VNIVERSITAT DE VALÈNCIA

Role of the microbiota in the defense against infections by Enterococci

Tesis Doctoral

Programa de Doctorado 3003 Bioquímica y Biomedicina

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Valencia, May 2017

Carles Úbeda Morant,
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INFORMA:

Que la presente memoria "Role of the microbiota in the defense against infections by Enterococci" ha sido realizada bajo su dirección en el Centro Superior de Investigación en Salud Pública, centro mixto de la Universidad de Valencia y del CSISP, y constituye su Tesis para optar al grado de Doctora por la Universitat de València.

Y para que así conste, en cumplimiento de la legislación vigente, presenta en el Departamento de Bioquímica y Biología Molecular de la Facultad de Ciencias Biológicas de la Universidad de Valencia la referida Tesis Doctoral, y firma el presente certificado.

Valencia, a 25 de Mayo de 2017.

Carles Úbeda Morant

Glossary

BEA	Bilis esculin agar (culture medium)
BHI	Brain heart infusion (culture medium)
BSI	Blood-stream Infection
CA	Community-associated
CDI	Clostridium difficile infection
CLABSI	Central line associated blood-stream infection
CR	Colonization resistance
CRISPR	Clustered regularly interspaced short palindromic repeats
DEPC	Diethylpyrocarbonate (used to inactivate RNAses)
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EHEC	Enterohemorrhagic Escherichia coli
HA	Hospital associated
HAI	Hospital associated infection
HGT	Horizontal gene transfer
HSCT	Hematopoietic stem cell transplant
IBD	Inflammatory bowel disease
IG	Intra-gastric
ILC	Innate lymphoid cell
ILC3	Innate lymphoid cell of type 3
LB	Luria-Bertani (culture medium)
LPS	Lipopolysaccharide
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
MLN	Mesenteric lymph nodes
MLST	Multi-locus sequence typing
mRNA	Messenger RNA

NaAc	Sodium acetate
NCBI	National Center for Biotechnology Information
NIAID	National Institute of Allergy and Infectious Disease
nr genes	Non-redundant genes
O/N	Overnight
ORF	Open reading frame
OTUs	Operational taxonomic units
P.O	Per os (orally)
PAI	Pathogenicity island
PBP	Penicillin binding protein
PBS	Phosphate buffer solution
PPP	Pentose phosphate pathway
PTS	Phosphotransferase system
R.A	Reumathoid arthritis
RM	Restriction-modification
RNA	Ribonucleic acid
RPKM	Reads per kilobase million
RT	Room temperature
SC	Sub-cutaneous
SCFA	Short-chain fatty acid
Tm	Temperature medium of hybridation
TPM	Transcripts per million
VRE	Vancomycin resistant Enterococcus
WHO	Word Health Organization

Acknowledgements

Esta tesis es el resultado de un largo camino, apasionante y a veces duro, en cual mucha gente ha participado. Con estas líneas quisiera mostrar mi agradecimiento a todas ellas.

Primero, quiero agradecer a mi director de tesis, Carles Úbeda. Por mi personalidad, creo que fuiste el mejor director de tesis posible! Me ha encantado la libertad de pensamiento, discusión (a veces largas), así como la posibilidad de experimentar vías alternativas. Al mismo tiempo, estabas presente en los momentos necesarios para poder sacar adelante los puntos críticos. Gracias por haber puesto tanta perseverancia en asegurar que tengamos una buena formación. Aunque fue complicado instaurar la costumbre, los múltiples journal clubs y labmeetings me han ayudado mucho a estructurar mi pensamiento científico, así como a exponer mis ideas.

A mis compañeros de trabajo, tanto los doctorandos de la antigua generación, cómo los nuevos. A Ana Dj, tienes una plaza especial para mi, cuántos años juntas. Ya lo has acabado, čestitka ! A Bea, eres una persona muy integrativa y creo que tu presencia ha hecho mucho para la cohesión de la gente en la sala. A Alejandra, me encanta trabajar contigo, todo es tan fácil! Tanto, que volvería a enseñarte a pinchar los ratones un domingo encantada! Majo, bienvenida en el grupo y gracias por preocuparte por los trámites de mi tesis. A Jorge, echo & echaré de menos nuestras conversaciones originales. Hablar contigo me ha ayudado muchísimo en un periodo difícil. A Pedro, me ha encantado tu integridad y buen humor. Espero que te vaya muy bien en USA y que volvamos a coincidir. A Leo, eres para mi un ejemplo de dedicación a la investigación. Gracias por estar siempre de buena voluntad cuando se necesita tu ayuda. A Galo, Alba, Majda y Irving, me ha gustado mucho el ambiente de la sala mientras estabais, que pena que os habéis ido en otros centros! Tenemos un billar pendiente. Cada uno que ha participado en el ambiente del CSISP, no os olvido AnaE, Raúl, Rodrigo y Letty , Maria, la fotografa oficial, Juán Angel, Paula, Gabriella.... Bob, eres muy seguro de ti y emprendedor, seguro que te sale muy bien el doctorado & que se vuelvan a organizar más salidas.

