechanisms of resistance of extensively drug resistant gram-negative bacteria**. *Antibiotics* 2019, **8**(2).
165. Bush K, Bradford PA: **Epidemiology of β-lactamase-producing pathogens**. *Clinical Microbiology Reviews* 2020, **33**(2):e00047-00019.
166. Shu J-C, Kuo A-J, Su L-H, Liu T-P, Lee M-H, Su IN, Wu T-L: **Development of carbapenem resistance in Pseudomonas aeruginosa is associated with OprD polymorphisms, particularly the amino acid substitution at codon 170**. *Journal of Antimicrobial Chemotherapy* 2017, **72**(9):2489-2495.
167. van Boxtel R, Wattel AA, Arenas J, Goessens WHF, Tommassen J: **Acquisition of carbapenem resistance by plasmid-encoded-AmpC-expressing Escherichia coli**. *Antimicrobial Agents and Chemotherapy* 2017, **61**(1):e01413-01416.
168. Bush K: **The evolution of beta-lactamases.** *Ciba Found Symp* 1997, **207:152-63; discussion 163-6. PMID: 9189640.**
169. Bush K: **Characterization of beta-lactamases**. *Antimicrobial agents and chemotherapy* 1989, **33**(3):259-263.

170. Bush K, Jacoby GA, Medeiros AA: **A functional classification scheme for beta-lactamases and its correlation with molecular structure**. *Antimicrobial Agents and Chemotherapy* 1995, **39**(6):1211.
171. Fisher JF, Meroueh SO, Mobashery S: **Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity**. *Chemical Reviews* 2005, **105**(2):395-424.
172. Massova I, Mobashery S: **Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases**. *Antimicrobial agents and chemotherapy* 1998, **42**(1):1-17.
173. Palzkill T: **Metallo-β-lactamase structure and function**. *Ann N Y Acad Sci* 2013, **1277**:91-104.
174. Wilke MS, Lovering AL, Strynadka NCJ: **β-Lactam antibiotic resistance: a current structural perspective**. *Current Opinion in Microbiology* 2005, **8**(5):525-533.
175. Bush K, Jacoby GA: **Updated functional classification of β-lactamases**. *Antimicrobial Agents and Chemotherapy* 2010, **54**(3):969.
176. Bradford PA: **Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat**. *Clinical Microbiology Reviews* 2001, **14**(4):933.
177. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K, Tenover FC: **Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae**. *Antimicrobial Agents and Chemotherapy* 2001, **45**(4):1151.
178. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK *et al*: **Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases**. *Lancet Infect Dis* 2013, **13**(9):785-796.
179. Kazmierczak KM, Bradford PA, Stone GG, de Jonge BLM, Sahm DF: **In vitro activity of ceftazidime-avibactam and aztreonam-avibactam against OXA-48-carrying Enterobacteriaceae isolated as part of the international network for optimal resistance monitoring (INFORM) global surveillance program from 2012 to 2015**. *Antimicrobial agents and chemotherapy* 2018, **62**(12):e00592-00518.
180. Jacoby GA: **AmpC beta-lactamases**. *Clinical microbiology reviews* 2009, **22**(1):161-182.
181. Bauernfeind A, Schweighart S, Chong Y: **Extended broad spectrum β-lactamase in Klebsiella pneumoniae including resistance to cephamycins**. *Infection* 1989, **17**(5):316-321.
182. Carfi A, Pares S, Duée E, Galleni M, Duez C, Frère JM, Dideberg O: **The 3-D structure of a zinc metallo-beta-lactamase from Bacillus cereus reveals a new type of protein fold**. *The EMBO Journal* 1995, **14**(20):4914-4921.
183. Concha NO, Rasmussen BA, Bush K, Herzberg O: **Crystal structure of the wide-spectrum binuclear zinc β-lactamase from Bacteroides fragilis**. *Structure* 1996, **4**(7):823-836.
184. Jenssen H, Hamill P, Hancock REW: **Peptide antimicrobial agents**. *Clinical Microbiology Reviews* 2006, **19**(3):491.
185. Kempf I, Fleury MA, Drider D, Bruneau M, Sanders P, Chauvin C, Madec J-Y, Jouy E: **What do we know about resistance to colistin in Enterobacteriaceae in avian and pig production in Europe?** *International Journal of Antimicrobial Agents* 2013, **42**(5):379-383.
186. Gunn JS: **The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more**. *Trends in Microbiology* 2008, **16**(6):284-290.

187. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X *et al*: **Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study**. *The Lancet Infectious Diseases* 2016, **16**(2):161-168.
188. Perrin-Guyomard A, Bruneau M, Houée P, Deleurme K, Legrandois P, Poirier C, Soumet C, Sanders P: **Prevalence of mcr-1 in commensal Escherichia coli from French livestock, 2007 to 2014**. *Eurosurveillance* 2016, **21**(6):30135.
189. Hinchliffe P, Yang QE, Portal E, Young T, Li H, Tooke CL, Carvalho MJ, Paterson NG, Brem J, Niumsup PR *et al*: **Insights into the mechanistic basis of plasmid-mediated colistin resistance from crystal structures of the catalytic domain of MCR-1**. *Sci Rep* 2017, **7**:39392-39392.
190. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y *et al*: **Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis**. *The Lancet Infectious Diseases* 2018, **18**(3):318-327.
191. Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM: **Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe**. *Clinical Microbiology and Infection* 2008, **14**:144-153.
192. Bevan ER, Jones AM, Hawkey PM: **Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype**. *Journal of Antimicrobial Chemotherapy* 2017, **72**(8):2145-2155.
193. Chong Y, Shimoda S, Shimono N: **Current epidemiology, genetic evolution and clinical impact of extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae**. *Infection, Genetics and Evolution* 2018, **61**:185-188.
194. Peirano G, Pitout JDD: **Extended-spectrum β-lactamase-producing Enterobacteriaceae: update on molecular epidemiology and treatment options**. *Drugs* 2019, **79**(14):1529-1541.
195. Banerjee R, Johnson JR: **A new clone sweeps clean: the enigmatic emergence of Escherichia coli Sequence Type 131**. *Antimicrobial Agents and Chemotherapy* 2014, **58**(9):4997.
196. Woerther P-L, Burdet C, Chachaty E, Andremont A: **Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M**. *Clinical Microbiology Reviews* 2013, **26**(4):744.
197. van den Bunt G, Liakopoulos A, Mevius DJ, Geurts Y, Fluit AC, Bonten MJM, Mughini-Gras L, van Pelt W: **ESBL/AmpC-producing Enterobacteriaceae in households with children of preschool age: prevalence, risk factors and co-carriage**. *Journal of Antimicrobial Chemotherapy* 2017, **72**(2):589-595.
198. Senchyna F, Gaur RL, Sandlund J, Truong C, Tremintin G, Kültz D, Gomez CA, Tamburini FB, Andermann T, Bhatt A *et al*: **Diversity of resistance mechanisms in carbapenem-resistant Enterobacteriaceae at a health care system in Northern California, from 2013 to 2016**. *Diagn Microbiol Infect Dis* 2019, **93**(3):250-257.
199. (Madrid) WTOU: **UNWTO tourism highlights**. In.; 2014.
200. Organization WT: **International Tourism Highlights, (https://www.e-unwto.org/doi/epdf/10.18111/9789284421152)**. 2019.
201. Laupland KB, Church DL, Vidakovich J, Mucenski M, Pitout JDD: **Community-onset extended-spectrum β-lactamase (ESBL) producing Escherichia coli: importance of international travel**. *Journal of Infection* 2008, **57**(6):441-448.