Además de los doctorandos, muchas más personas del CSISP han estado presentes estos años; gracias a vosotros por estos años compartidos y hacer del CSISP un lugar agradable - aunque haya que respetar las normas, por supuesto ! A Concha, me has salvado la vida con el número de placas que me has hecho ! No sé como habría hecho todos mis experimentos sin tu ayuda. A Alejandro Artacho, me has ayudado muchísimo con R; gracias por tus consejos en estadística y más general en como hacer lo que quería.

A Núria, gracias por todo tu trabajo y siempre intentar encajar las cosas incluso cuando las queremos para ya. Pepa, Alma, gracias por los consejos experimentales para la manipulación del RNA o otros. Arantxa, gracias por los consejos y la súper idea del aceite a modo API para conservar la anaerobiosis. A Pascual, gracias por preocuparte por mi y intentar arreglar estos malditos ordenadores.

Quiero agradecer especialmente a la gente del animalario, con quienes he pasado mucho tiempo y que siempre han intentado encontrar una solución para conciliar las normas de seguridad y nuestras necesidades experimentales. A Inma Noguera, por preocuparte tanto por mis ratones, ayudarme cuando lo necesitaba y enseñarme nuevas técnicas. A Armand, por el humor, las conversaciones de escalada y el canto de opera. Ha sido un placer empezar en el P2 contigo. A Inma Ballester, por seguir cuidando de mis ratoncitos.

Quiero agradecer también a las personas del laboratorio de José Penadés que nos han acogido al principio, cuando el P2 del animalario de la UV no existía aún.

Pensando en la gente fuera del CSISP que me ha ayudado, no puedes faltar, Leo ! Muchísima gracias por todos los esfuerzos para analizar los datos de metabolómica. Ha sido muy estresante pero al final todo ha salido muy bien!

To Karina Xavier, for opening her doors at the Gulbenkian Institute in Oeiras. Thanks for trying to find a solution for the different problems that arose, and for finding an anaerobic chamber, even if it was not something easy. To Jess, for all the times together. Thank you so much for facilitating with Joana my travel to Buenos Aires! Ozhan, Pol, Ana Rita, Rita y Joana, thank you for sharing these months with me.

Tengo también un pensamiento para mis estudiantes de final de carrera, especialmente el primero y el artista, esto va para vosotros Rafa y Iván. Rafa, he aprendido muchísimo al enseñarte cosas que ninguno de los dos había hecho jámas ! Me ha encantado tu determinación; era muy divertido enseñarte y utilizar el subjuntivo para explicarte que tenías que hacer... qué mayor te hacía sentir ! Iván, eres simplemente impresionante. Tan meticuloso que puedes hacer cualquier cosa, te saldrá todo bien por defecto. Me ha encantado trabajar contigo, hablar de dibujo y cualquier cosa, tienes tantos intereses y entusiasmo que das ganas de clonarse para poder hacer más cosas.

Mucha gente ha participado en mi orientación científica, algo que no era algo obvio con una formación de farmaceútica.

A Leopoldo García Sancho, con quién he entrado en el mundo de la investigación. Gracias por acogerme como un miembro del laboratorio cuando sólo hacia una beca de colaboración. Gracias Ana Crespo, Mercedes Vivas y los otros miembros del grupo, por hacerme sentir parte de ello.

A Bea, mi compañera de piso de Madrid, has sido como una hermana.

A Isabel, por siempre haber estado a mi lado, por corregir mi castellano y por entenderme y aceptarme tal como soy, tan diferente de tu educación.

A José, por las horas de salsa.

A Paco, por los sueños,

A Enrique, por el apoyo y devolverme la fe en los hombres,

Y después de mi llegada a Valencia, gracias a las personas del departamento de Parasitología de la UV,

A Maria Trelis, por dirigir mi tesis de Máster y ser tan buena persona. Espero que quedemos en contacto.

A Alba Cortes, gracias por enseñarme tanto y toda tu paciencia. Por los western blot y la suerte del principiante !

Estos momentos en Valencia han sido también una época de descubrimiento personal.

A Alberto, testigo mío, por la escalada, tu energía y optimismo sin fin. He aprendido muchisimo de tí, me has hecho replantearme mi visión de la vida y de qué es realmente importante, aunque a veces me cueste aplicarlo día a día.

To Marija, it was all of an experience to live with you ! You made me realize I did not have to be so reserved. And Jordi, I love your tip of craziness. “Diffund the Sandrinish, Jordi”.

A Alex, por los redbulls compartidos y el deportes en los grandes momentos de estrés,

A Maria, por las charlas hippies, la energía, y las carreras. A ver si hacemos esta maratón !

A Mila, Voro, Jordi, Alba y mi nueva familia tan amplia, gracias por haberme acogido a brazos abiertos desde el primer instante.

A mes frères, parce qu'un monde sans vous serais inimaginable. A Estelle, Cristina et les petits, Axel, Elena et Hugo, pour le futur et une famille unie.

A mes parents, Martine y Christian. Merci pour la tenacité et la capacité à surpasser n'importe quel obstacle. C'est une qualité trés importante, dans la vie en général et spécialement pour faire un doctorat. Je sais également combien vous avez pensé à moi, même dans la distance, et que vous auriez voulu pouvoir m'appuyer plus. Ce doctorat vous est dédié, d'une certaine manière c'est l'aboutissement de nombreux efforts et sacrifices pour vous aussi.

Este camino habría sido muy duro sin un soporte moral de acero, un compañero para hablar de ciencias y quejarse cuando las cosas no salen, y más que todo, compartir mucho mucho amor. A mi marido, muchas gracias por todo esto Marc.

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Abstract

Antibiotic resistant bacteria, such as vancomycin-resistant *Enterococcus* (VRE) are an increasing problem in hospitalized patients and commonly cause infections following antibiotic therapy. Infections with VRE generally begin by colonization of the intestinal tract. In normal conditions, our gut is colonized by hundreds of commensal bacterial species, the microbiota, that suppress intestinal colonization by VRE, a phenomenon known as "colonization resistance" (CR). However, administration of antibiotics alters the composition of the microbiota, which allows VRE to densely colonize the intestine and subsequently disseminate to the bloodstream where it can put in serious danger the life of the patient. Thus, understanding how and which members of the microbiota confer CR and how antibiotics promote intestinal colonization by VRE, is crucial if we want to prevent VRE infections. Unfortunately, the absence of techniques to study complex bacterial populations has hampered, until the last recent years, the research in this clinically relevant field. For this reason, it is not completely understood how antibiotics change the microbiota and promote infections by VRE, which are the members of the microbiota that are key for conferring colonization resistance against VRE and the mechanisms by which they confer protection.

Thus in this thesis, the main objectives proposed are (i) to understand how antibiotics change the composition of the microbiota and subsequently promote intestinal colonization by VRE, (ii) identify commensal bacterial species that are key for conferring protection and (iii) study mechanisms by which these commensal bacteria may confer protection against VRE.

We first studied the effect of oral vancomycin treatment on the human gut microbiota through 16s rRNA high-throughput sequencing. This antibiotic is frequently given to patients to treat *Clostridium difficile* infections and subsequently can promote secondary infections by VRE. Our analysis showed that vancomycin promotes drastic changes on the composition of the microbiota, including the depletion of all bacterial species from the phylum Bacteroidetes, one of the most prevalent phyla inhabiting in the human intestinal tract. Moreover, our analysis indicates that the microbiota never recovers its baseline state, even after 22 weeks post-antibiotic cessation. Importantly, the microbiota recovery rate was different depending on the subject analyzed. While some patients recovered most of their baseline bacterial species, up to 89% of their baseline bacterial species were not recovered in other patients. Moreover, using a mouse model we were able to demonstrate that the microbiota recovery rate upon vancomycin treatment was clinically relevant since a lower microbiota recovery upon vancomycin cessation is associated with an increased susceptibility to VRE intestinal colonization.

Subsequently, using the same VRE infection mouse model, we analyzed the impact of other antibiotics of different spectrum (ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin, besides vancomycin) on the gut microbiota composition and on the VRE colonization capacity, both during the treatment and two weeks after antibiotic cessation. Our analysis shown, as expected, that different antibiotics promoted different changes in the composition of the microbiota, being vancomycin and clindamycin those that promoted a higher number of changes, while neomycin or ciprofloxacin had a minor effect on the microbiota composition. The changes in the composition of the microbiota were associated with the capacity of VRE to colonize the intestinal tract since those antibiotics that promote more alterations on the microbiota allowed a higher level of VRE intestinal colonization. Subsequently, applying correlation analysis and Linear Discriminatory analysis, we were able to identify several bacterial taxa that are associated with VRE resistance. These taxa include the genera *Alistipes*, *Barnesiella*, *Oscillibacter* and some members of the families Lachnospiraceae and Ruminococcaceae. We next isolated and administered these bacteria to vancomycin-treated mice to test their capability to restore colonization resistance. We demonstrated that the administration of a combination of 4 bacterial isolates to mice (*Alistipes*, *Barnesiella*, *Oscillibacter* and a bacterium from Ruminococcaceae family) drastically diminished the capacity of VRE to colonize the intestinal tract of antibiotic-treated mice.