202. Steffen R, Hill DR, DuPont HL: **Traveler's diarrhea: a clinical review**. *JAMA* 2015, **313**(1):71-80.
203. Kean BH: **The diarrhea of travelers to mexico**. *Annals of Internal Medicine* 1963, **59**(5_Part_1):605-614.
204. Gorbach SL, Kean BH, Evans DG, Evans DJ, Bessudo D: **Travelers' diarrhea and toxigenic Escherichia coli**. *New England Journal of Medicine* 1975, **292**(18):933-936.
205. Hill DR, Beeching NJ: **Travelers' diarrhea**. *Current Opinion in Infectious Diseases* 2010, **23**(5).
206. Hagmann S, Neugebauer R, Schwartz E, Perret C, Castelli F, Barnett ED, Stauffer WM: **Illness in children after international travel: analysis from the GeoSentinel Surveillance Network**. *Pediatrics* 2010, **125**(5):e1072.
207. DuPont HL, Reves RR, Galindo E, Sullivan PS, Wood LV, Mendiola JG: **Treatment of travelers' diarrhea with trimethoprim/sulfamethoxazole and with trimethoprim alone**. *New England Journal of Medicine* 1982, **307**(14):841-844.
208. Pitzurra R, Steffen R, Tschopp A, Mutsch M: **Diarrhoea in a large prospective cohort of European travellers to resource-limited destinations**. *BMC Infectious Diseases* 2010, **10**(1):231.
209. Ruppé E, Armand-Lefèvre L, Estellat C, Consigny P-H, El Mniai A, Boussadia Y, Goujon C, Ralaimazava P, Campa P, Girard P-M *et al*: **High rate of acquisition but short duration of carriage of multidrug-resistant Enterobacteriaceae after travel to the tropics**. *Clinical Infectious Diseases* 2015, **61**(4):593-600.
210. Hassing RJ, Alsma J, Arcilla MS, van Genderen PJ, Stricker BH, Verbon A: **International travel and acquisition of multidrug-resistant Enterobacteriaceae: a systematic review**. *Eurosurveillance* 2015, **20**(47):30074.
211. Arcilla MS, van Hattem JM, Haverkate MR, Bootsma MCJ, van Genderen PJJ, Goorhuis A, Grobusch MP, Lashof AMO, Molhoek N, Schultsz C *et al*: **Import and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study**. *The Lancet Infectious Diseases* 2017, **17**(1):78-85.
212. Kuenzli E, Jaeger VK, Frei R, Neumayr A, DeCrom S, Haller S, Blum J, Widmer AF, Furrer H, Battegay M *et al*: **High colonization rates of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli in Swiss travellers to South Asia– a prospective observational multicentre cohort study looking at epidemiology, microbiology and risk factors**. *BMC Infectious Diseases* 2014, **14**(1):528.
213. Hilty M, Betsch BY, Bögli-Stuber K, Heiniger N, Stadler M, Küffer M, Kronenberg A, Rohrer C, Aebi S, Endimiani A *et al*: **Transmission dynamics of extended-spectrum β-lactamase–producing Enterobacteriaceae in the tertiary care hospital and the household setting**. *Clinical Infectious Diseases* 2012, **55**(7):967-975.
214. Vento TJ, Cole DW, Mende K, Calvano TP, Rini EA, Tully CC, Zera WC, Guymon CH, Yu X, Cheatle KA *et al*: **Multidrug-resistant gram-negative bacteria colonization of healthy US military personnel in the US and Afghanistan**. *BMC Infectious Diseases* 2013, **13**(1):68.
215. Schimpff S: **Gram-negative bacteremia.** *Support Care Cancer* 1993, **1(1):5-18. doi: 10.1007/BF00326634. PMID: 8143100.**
216. Christaki E, Giamarellos-Bourboulis EJ: **The complex pathogenesis of bacteremia: from antimicrobial clearance mechanisms to the genetic background of the host**. *Virulence* 2014, **5**(1):57-65.

217. von Dach E, Albrich WC, Brunel A-S, Prendki V, Cuvelier C, Flury D, Gayet-Ageron A, Huttner B, Kohler P, Lemmenmeier E *et al*: **Effect of C-reactive protein-guided antibiotic treatment duration, 7-day treatment, or 14-day treatment on 30-day clinical failure rate in patients with uncomplicated Gram-negative bacteremia: a randomized clinical trial**. *JAMA* 2020, **323**(21):2160-2169.
218. Bell BG, Schellevis F, Stobberingh E, Goossens H, Pringle M: **A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance**. *BMC infectious diseases* 2014, **14**:13-13.
219. Fuchs BB, Tharmalingam N, Mylonakis E: **Vulnerability of long-term care facility residents to Clostridium difficile infection due to microbiome disruptions**. *Future Microbiology* 2018, **13**(13):1537-1547.
220. Stripling J, Rodriguez M: **Current evidence in delivery and therapeutic uses of fecal microbiota transplantation in human diseases—Clostridium difficile disease and beyond**. *The American Journal of the Medical Sciences* 2018, **356**(5):424-432.
221. Eiseman B, Silen W, Bascom G, Kauvar A: **Fecal enemaas an adjunct in the treatment of pseudomembranousenterocolitis.** *Surgery* 1958, **44:854-9; PMID:13592638**.
222. Khoruts A: **Fecal microbiota transplantation-early steps on a long journey ahead**. *Gut Microbes* 2017, **8**(3):199-204.
223. Drekonja D, Reich J, Gezahegn S, Greer N, Shaukat A, MacDonald R, Rutks I, Wilt TJ: **Fecal microbiota transplantation for Clostridium difficile infection**. *Annals of Internal Medicine* 2015, **162**(9):630-638.
224. Wilson BC, Vatanen T, Cutfield WS, O'Sullivan JM: **The super-donor phenomenon in fecal microbiota transplantation**. *Frontiers in Cellular and Infection Microbiology* 2019, **9**(2).
225. Mattila E, Uusitalo–Seppälä R, Wuorela M, Lehtola L, Nurmi H, Ristikankare M, Moilanen V, Salminen K, Seppälä M, Mattila PS *et al*: **Fecal transplantation, through colonoscopy, is rffective therapy for recurrent Clostridium difficile infection**. *Gastroenterology* 2012, **142**(3):490-496.
226. Tian H, Ge X, Nie Y, Yang L, Ding C, McFarland LV, Zhang X, Chen Q, Gong J, Li N: **Fecal microbiota transplantation in patients with slow-transit constipation: A randomized, clinical trial**. *PLOS ONE* 2017, **12**(2):e0171308.
227. Lee CH, Belanger JE, Kassam Z, Smieja M, Higgins D, Broukhanski G, Kim PT: **The outcome and long-term follow-up of 94 patients with recurrent and refractory Clostridium difficile infection using single to multiple fecal microbiota transplantation via retention enema**. *European Journal of Clinical Microbiology & Infectious Diseases* 2014, **33**(8):1425-1428.
228. Youngster I, Russell GH, Pindar C, Ziv-Baran T, Sauk J, Hohmann EL: **Oral, capsulized, frozen fecal microbiota transplantation for relapsing Clostridium difficile infection**. *JAMA* 2014, **312**(17):1772-1778.
229. Wang S, Xu M, Wang W, Cao X, Piao M, Khan S, Yan F, Cao H, Wang B: **Systematic review: adverse events of fecal microbiota transplantation**. *PLOS ONE* 2016, **11**(8):e0161174.
230. Taur Y, Coyte K, Schluter J, Robilotti E, Figueroa C, Gjonbalaj M, Littmann ER, Ling L, Miller L, Gyaltshen Y *et al*: **Reconstitution of the gut microbiota of antibiotic-treated patients by autologous fecal microbiota transplant**. *Sci Transl Med* 2018, **10**(460):eaap9489.

231. Ianiro G, Rossi E, Thomas AM, Schinzari G, Masucci L, Quaranta G, Settanni CR, Lopetuso LR, Armanini F, Blanco-Miguez A *et al*: **Faecal microbiota transplantation for the treatment of diarrhoea induced by tyrosine-kinase inhibitors in patients with metastatic renal cell carcinoma**. *Nature Communications* 2020, **11**(1):4333.
232. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, Sokol H, Arkkila P, Pintus C, Hart A *et al*: **European consensus conference on faecal microbiota transplantation in clinical practice**. *Gut* 2017, **66**(4):569-580.
233. Vermeire S, Joossens M, Verbeke K, Wang J, Machiels K, Sabino J, Ferrante M, Van Assche G, Rutgeerts P, Raes J: **Donor species richness determines faecal microbiota transplantation success in inflammatory bowel disease**. *Journal of Crohn's and Colitis* 2016, **10**(4):387-394.
234. Kump P, Wurm P, Gröchenig HP, Wenzl H, Petritsch W, Halwachs B, Wagner M, Stadlbauer V, Eherer A, Hoffmann KM *et al*: **The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis**. *Aliment Pharmacol Ther* 2018, **47**(1):67-77.
235. Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, Brown EM, Schroeter K, Allen-Vercoe E: **Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut**. *Microbiome* 2013, **1**(1):3.
236. **Resistance in Gram-negative organisms: studying intervention strategies, https://cordis.europa.eu/project/id/282512/reporting**. In.
237. Huttner BD, de Lastours V, Wassenberg M, Maharshak N, Mauris A, Galperine T, Zanichelli V, Kapel N, Bellanger A, Olearo F *et al*: **A 5-day course of oral antibiotics followed by faecal transplantation to eradicate carriage of multidrug-resistant Enterobacteriaceae: a randomized clinical trial**. *Clinical Microbiology and Infection* 2019, **25**(7):830-838.
238. Huttner B, Haustein T, Uçkay I, Renzi G, Stewardson A, Schaerrer D, Agostinho A, Andremont A, Schrenzel J, Pittet D *et al*: **Decolonization of intestinal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae with oral colistin and neomycin: a randomized, double-blind, placebo-controlled trial**. *Journal of Antimicrobial Chemotherapy* 2013, **68**(10):2375-2382.
239. Caballero S, Carter R, Ke X, Sušac B, Leiner IM, Kim GJ, Miller L, Ling L, Manova K, Pamer EG: **Distinct but spatially overlapping intestinal niches for vancomycin-resistant Enterococcus faecium and carbapenem-resistant Klebsiella pneumoniae**. *PLOS Pathogens* 2015, **11**(9):e1005132.
240. Crum-Cianflone NF, Sullivan E, Ballon-Landa G: **Fecal microbiota transplantation and successful resolution of multidrug-resistant-organism colonization**. *Journal of clinical microbiology* 2015, **53**(6):1986-1989.
241. Biliński J, Grzesiowski P, Muszyński J, Wróblewska M, Mądry K, Robak K, Dzieciątkowski T, Wiktor-Jedrzejczak W, Basak GW: **Fecal microbiota transplantation inhibits multidrug-resistant gut pathogens: preliminary report performed in an immunocompromised host**. *Arch Immunol Ther Exp (Warsz)* 2016, **64**(3):255-258.
242. Keith JW, Pamer EG: **Enlisting commensal microbes to resist antibiotic-resistant pathogens**. *J Exp Med* 2019, **216**(1):10-19.
243. Elvers KT, Wilson VJ, Hammond A, Duncan L, Huntley AL, Hay AD, van der Werf ET: **Antibiotic-induced changes in the human gut microbiota for the most commonly**