We next performed metatranscriptomic and metabolomic analysis to identify *in vivo* functions expressed and metabolites produced by the identified protective commensal bacteria in order to determine possible mechanisms by which these bacteria could be suppressing VRE intestinal colonization (e.g. production of inhibitory molecules, competition for nutrients). In addition, we also analyzed the *in vivo* transcriptome of VRE to determine the functions express by this pathogen to colonize the intestinal tract, and we performed nutrient arrays to identify nutrients that the pathogen could be using for its growth in the intestinal tract. The analysis performed determined that (i) oral inoculation of mice with the protective bacterial mixture restores the expression of genes encoding for transporters that internalize saccharides (cellobiose, N-acetyl-galactosamine, N-acetyl-glucosamine) and amino acids (serine), whose expression was diminished upon antibiotic treatment. (ii) Increased expression of these genes was associated with diminished intestinal availability of several nutrients including saccharides (i.e. cellobiose) and amino acids (i.e. serine, proline, leucine and threonine). (iii) Some of the nutrients consumed upon administration of the protective commensal bacteria may be crucial for VRE growth in the intestinal tract. Indeed, analysis of the VRE transcriptome in colonized mice revealed that VRE highly express *in vivo* transporters for the internalization of saccharides (i.e. cellobiose, N-acetyl-galactosamine, N-acetyl-glucosamine) and several branched-chain aminoacids (i.e. proline). Moreover, out of 190 carbon sources tested, the saccharides cellobiose, N-acetyl-glucosamine and N-acetyl-galactosamine are among the 10 carbon sources that promote the highest VRE growth under anaerobic conditions. Thus, although additional experiments should be performed for validation, our results support a model in which the administered protective bacteria decrease the intestinal levels of nutrients that can be utilized by VRE for growth, which reduces the capacity of this pathogen to colonize the intestinal tract.

1. Introduction

1.1 Nosocomial diseases

According to the last report of the World Health Organization (WHO), a nosocomial infection — also called "hospital-acquired infection" can be defined as: (i) an infection acquired in the hospital by a patient who was admitted for a reason other than that infection [1], (ii) an infection occurring in a patient in a hospital or other health-care facility in whom the infection was either present or incubating at the time of admission. This also includes infections acquired in the hospital but appearing after the discharge, and occupational infections among staff of the facility [2].

These nosocomial infections have considerable economic costs, mainly by increasing the length of the stay for infected patients and by the additional treatments required [3–5]. Moreover, it is one of the leading causes of death, at least in the USA [6]. It occurs worldwide and affects both developed and resource-limited countries [2].

1.1.1 Relevance

Hospitals are crowded environments where both infected people and people with increased risk of infection congregate.

Many factors promote infection among hospitalized patients: decreased immunity among patients; the increasing variety of medical procedures and invasive techniques creating potentially new routes of infection; and the transmission of drug-resistant bacteria among crowded hospital populations, where poor infection control practices may facilitate transmission.

According to these factors, infection rates are higher among patients with increased susceptibility because of extreme ages (very young or old hospitalized people), underlying disease, or chemotherapy. Any factor that affects the immunity of the patients, like malnutrition, or that increases their hospitalization, time, such as chronic disease (diabetes mellitus, renal failure, malignant tumors, leukemia, or the acquired immunodeficiency syndrome) is susceptible to increase their risk to suffer infections by an opportunistic pathogen [7]. Infections by opportunistic pathogens involve organisms that are normally innocuous but that may become pathogenic due to the specific situation of the patient.

Besides the immunocompromised state of the patient that increases its susceptibility to suffer an opportunistic infection, the invasive medical techniques, which disrupt the physical protection of the host, can generate access to an otherwise sterile part of the body [8]. Indeed, in an European study conducted in 2011, the most frequently reported hospital-associated infections (HAIs) types were surgical site infections (19.6%), respira-

tory tract infections (pneumonia 19.4% and lower respiratory tract 4.1%), urinary tract infections (19.0%) and bloodstream infections (10.7%) [7].