prescribed antibiotics in primary care in the UK: a systematic review. *BMJ Open* 2020, **10**(9):e035677.
244. Gosalbes MJ, Vázquez-Castellanos JF, Angebault C, Woerther P-L, Ruppé E, Ferrús ML, Latorre A, Andremont A, Moya A: **Carriage of Enterobacteria producing extended-spectrum β-lactamases and composition of the gut microbiota in an Amerindian community**. *Antimicrobial Agents and Chemotherapy* 2016, **60**(1):507.
245. Kampmann C, Dicksved J, Engstrand L, Rautelin H: **Composition of human faecal microbiota in resistance to Campylobacter infection**. *Clinical Microbiology and Infection* 2016, **22**(1):61.e61-61.e68.
246. Walters WA, Reyes F, Soto GM, Reynolds ND, Fraser JA, Aviles R, Tribble DR, Irvin AP, Kelley-Loughnane N, Gutierrez RL *et al*: **Epidemiology and associated microbiota changes in deployed military personnel at high risk of traveler's diarrhea**. *PLOS ONE* 2020, **15**(8):e0236703.
247. Iljazovic A, Roy U, Gálvez EJC, Lesker TR, Zhao B, Gronow A, Amend L, Will SE, Hofmann JD, Pils MC *et al*: **Perturbation of the gut microbiome by Prevotella spp. enhances host susceptibility to mucosal inflammation**. *Mucosal Immunology* 2021, **14**(1):113-124.
248. De Filippis F, Pasolli E, Tett A, Tarallo S, Naccarati A, De Angelis M, Neviani E, Cocolin L, Gobbetti M, Segata N *et al*: **Distinct genetic and functional traits of human intestinal Prevotella copri strains are associated with different habitual diets**. *Cell Host & Microbe* 2019, **25**(3):444-453.e443.
249. Pasolli E, Schiffer L, Manghi P, Renson A, Obenchain V, Truong DT, Beghini F, Malik F, Ramos M, Dowd JB *et al*: **Accessible, curated metagenomic data through ExperimentHub**. *Nature Methods* 2017, **14**(11):1023-1024.
250. Tett A, Huang KD, Asnicar F, Fehlner-Peach H, Pasolli E, Karcher N, Armanini F, Manghi P, Bonham K, Zolfo M *et al*: **The Prevotella copri complex comprises four distinct clades underrepresented in Westernized populations**. *Cell Host & Microbe* 2019, **26**(5):666-679.e667.
251. Pop M, Paulson JN, Chakraborty S, Astrovskaya I, Lindsay BR, Li S, Bravo HC, Harro C, Parkhill J, Walker AW *et al*: **Individual-specific changes in the human gut microbiota after challenge with enterotoxigenic Escherichia coli and subsequent ciprofloxacin treatment**. *BMC Genomics* 2016, **17**(1):440.
252. Youmans BP, Ajami NJ, Jiang Z-D, Campbell F, Wadsworth WD, Petrosino JF, DuPont HL, Highlander SK: **Characterization of the human gut microbiome during travelers' diarrhea**. *Gut Microbes* 2015, **6**(2):110-119.
253. Casals-Pascual C, Vergara A, Vila J: **Intestinal microbiota and antibiotic resistance: perspectives and solutions**. *Human Microbiome Journal* 2018, **9**:11-15.
254. Walker C, Preshaw PM, Novak J, Hefti AF, Bradshaw M, Powala C: **Long-term treatment with sub-antimicrobial dose doxycycline has no antibacterial effect on intestinal flora**. *Journal of Clinical Periodontology* 2005, **32**(11):1163-1169.
255. Rashid M-U, Panagiotidis G, Bäckström T, Weintraub A, Nord CE: **Ecological impact of doxycycline at low dose on normal oropharyngeal and intestinal microflora**. *International Journal of Antimicrobial Agents* 2013, **41**(4):352-357.
256. Heimdahl A, Nord C: **Influence of doxycycline on the normal human flora and colonization of the oral cavity and colon.** *Scand J Infect Dis* 1983, **15(3):293-302. doi: 10.3109/inf.1983.15.issue-3.10. PMID: 6648373.**

257. Mättö J, Alakomi H-L, Saarela M, Suihko M-L, Maukonen J: **Influence of doxycycline therapy on the diversity and antibiotic susceptibility of human intestinal bifidobacterial population**. In: *American Society for Microbiology 107th General Meeting, Toronto, Canada, 21 - 25 May 2007*. edn.; 2007.
258. (SPILF) Sdpidlf: **Health recommendations for travellers (for health professionals) - https://afpa.org/content/uploads/2019/05/HCSP-Recommandations-sanitaires-voyageurs-2019.pdf**. In.; 2019.
259. Li SS, Zhu A, Benes V, Costea PI, Hercog R, Hildebrand F, Huerta-Cepas J, Nieuwdorp M, Salojärvi J, Voigt AY *et al*: **Durable coexistence of donor and recipient strains after fecal microbiota transplantation**. *Science* 2016, **352**(6285):586.
260. Bar-Yoseph H, Carasso S, Shklar S, Korytny A, Even Dar R, Daoud H, Nassar R, Maharshak N, Hussein K, Geffen Y *et al*: **Oral capsulized fecal microbiota transplantation for eradication of carbapenemase-producing Enterobacteriaceae colonization with a metagenomic perspective**. *Clinical Infectious Diseases* 2020.
261. Mizuno S, Masaoka T, Naganuma M, Kishimoto T, Kitazawa M, Kurokawa S, Nakashima M, Takeshita K, Suda W, Mimura M *et al*: **Bifidobacterium-rich fecal donor may be a positive predictor for successful fecal microbiota transplantation in patients with irritable bowel syndrome**. *Digestion* 2017, **96**(1):29-38.
262. Jouhten H, Ronkainen A, Aakko J, Salminen S, Mattila E, Arkkila P, Satokari R: **Cultivation and genomics prove long-term colonization of donor's Bifidobacteria in recurrent Clostridioides difficile patients treated with fecal microbiota transplantation**. *Frontiers in Microbiology* 2020, **11**(1663).
263. Sultana R, McBain AJ, O'Neill CA: **Strain-dependent augmentation of tight-junction barrier function in human primary epidermal keratinocytes by Lactobacillus and Bifidobacterium lysates**. *Applied and environmental microbiology* 2013, **79**(16):4887-4894.
264. Bai T, Zhang L, Wang H, Qian W, Song J, Hou X: **Fecal microbiota transplantation is effective in relieving visceral hypersensitivity in a postinfectious model**. *BioMed Research International* 2018, **2018**:3860743.
265. Hudson LE, Anderson SE, Corbett AH, Lamb TJ: **Gleaning insights from fecal microbiota transplantation and probiotic studies for the rational design of combination microbial therapies**. *Clinical Microbiology Reviews* 2017, **30**(1):191.
266. Allen SJ, Wareham K, Wang D, Bradley C, Hutchings H, Harris W, Dhar A, Brown H, Foden A, Gravenor MB *et al*: **Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial**. *The Lancet* 2013, **382**(9900):1249-1257.
267. Schubert AM, Rogers MAM, Ring C, Mogle J, Petrosino JP, Young VB, Aronoff DM, Schloss PD: **Microbiome data distinguish patients with Clostridium difficile infection and non-C. difficile-associated diarrhea from healthy controls**. *mBio* 2014, **5**(3):e01021-01014.
268. Min S, Kim S, Cho S-W: **Gastrointestinal tract modeling using organoids engineered with cellular and microbiota niches**. *Experimental & Molecular Medicine* 2020, **52**(2):227-237.