Another important factor to take into account when considering the nosocomial diseases are the organisms themselves. The probability of exposure leading to infection depends partly on the characteristics of the microorganisms, including their resistance to antimicrobial agents, intrinsic virulence, and amount of the infectious agent. According to the last surveillance report of the European center for disease prevention and control (ECDC), the microorganisms most frequently isolated from HAIs were, in decreasing order, *Escherichia coli* (15.9%), *Staphylococcus aureus*(12.3%), *Enterococcus* spp. (9.6%), *Pseudomonas aeruginosa* (8.9%) *Klebsiella* spp. (8.7%), Coagulase-negative staphylococci (7.5%), *Candida albicans* (6.1%), *Clostridium difficile* (5.4%), *Enterobacter* spp.(4.2%), *Proteus* spp. (3.8%) and *Acinetobacter* spp. (3.6%). The predominant families of microorganisms were gram-positive cocci in surgical site and bloodstream infections, Enterobacteriaceae in urinary tract infections, non-fermenting gram-negative bacteria (especially *P aeruginosa* and *A.baumanni*) in respiratory tract infections and anaerobes (especially *C. difficile*) were the most frequently reported family in gastrointestinal tract infections [7].

Among the pathogens previously cited, a large number have acquired some antibiotic resistance gene that complicates their treatment. This is the case for example of *Enterococcus faecium*, the microorganism studied in this thesis, that shows a high prevalence of multi-resistant strains. Regarding this aspect, the hospital environment is especially dangerous due to the intensive use of antibiotics. Indeed, the constant environmental concentration and usage of antibiotics promote the emergence of multidrug-resistant strains through selection and exchange of genetic resistance elements [6].

The environmental properties of the hospital facilitate the transmission of nosocomial bacteria and the development of antimicrobial resistances. This can result in multiresistant bacterial infections, with a bad prognostic, since we have very limited treatment options. Thus, it is primordial to investigate new alternative treatments to combat these type of infections.

1.1.2 Antibiotics

As we mentioned before, the antimicrobial resistance problem awoke a huge concern in public health sector and in the population in general as it is extensively broadcast. For this same reason it is important to highlight to which extent the antibiotics have greatly helped humanity, diminishing the rate of mortality for infectious diseases (death at birth, in childhood, in case of wound,...).

The use of antibiotics in human society by means of the diet or of specific medicinal plants is in fact very ancient. It was possible to detect tetracycline in the human skeletal remains from ancient Sudanese Nubia dating back to 350–550 CE, although we cannot be sure whether they specifically ingested it to combat an infection or if it was present in their normal diet [9, 10]. Another example is the use for millennia of medical plants with active compounds in traditional Chinese medicine. Thus, even if the antimicrobial resistance was really described as a problem since last century, the human exposure to antibiotics is far older.

The modern "antibiotic era", started approximately with the XX century, with the synthesis in 1909 by Erlich of an organoarsenic derivative to treat syphilis, the discovery

of Fleming's penicillin in 1928, and the use of the precursors of sulfonamide [11]. Compounds from most of the antibiotic classes that we use nowadays were isolated by the early sixties. In chronological order, β-lactames, sulfonamides, aminoglycosides, tetracyclines, chloramphenicol, macrolides, glycopeptides, ansamycins and, in the early sixties, quinolones were isolated. Since then, relatively few compounds of new classes have been discovered or they are a synthetic derivative of compounds from other classes, modified to overcome resistance, to achieve a better distribution or a more specific spectrum. For example, the streptogramins quinupristin/dalfopristin are hydro soluble derivatives of pristinamycin, discovered in 1965 [12, 13]. Glycylcyclines (such as tigecycline) are derivatives of tetracycline but alterations to the molecule resulted in an expanded spectrum of activity and decreased susceptibility to the development of resistance when compared with other tetracycline antibiotics [14].

Table 1.1: Summary of mode of action, class and spectrum of antibiotics.

Mode action	**Class**	**Example**	**Spectrum**
depolarization of cell wall	lipopeptides	daptomycin	Gram-positive
inhibition of cell membrane function	polymixins	colistin	Gram-negative
inhibition of cell wall synthesis	β-lactams	ampicillin	Broad-spectrum
	glycopeptides	vancomycin	Gram-positive
inhibition of de novo folate synthesis	sulfonamide	thrimetoprim-sulfamethoxazole	Broad-spectrum 1
inhibition of DNA replication	nitroimidazole	metronidazol	Anaerobic bacteria
	quinolones	ciprofloxacin	Broad-spectrum
inhibition of ribosomal protein synthesis (30S)	tetracycline	tetracycline	Broad-spectrum
	aminocyclitol	spectinomycin	Broad-spectrum 1
	aminocyclitol-aminoglycoside	neomycin	Broad-spectrum 1
inhibition of ribosomal protein synthesis (50S)	amphenicol	chloramphenicol	Broad-spectrum
	streptogramin	dalfopristin/quinupristin	Gram-positive
	oxazolidinone	linezolid	Gram-positive
	macrolide	erythromycin	Gram-positive, 2
	lincosamide	clindamycin	Gram-positive, 2
inhibition of RNA synthesis	rifamycins	rifampin	Specific use 3

Compilation of information from [15,16] 1: only aerobic bacteria, 2: also against anaerobic bacteria 3: used mainly against *Mycobacterium tuberculosis*

The classification of antibiotics is done according to their molecular structure. All of them exert their effect by either one of 4 different mechanisms, namely (i) inhibition of DNA replication, (ii) inhibition of RNA synthesis, (iii) inhibition of cell wall synthesis or (iv) inhibition of protein synthesis (Table 1.1) [15]. They can be either bacteriostatic or bactericidal, changing the duration of the treatment required to achieve the effect. According to their molecular structure, they will display a different spectrum of action. This aspect is especially important as nowadays it is broadly described that antibiotics do no only act against one specific pathogen but also get rid of a larger spectrum of commensal bacteria at the same time. As we will later discuss, a side effect of the antibiotic treatments is the alteration of the commensal bacteria, which can result in an increased susceptibility to a range of bacterial infections [17].

Within the same class of antibiotic, the compounds vary in their pharmacokinetic characteristics. The election depends obviously on the specific characteristics and requirements of the patient. Concerning the present work, we are specially interested into the metabolization and way of excretion (biliary/urinary excretion). Indeed, antibiotics that are excreted biliary in an active form are susceptible to alter the gut microbiota no matter the way of administration (IV, SC,...), possibly increasing the host susceptibility to intestinal colonizations by bacterial pathogens and promoting the selection of resistant strains as the concentration reached are sub-lethal.

An important feature of the modern "antibiotic era" is the extent to which bacteria, pathogens and commensals, are exposed to antibiotics and the conditions of use that promote the selection and widespread of resistances. As mentioned before, the use of antibiotic substances is documented before the "antibiotic era" and, moreover, genes conferring resistance to several classes of antibiotics already existed in the nature [18]. However, the increased use of antibiotics as well as their inappropriate use has promoted the selection of resistant strains. Among the inappropriate uses, we can cite (i) the no compliance of antibiotic treatment by patients, (ii) the discharge of antibiotics and other wastes (i.e. biocides such as chlorhexidine or metals) in the environment and (iii) the administration of antibiotics in livestock food as a growth promoter. In these cases, the concentration of the antibiotic reached are lower than the effective one, promoting the selection of resistance [11, 19]. As a consequence, some bacteria have acquired a broad resistance to the majority of the antibiotic commonly used. For example, it has been demonstrated that multidrug-resistant *Enterococcus*, similar to clinical isolates, can be isolated from pigs farm environment [20]. As we mentioned in the previous paragraph, infections by multidrug-resistant bacteria are difficult to treat. Then, in an attempt to tackle the increasing problem of multi-resistance, the European commission has banned in 2006 the use of antimicrobials for growth promotion in animal husbandry [21].

In summary, the antibiotics have improved our life expectancy but their intensive use and misuse have promoted the selection of multi-resistant bacteria, really complicated to combat as they are resistant to most of the therapeutic options we have. This is the case of the pathogen I did study in the present thesis, vancomycin-resistant *Enterococcus* (VRE), that, as specified by its name, is resistant to vancomycin in addition to other antibiotics.

1.2 *Enterococcus*

Among the multi-resistant organisms that arose in the last decades, *Enterococcus* has acquired an especially broad spectrum of resistance to antibiotics. As we mentioned before, *Enterococcus* is the third main microorganism responsible for HAIs in Europe, and the fifth one in the USA [22]. Because of its high prevalence, *Enterococcus* is already an important health concern, being *E. faecalis* the most abundant one, followed by *E. faecium*. However, as we will discuss it later, the problem of the multi-resistance is changing its epidemiology, with an increase in infection by multi-resistant *E. faecium*.

1.2.1 Characteristics

The enterococci are gram-positive, non-spore-forming, facultative anaerobic oval cocci arranged in pairs or chains of various lengths; they are especially resistant, with a particular ability to survive under harsh conditions (including high salt concentrations) and at a wide range of temperatures (from 10 °C to >45 °C) [23]. They typically grow in broth containing 6.5% NaCl, and hydrolyze esculin in the presence of 40% bile salts [24]

Almost all of them are catalase reaction negative, and do not express a complete cytochrome. They are usually homofermentative, producing lactic acid as the end product of glucose fermentation, without production of gas [25, 26]. Some species are motile,

such as *E. gallinarum* and *E. casseliflavus* [27]. Pigmentation is a variable trait in the genus *Enterococcus*, with a few yellow-pigmented species [27,28], pigmented species being commonly found among plants [29].

1.2.2 Habitats and nutritional requirements

Currently, the genus *Enterococcus* consists of 56 species described in the NCBI database (21 January 2017), although the vast majority of clinical enterococcal infections in humans are caused by *Enterococcus faecalis* and *E. faecium*.