Appendix

Supplementary Materials of Chapter 2 of Results section.

Effects of antibiotic duration on the intestinal microbiota and resistome: the PIRATE RESISTANCE project, a cohort study nested within a randomized trial

Stefano Leo, Vladimir Lazarevic, Elodie von Dach, Laurent Kaiser, Virginie Prendki, Jacques Schrenzel, Benedikt D. Huttner, Angela Huttner

Supplementary Materials

Supplementary Results

Resistome changes in baseline population

We extended the analyses to 16 patients excluded from the per-protocol group (baseline population; Figure S1; Table S2). Since D30 faecal samples were missing or otherwise ineligible for per-protocol analyses in this group, we compared their D7 and D14 resistomes: there were no clear trends of differences between short versus long treatment (Figure S8).

Supplementary Methods

DNA extraction, library preparation and whole-metagenome shotgun sequencing

DNA was extracted from about 150 mg of stool (95–300 mg) using Quick-DNA faecal/Soil Microbe Miniprep Kit (Zymo) and eluted in 55 µL of water. Bacterial and human DNA were quantified by qPCR and stored at –20°C. The concentration of human DNA was determined by qPCR experiments targeting human β-actin genes on a Bio-Rad CFX96 qPCR system. The assay was performed in 20 µL of ABsolute qPCR Mix (Thermo Scientific) containing 300 nM each forward and reverse β-actin primers, 200 nM β-actin probe and 1 µL of non-diluted DNA extract. The amplification parameters were as follows: 95 °C/15 min, followed by 95 °C/15 s and 60 °C/60 s for 42 cycles. Human genomic DNA used at known concentrations to generate the reference curve and β-actin primers and probe were from the Applied Biosystems TaqMan β-Actin Control Reagents kit (Thermo Fisher Scientific).

The concentration of bacterial DNA was determined using primers targeting the V3 region of bacterial 16S rRNA genes (*Escherichia coli* positions 338–534) on an Mx3005P qPCR system (Agilent) as previously described (1). One microliter of the 1/10 diluted DNA extract was added to the qPCR reaction mix. The cycling conditions included an initial step of 10 min at 95 °C followed by 40 cycles of 95 °C for 5 s and 68 °C for 20 s. All reactions were carried out in duplicate and the reference curves for DNA absolute quantitation were obtained using known concentrations of genomic DNA of *E. coli* strain DH5α.

Four no-sample controls were performed by extracting DNA using the same extraction procedure but omitting the addition of stool sample. After DNA fragmentation by sonication, the sequencing library was constructed using TruSeq Nano DNA sample preparation kit (Illumina) under conditions aimed at producing the insert size of 250 bp. Library was sequenced (2 x 150) on a NovaSeq 6000 System (Illumina) at Fasteris (Plan-les-Ouates, Switzerland).

Quality filtering, dereplication and taxonomic assignment

The quality of fastq files was inspected with FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were scanned with Trimmomatic v0.36 (2) with a sliding window of 10 nt. Reads were trimmed when the average quality within the window fell below a Phred score of 30 and filtered out if their length before or after trimming step was <120 nt. Quality filtered reads were dereplicated with in-house Perl script. Reads which were classified by CLARK v1.2.5 (3) to the species *Homo sapiens* using a confidence score of 0.5, were removed. For this step, the human genome reference assembly vGRCh38.p7 was used. Dereplicated reads classified as non-human were then assigned by CLARK to bacterial, viral and fungal species when a confidence score of 0.8 was obtained. Eventually reads that remained unclassified, were analysed with CLARK and assigned to phage genomes with a confidence score of 0.8. The relative abundance of bacterial species was expressed as percentage of total number of reads mapping to bacteria. The abundance of phages was, instead, computed by dividing the total number of reads mapping to phages with the total number of dereplicated non-human reads; such ratio was then multiplied by one million (counts per million; CPM).

Filtering of bacterial species

We filtered out species for which the ratio between mean relative abundance in negative extraction controls and mean relative abundance in test samples was superior to 1. We retained 3,020 of 5,373 (56%) bacterial species for further analyses. On average, 5.6 million read pairs per sample were mapped to those selected species.

Identification of genes encoding antibiotic resistance

Dereplicated and quality-filtered forward non-human reads were mapped against Resfinder database (4) by USEARCH v11.0.667 (5) using the following settings: -id 0.9 -strand both -top_hits_only -mincols 100 -maxaccepts 20.

Generally, one read was associated with one best hit (single best hit). In case one read mapped to more than one best hit (multiple best hits), we chose the hit that was most abundant in the sample. When two or more best hits had the same abundance for a given read, their names were concatenated and considered as a new hit. Best hits were labelled with the acronym combining the gene name(s) and corresponding accession number(s). Roughly 5,800 forward reads per sample were classified to 904 different ARG hits. Importantly, we used forward reads for two reasons: first the sequencing quality of forward reads was better than reverse reads thereby allowing a

more reliable detection of ARGs; second, mapping both forward and reverse reads might lead to spurious hits. For 456/904 [50.4%], identified ARGs resulted from combining equally abundant multiple best hits.

In the present study, the ARG class takes the name of the antibiotic class against which a given gene encodes resistance. We assigned each hit to the corresponding ARG class by querying its label against the table reported in the ResFinder file "phenotype.txt". When the hit was not assigned to any class in "phenotype.txt", we extracted the gene's full acronym from the hit label and queried it against the table reported in the ResFinder "notes.txt" file. If thereafter no association was identified, antibiotic class was manually assigned. Importantly, best hits of the same read could be assigned to more than one ARG class. When this happened, the classes' names were combined and treated as a new class.

The abundance of ARG and classes was normalized (per sample) to bacteria-assigned reads: read counts assigned to an ARG or to an ARG class in a given sample were divided by the total number of reads classified to bacteria. The ratio was then multiplied by one million (counts per million; CPM).

To gain a temporal resolution of resistome changes, we analysed the relative abundance of ARGs encoding resistance against five of the antibiotic classes used in the study by treatment duration and distance from last treatment day. For each antibiotic class, we considered only patients receiving antibiotics of that class.

Databases

The two databases used to classify reads to bacteria, archaea, viruses and fungi, and to phages, respectively, were downloaded from NCBI on 13 December 2019. The first contained 5,550 bacterial, 257 archaeal, 1,000 viral and 288 fungal genomic sequences, the second 2,450 and 23 genomes belonging to the orders of Caudovirales and Ligamenvirales, respectively. The ResFinder database was downloaded on 7 January 2020.

Statistical analyses

Only complete data on the variables accounted in a given analyses were considered. To analyse global differences between bacterial communities, we performed principal coordinates analysis (PCoA) in R v3.2.3 using the vegan v2.3-5 package (6). Bray-Curtis dissimilarity was computed on square-rooted species relative abundance with vegdist (vegan). Samples coordinates were computed with betadisper function (vegan). Shannon diversity was computed with diversity R function (vegan). The formula used for the diversity index computation is $H = \sum_{i=1}^{n} p_i \ln p_i$ *w*here p_i represents the relative abundance of the i^{th} species (the number of

reads mapping to the i^{th} species, divided by the total number of reads). For species richness we considered the number of species with read counts > 0 in a given sample. Counts of reads classified to bacterial species were rarefied to 500,000 with rarefy function (vegan) prior to computation of Shannon and richness indices.

To investigate differences in microbiota composition between groups of patients, we applied permutational analysis of variance (pairwise PERMANOVA, with 9,999 permutations, unrestricted permutation of raw data and Type III sums of squares) based on the Bray-Curtis similarity matrices of square-root-transformed species' relative abundance. PERMANOVA was performed in PRIMER v7 (PRIMER-E Ltd, Plymouth, UK).

We defined differentially abundant species if Wilcoxon signed-rank and/or Wilcoxon rank-sum tests were associated with a p value <.05. Data visualization was performed in R with ggplot2 v3.3.2 package.

Supplementary Figures

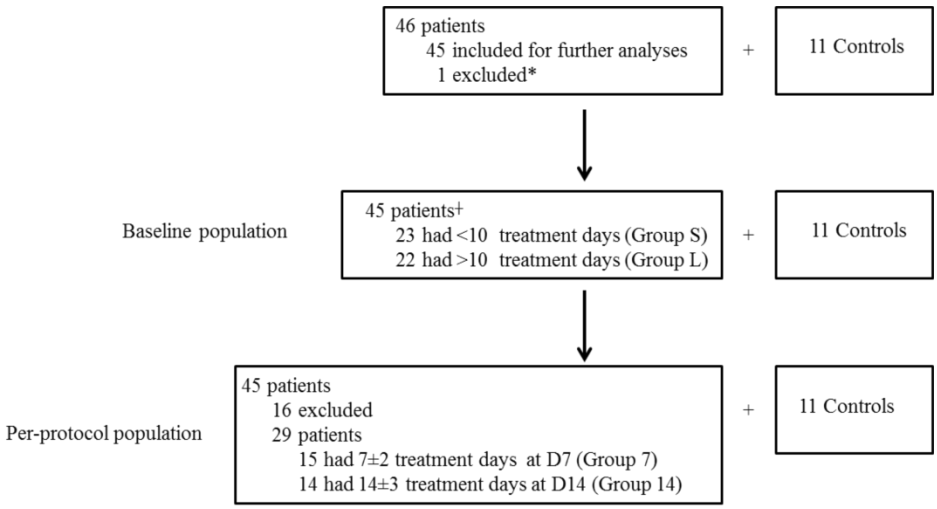

Figure S1. Patient cohort. Flow chart depicting the selection of patients for the analyses. *Patient 159 was excluded because of the 22 days of pre-treatment with vancomycin. ┼Time points D30 and/or D90 of patients 183 and 197 were not included as they were treated later than D14. The 'per-protocol' population included case patients with the following criteria: (1) ≥2 faecal samples, one being a baseline sample (D7 or D14) and the other a D30 sample (primary outcome); (2) no further receipt of antibiotics after D14, and (3) treatment duration of either 7 (±2) or 14 (±3) days.

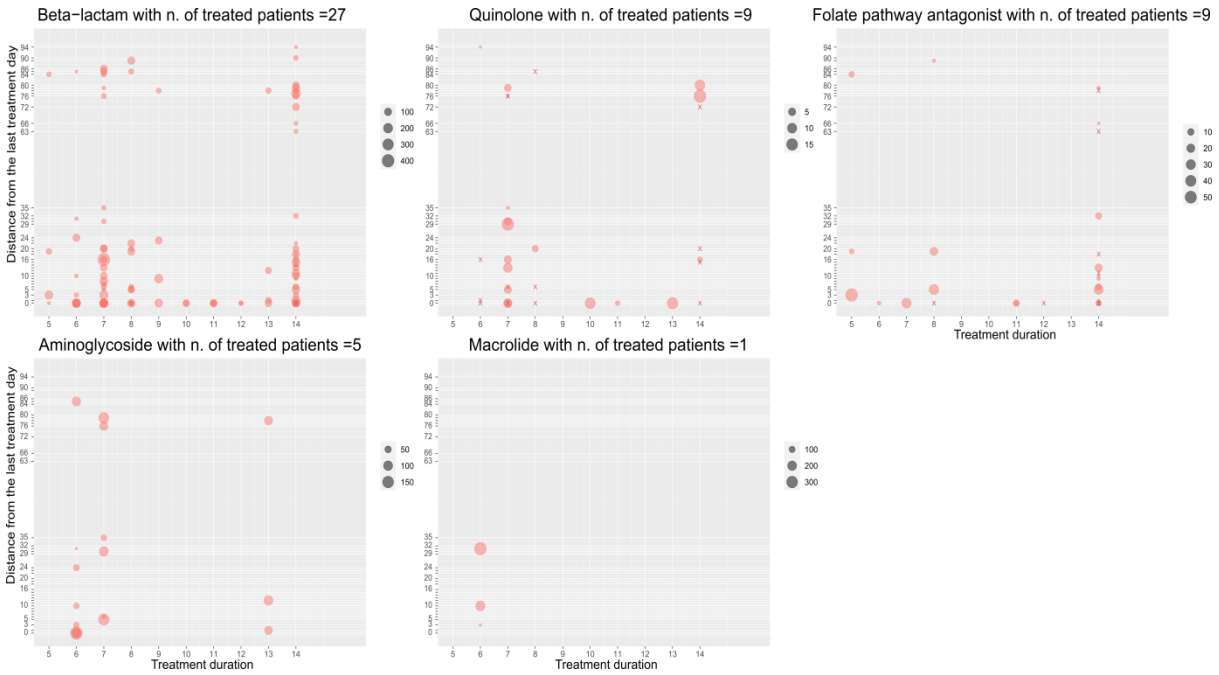

Figure S2. Temporal changes of the resistome. Bubble plots reporting the distance from the last antibiotic treatment day versus the treatment duration. The size of dots corresponds to the read counts of genes encoding resistance for a given antibiotic class per million of bacterial reads. For each plot, only samples from patients receiving antibiotics from a given class were reported. When an X is represented, it means that no counts were found for that patient.

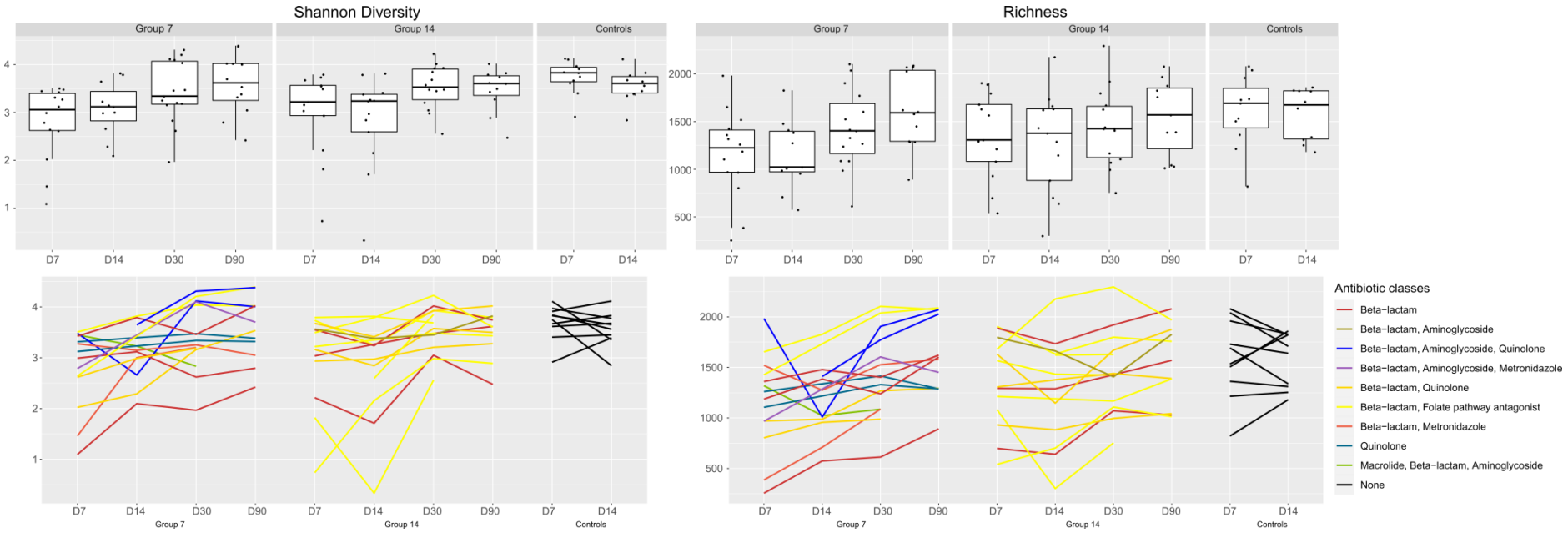

Figure S3. Ecological indices. Box plots (top row) and line plots (bottom row) report the dynamics of changes for ecological indexes. Line plots represent the changing observed in each patient and are coloured according to the antibiotic class used in the treatment. See also Table S4.