Enterococci are ubiquitous in guts but they constitute a small proportion of the gut consortium, typically comprising less than 1% of the adult microbiota [29]. They are regarded as harmless commensals. However, they can cause invasive diseases when the commensal relationship with the host is disrupted. Principally, Enterococci can cause urinary tract infections, bloodstream infections, endocarditis, and are also associated with peritonitis and intra-abdominal abscesses. [30]

In addition to the broadly known human association, the genus *Enterococcus* consists of species distributed in the whole animal reign as well as in various biotopes of the environment.

Different species of *Enterococcus* have been isolated from the gut of insects, birds, reptiles and mammals. In a large variety of insects, including beetles, flies, bees, termites, and worms, *E. faecalis* and *E. faecium* are predominant while other species occur at lower prevalence [31]. As mentioned before, *Enterococcus* has also been isolated from reptiles [32], birds [33] and a wide range of mammals such as donkeys [34], mouse, rat, guinea pig, rabbit, dog, rat, chicken, fly, and monkey [35]. In a thorough analysis of the occurrence of enterococci in animals and natural environments conducted in 1963, Mundt and colleagues [32] described a high prevalence of *Enterococcus* in the feces of mammals (71% of 216 samples) and reptiles (86% of 70 samples), and a lower prevalence in birds (32% of 22 samples) [32]. A striking fact is that enterococci occurred only sporadically among the primarily herbivorous mammals, whereas it appeared to naturally colonize rodents and larger animals with diversified diets [32]. The most commonly encountered enterococcal species in the gut of mammals are *E. faecalis, E. faecium, E. hire,* and *E. durans*. Others species are found only occasionally [36].

Besides its wide distribution in the animal kingdom, *Enterococcus* has been isolated in various types of plants [37,38], algae [39], beach wrack [40,41], submerged vegetation [42,43], flowering plants [37], and forage crops [44,45]. Recently, some *Enterococcus* species have also been recovered from both fresh and marine water [45–47], fresh or marine water sediments [48,49], soils [45], and sand [50,51]. Thus, *Enterococcus* is quite ubiquitous, as illustrated in the figure 1.1.

Enterococcus serves to monitor microbiological water quality, as an assessment of fecal contamination. Thereupon, it is difficult to conclude whether the environmental presence of *Enterococcus* is transient, due to fecal contamination and the high resistance of the bacteria or whether the environment constitutes a habitat where the bacteria is actually able to grow.

In any case, the presence of the genus *Enterococcus* in insects, reptiles, birds and mammals suggests a very ancient association with the gut environment, before the diversification of these different taxa [52]. Several characteristics of the genus *Enterococcus*