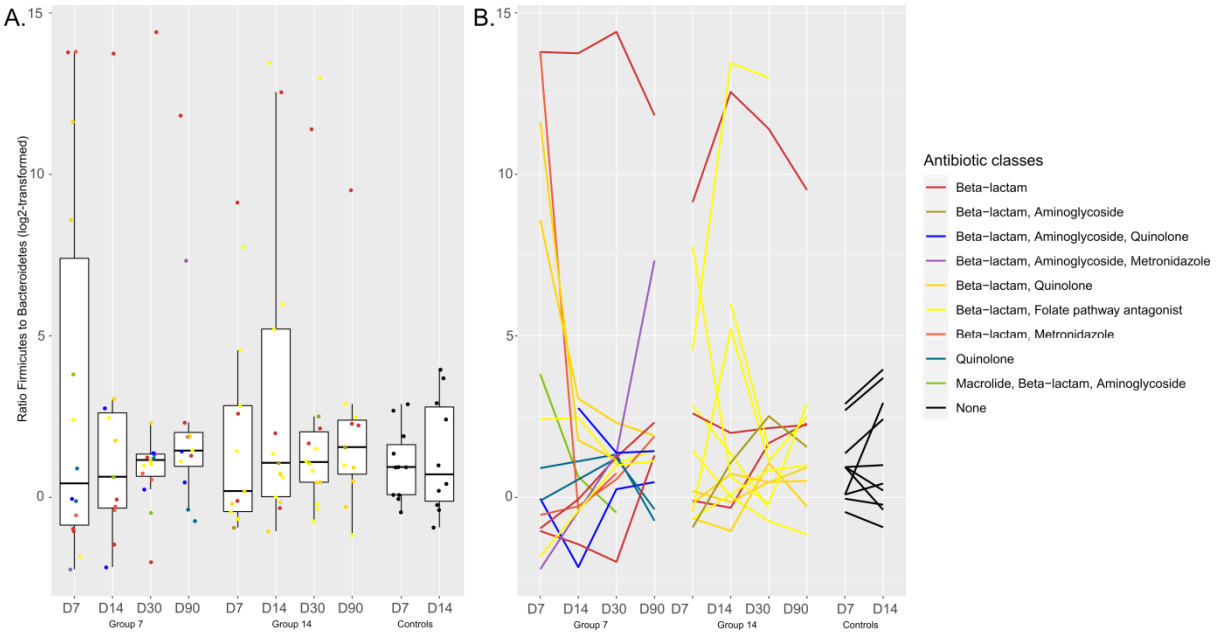

Figure S4. Ratio of Firmicutes to Bacteroidetes. A. Boxplots combined with dot plots showing the temporal variation of the ratio in groups 7, 14 and in controls. Case patients showed a dispersion of ratio values, represented by box plot IQR1-3 and whiskers, at D7 and D14 than at later time points. B. Line plots representing the temporal changes of the ratio for each patient. Lines are coloured according to the classes of the antibiotics used in the treatments. The ratio was log2 transformed before plotting.

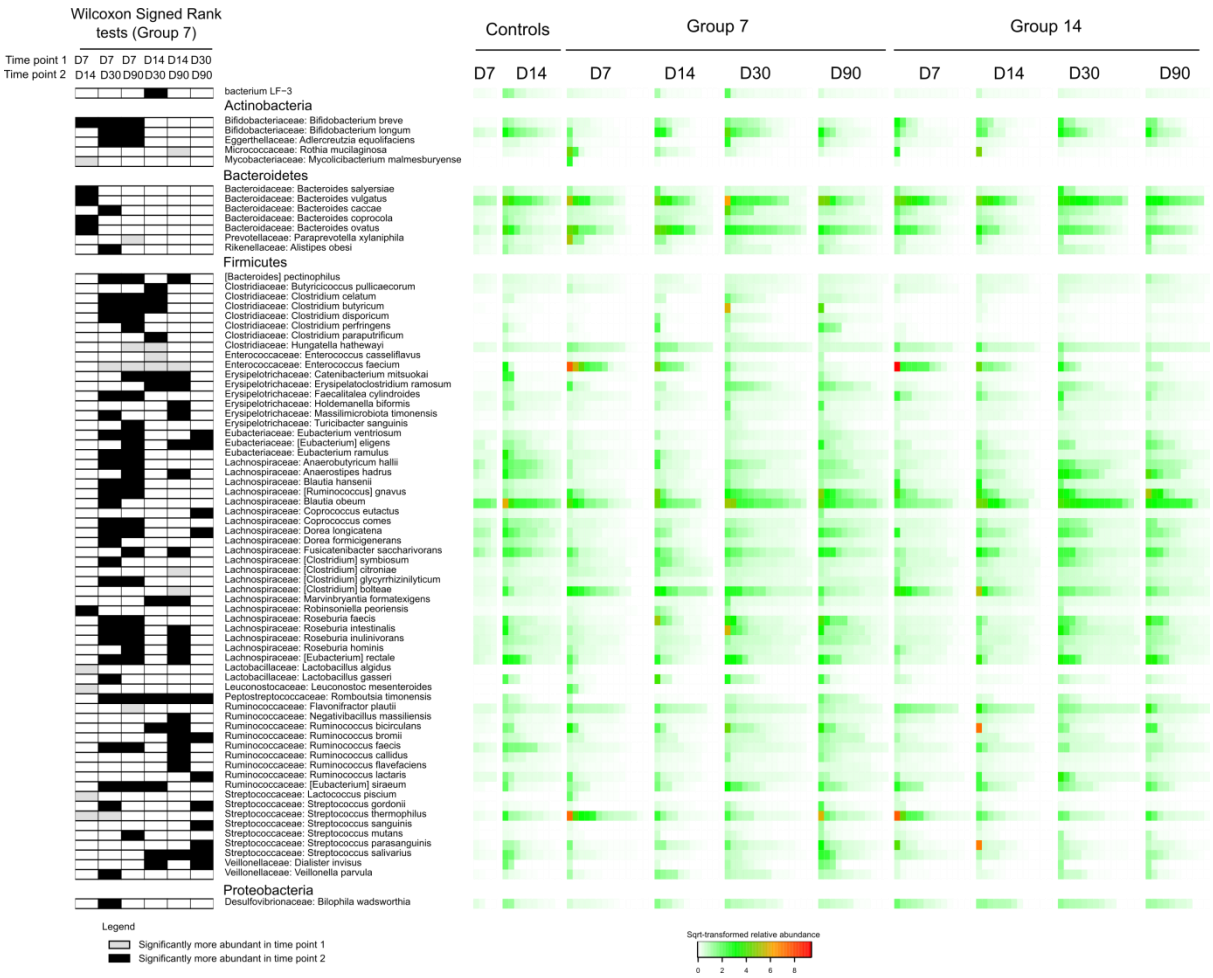

Figure S5. Differentially abundant species at different time points, 7-day group. For each of the represented species, we report the significance of differences in the relative abundance between time points (heat map on the left), the relative abundance in the 7- and 14-day groups (heat map on the right). We reported species with differences in relative abundance between time points associated with a significant Wilcoxon signed rank test ($p < .05$), a fold change ≥ 2 in the relative abundance and a mean relative abundance $\geq 0.1\%$ in at least one of the compared groups. Heat map on the left: coloured cells report significant differences ($p < .05$) associated with a ≥ 2 fold change in the relative abundance whereas white cells correspond to other cases. Heat map on the right: square-root-transformed relative abundance is colour-scaled as indicated in the legend.

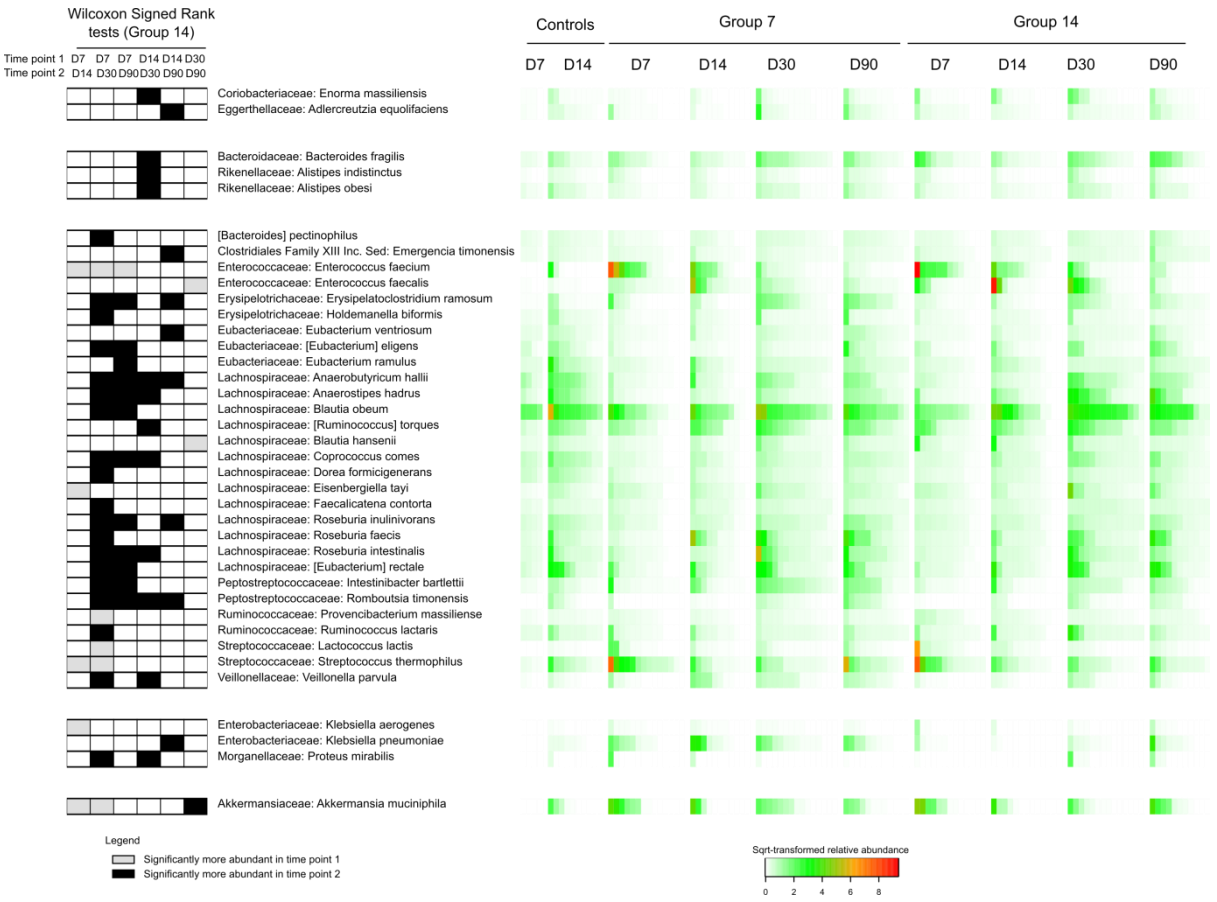

Figure S6. Differentially abundant species at different time points, 14-day group. Same as Figure S5 except that data are reported for the 14-day group.

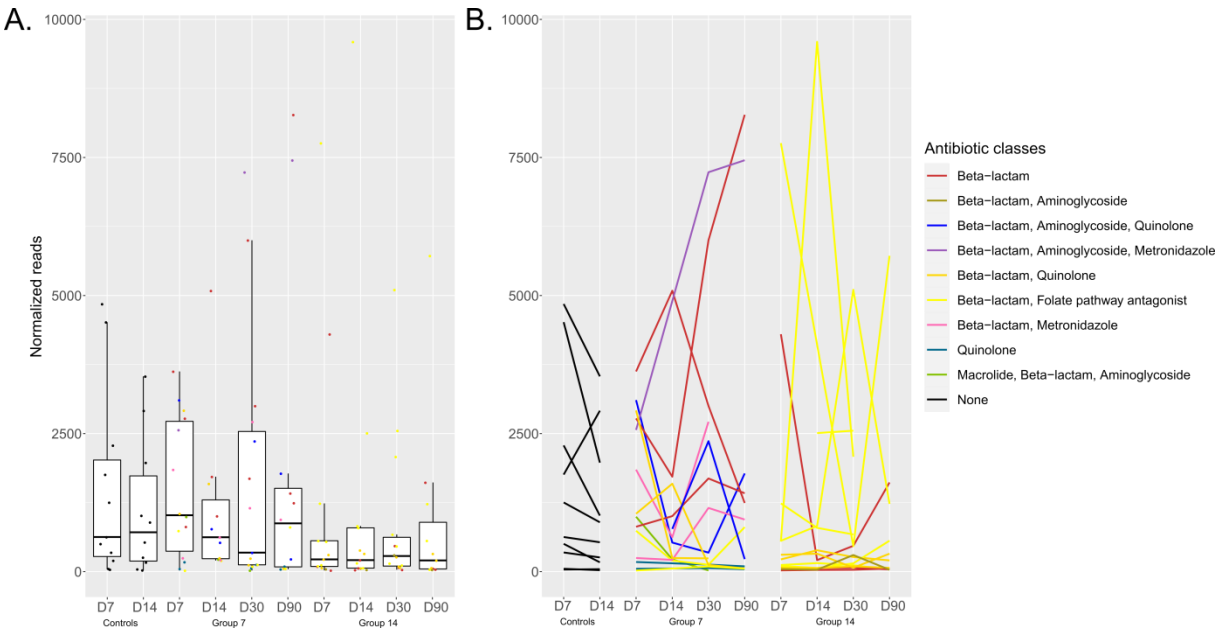

Figure S7. Relative abundance of detected phages. A. Boxplots reporting the normalized reads mapping to phages for each time point of each patient group. Boxplots are combined with dots representing singular values from each sample. Dots are coloured by antibiotic class received. B. Line plots represent the changes observed in

each patient and are coloured as panel A. Reads mapping to phages were divided by non-human dereplicated reads and the ratio was multiplied by one million (counts per million; CPM).

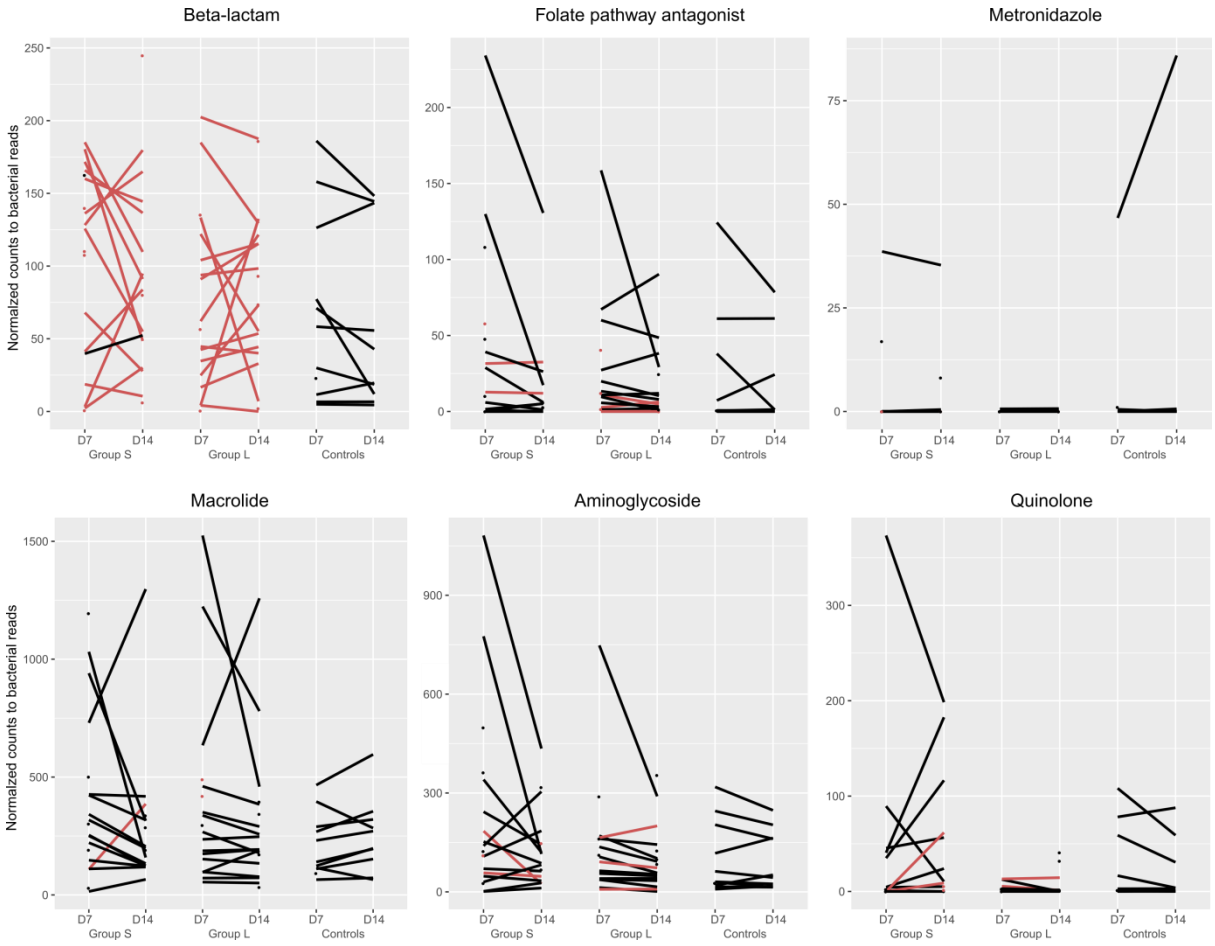

Figure S8. Resistome changes in patients from the baseline population dataset. Reads mapping to genes giving resistance to a given class of antibiotics were summed up and normalized to the number of reads mapping to bacteria (counts per million; CPM). Normalized counts are plotted versus time points D7 and D14 of patients from group S (≤10 days of antibiotic therapy), group L (>10 day of antibiotic therapy) and controls. For each class of antibiotics, red lines represent changes for patients that were administered that class, whereas black ones do for those patients that were not. No genes giving resistance to metronidazole were detected in patients administered with this antibiotic.

Supplementary Tables

Table S1. Characteristics of the study cohort. "n"= total number of patients.

Characteristic		**Full cohort** (n=46)	**Baseline population** (n=45)	**Per-protocol population** (n=29)	**Controls** (n=11)
Age	Median	83	83	83	73
	Range	39 - 96	39 - 96	39 - 94	36 - 86
Charlson Comorbidity Index*	Median	4	4	4	6
	Range	0 - 7	0 - 7	0 - 7	2-11
Gender	Female (%)	32 (69.6)	31 (68.9)	20 (69)	5 (45.5)
	Male (%)	14 (30.4)	14 (31.1)	9 (31)	6 (54.5)
Diseases	Diabetes mellitus (%)	10 (22)	9 (20)	3 (10)	5 (45)
	Congestive heart disease (%)	8 (17)	8 (18)	5 (17)	2 (18)
	Kidney disease (%)	7 (15)	6 (13)	6 (21)	3 (27)
	Liver disease (%)	1 (2)	1 (2)	0 (0)	3 (27)
	Chronic pulmonary disease (%)	1 (2)	1 (2)	1 (3)	2 (18)
	Connective-tissue disease (%)	1 (2)	1 (2)	0 (0)	1 (9)
	Cancer (%)	0 (0)	0 (0)	0 (0)	4 (36)
	Cerebrovascular disease (%)	0 (0)	0 (0)	0 (0)	4 (36)
	Metastatic solid tumor (%)	0 (0)	0 (0)	0 (0)	2 (18)
	End-organ damage due to DM (%)	0 (0)	0 (0)	0 (0)	2 (18)
	Myocardial infarction (%)	0 (0)	0 (0)	0 (0)	2 (18)
	Alcoholism (%)	0 (0)	0 (0)	0 (0)	2 (18)
	Leukemia (%)	0 (0)	0 (0)	0 (0)	1 (9)
	Hemiplegia (%)	0 (0)	0 (0)	0 (0)	1 (9)
	Ulcer disease (%)	0 (0)	0 (0)	0 (0)	1 (9)
	Dementia (%)	0 (0)	0 (0)	0 (0)	1 (9)

*****The index was computed with the online tool available at: https://www.mdcalc.com/charlson-comorbidity-index-cci

Table S2. Summary table reporting the time points samples for each patient, patient's assignment to a given group and the antibiotic therapy. X = faeces sampled; red cells= faecal sample excluded from the analyses; grey cells = faecal sample not collected. AMK: amikacin; GEN: gentamicin; AMX: amoxicillin; CRO: ceftriaxone; CXM: cefuroxime; FEP: cefepime; IPM: imipenem; PIP: piperacillin; CIP: ciprofloxacin; LVX: levofloxacin; SXT: sulfamethoxazole (in combination with trimethoprim); CLR: clarithromycin; MTZ: metronidazole. Patient 159 was excluded because of the 22 days of pre-treatment with vancomycin. Time points D30 and/or D90 of patients 183 and 197 were not included as they were treated later than D14.

	Per-protocol population					**Baseline population**				
	Group 7 vs **Group 14**					**Group S (<10)** vs **Group L (10+)**				
Patient ID	D7	D14	D30	D90		D7	D14	D30	D90	Antibiotic therapy
158	X	X	X			X	X	X		CLR, AMX, AMK
159	X	X	X	X		X	X	X	X	AMX, GEN, PIP, CIP
160	X	X	X	X		X	X	X	X	CRO, SXT
173	X	X	X	X		X	X	X	X	CRO, AMX
179	X	X	X			X	X	X		PIP, CRO, SXT
181	X	X				X	X			CRO, AMX
182	X		X	X		X		X	X	CIP
183	X	X	X			X	X	X		CRO, MTZ, CXM
184	X	X	X			X	X	X		CRO, SXT
185	X	X	X	X		X	X	X	X	CRO, SXT
188	X	X	X	X		X	X	X	X	CRO, AMX
189	X	X	X	X		X	X	X	X	AMX, GEN, CRO, CIP
190	X	X	X	X		X	X	X	X	PIP, IPM
191	X					X				CRO, CLR, SXT
195	X	X	X	X		X	X	X	X	CRO, AMX

	Per-protocol population					Baseline population				
	Group 7 vs Group 14					Group S (<10) vs Group L (10+)				
Patient ID	D7	D14	D30	D90		D7	D14	D30	D90	Antibiotic therapy
197		X	X	X			X	X	X	CRO, MTZ, IPM, AMX
198	X	X	X			X	X	X		CRO, MTZ, IPM
199	X	X				X	X			IPM, AMK, FEP, SXT
200	X					X				IPM, CRO
201	X	X				X	X			LVX, CRO
203	X	X				X	X			CRO, AMX, CIP
204	X	X				X	X			AMX, CRO, CIP
206	X	X	X	X		X	X	X	X	CRO, CIP
207		X	X				X	X		CRO, SXT
208	X	X	X	X		X	X	X	X	CRO, MTZ
210	X	X	X	X		X	X	X	X	CRO, CIP
211	X	X	X	X		X	X	X	X	PIP, AMX, SXT
213	X	X	X	X		X	X	X	X	CRO, CIP
215	X		X	X		X		X	X	LVX, CIP
217	X	X	X	X		X	X	X	X	PIP, CXM
218	X		X	X		X		X	X	CRO, SXT
219	X	X	X	X		X	X	X	X	AMX, CRO
221	X		X	X		X		X	X	CRO, AMK, MTZ, AMX
222	X					X				AMX, CLR, SXT

	Per-protocol population					Baseline population				
	Group 7 vs Group 14					Group S (<10) vs Group L (10+)				
Patient ID	D7	D14	D30	D90		D7	D14	D30	D90	Antibiotic therapy
226	X	X	X			X	X	X		CRO, CIP
227	X		X	X		X		X	X	CRO, SXT
228	X	X	X	X		X	X	X	X	CRO, CIP, IPM
229		X	X	X			X	X	X	AMX, GEN, PIP, CIP
230		X					X			CRO, PIP
231	X	X				X	X			CRO, AMK
232	X	X				X	X			AMX, PIP
233	X					X				CRO, MTZ
234	X	X	X	X		X	X	X	X	AMX, CRO, GEN
237	X	X	X	X		X	X	X	X	CRO, PIP, SXT
240	X	X				X	X			CIP
241	X	X				X	X			AMX, CRO, SXT

Table S3. *Pairwise PERMANOVA tests between time points of control and of treated patients from group 7 or 14.*

Control time point	Patient time point	Patient group	t	p
D7	D7	Group 7	1.769	**0.0012**
D7	D14	Group 7	1.552	**0.0024**
D7	D30	Group 7	1.342	**0.0223**
D7	D90	Group 7	1.175	0.109
D7	D7	Group 14	1.642	**0.0011**
D7	D14	Group 14	1.386	**0.0162**
D7	D30	Group 14	1.275	**0.0469**
D7	D90	Group 14	1.144	0.1616
D14	D7	Group 7	1.596	**0.0127**
D14	D14	Group 7	1.411	**0.0102**
D14	D30	Group 7	1.237	0.0533
D14	D90	Group 7	1.1	0.2081
D14	D7	Group 14	1.451	**0.015**
D14	D14	Group 14	1.217	0.0903
D14	D30	Group 14	1.107	0.1858
D14	D90	Group 14	1.031	0.3349

Table S4. Summary table of Shannon diversity and richness. Median values of ecological indices are reported for each time point of each patient group. Statistics (W and p values) obtained with Wilcoxon Rank Sum is showed for the each pair of patient groups reported in a given row.

Shannon diversity					
				Statistics	
Group 1	**Median**	**Group 2**	**Median**	**W**	**p**
D7 group 7	3.06	D7 group 14	3.22	65	0.2199
D7 group 7	3.06	D7 Controls	3.83	12	**0.0001**
D14 group 7	3.12	D14 group 14	3.24	75	0.8646
D14 group 7	3.12	D14 Controls	3.61	26	**0.0430**
D30 group 7	3.34	D30 group 14	3.53	95	0.6830
D90 group 7	3.62	D90 group 14	3.60	75	0.6075
D7 group 14	3.22	D7 Controls	3.83	23	**0.0039**
D14 group 14	3.24	D14 Controls	3.61	25	**0.0121**
Richness					
				Statistics	
Group 1	**Median**	**Group 2**	**Median**	**W**	**p**
D7 group 7	1224	D7 group 14	1307	71	0.3499
D7 group 7	1224	D7 Controls	1692	31	**0.0108**
D14 group 7	1023	D14 group 14	1378	59	0.4940

D14 group 7	1023	D14 Controls	1674.5	22.5	**0.0242**
D30 group 7	1403	D30 group 14	1425.5	100	0.8467
D90 group 7	1592	D90 group 14	1570	73	0.6890
D7 group 14	1307	D7 Controls	1692	46	0.1500
D14 group 14	1378	D14 Controls	1674.5	39	0.1151

References

1. Lazarevic V, Gaïa N, Girard M, Schrenzel J. Decontamination of 16S rRNA gene amplicon sequence datasets based on bacterial load assessment by qPCR. BMC Microbiology. 2016;16:73.
2. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
3. Ounit R, Wanamaker S, Close TJ, Lonardi S. CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers. BMC Genomics. 2015;16(1):236.
4. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. Journal of Antimicrobial Chemotherapy. 2012;67(11):2640-4.
5. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.
6. Jari Oksanen FGB, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B., O'Hara GLS, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner. vegan: Community Ecology Package. R package version 2.5-6. https://CRAN.R-project.org/package=vegan. 2019.