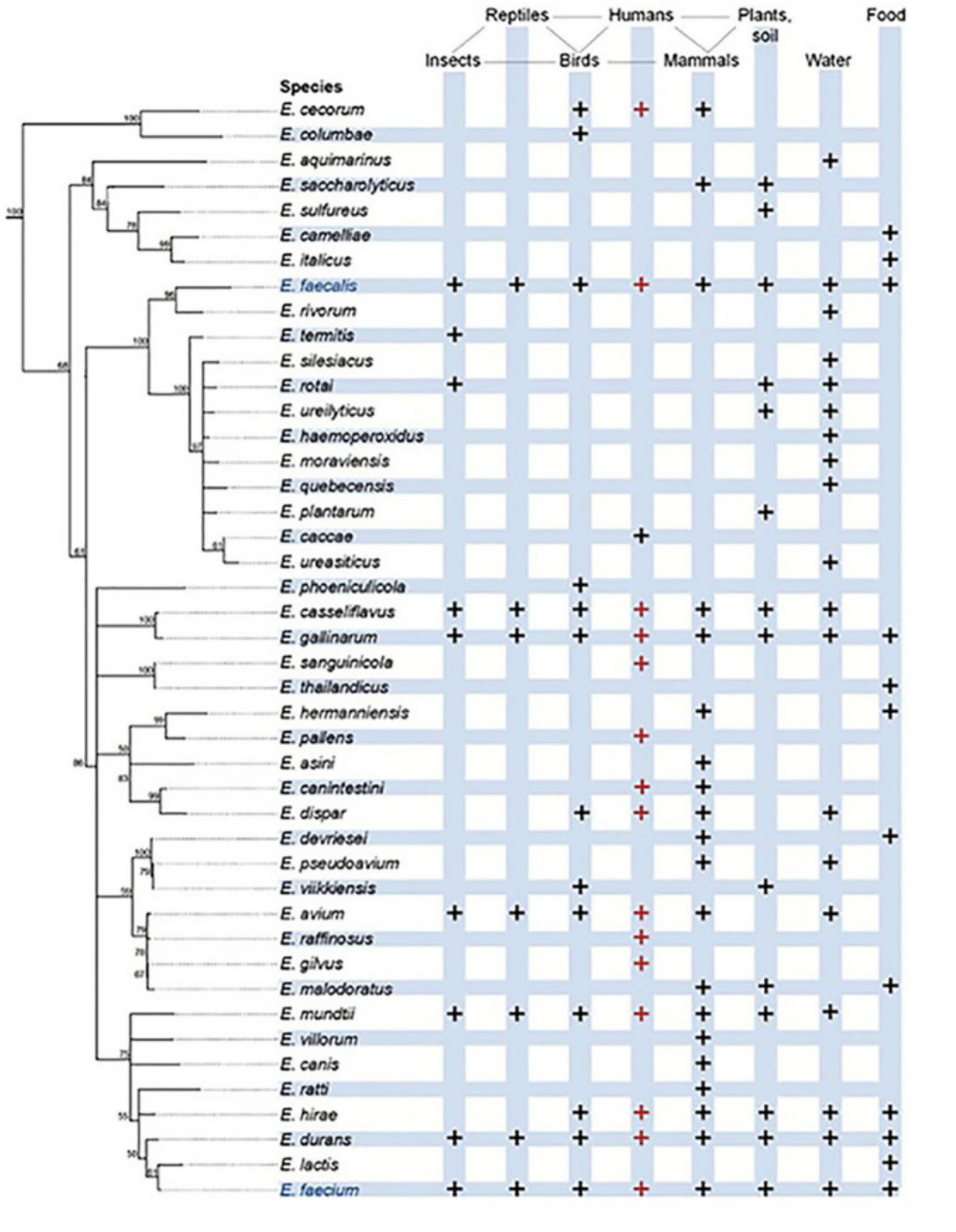


Figure 1.1: Distribution in nature of species of the genus Enterococcus. The dendrogram shows phylogenic relationships. The sources of isolation are indicated for each species. A simplified food chain is shown. Red and black symbols indicate species that have been described in human infections or colonization, respectively. Reproduced from [29].

advocate for an important adaptation to the gut environment. For example, *Enterococcus* have relatively reduced genomes (from 2.7 Mb to 3.6 Mb across species sequenced until 2014) [52]. A reduced genome is a distinctive feature of symbionts that do not have to express the full range of functions expected for their own survival as they obtain some specific nutrient from the host. Thus, the reduced genome of *Enterococcus* suggest their adaptation to a specific ecological niche.

Table 1.2: Enterococcus auxotrophy for amino acids.

Amino acid	**E. faecium ATCC 8043**	**E. faecalis V583**
L-Arginine	+	-
L-Isoleucine	+	-
L-Leucine	+	-
L-Threonine	-	+
L-Histidine	-	-
L-Methionine	-	-
L-Tryptophan	-	-
L-Valine	-	-

Comparison for the amino acid requirement from *E. faecium* [53] and *E. faecalis* [54]. -: auxotrophy, +: the bacteria can synthesize the aminoacid.

Indeed, *Enterococcus* is known to be auxotroph for various amino acids, although there is an important variation between species. For example, *Enterococcus faecalis* V583 is known to be auxothroph for L-arginine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-tryptophane and L-valine whereas *E. faecium* ATCC8043 absolutely requires L-histidine, L-methionine, L-treonine, L-tryptophane and L-valine (Table 1.2) [53, 54]. They also require some vitamins to grow. For example, *E. faecium* ATCC8043 does require folate, pyridoxal, D-pantothenic acid, nicotinic acid and D-biotin [53, 55]. Regarding their capacity to use carbohydrate sources, *Enterococcus* express few enzymes to catabolize complex carbohydrates, they do have a β-glucosidase but no β-glucuronidase and only *E. faecium* possess an α-galactosidase [56]. On the other hand, they can use an important number of monosaccharides to grow (Table 1.3) [56, 57].

The important nutritive requirement of the genus *Enterococcus* and its presence in the gut of very diverse animals suggest an adaptation to the gut environment. Thus, its broad distribution in the environment, exposed to very different conditions, is quite surprising. Nowadays, it is not clear whether this genus live freely in the environment (and how), being actually able to grow and divide, or whether it can only survive.

The wide distribution of *Enterococcus* increases the possibility of acquisition and selection of resistant genes in the environment contaminated with sub-lethal antibiotic concentration and where, moreover, the resistance genes are being poured through human and livestock waste water.

Indeed, several vancomycin-resistant *Enterococcus* were isolated in the wild, from fishes, Iberian wolf and lynx [58–60]. Nowadays, it is not clear to what extent the generation of multi-resistant bacteria in the environment contribute to the spread of antibiotic resistance in the hospital. As we will see later, the infection by *Enterococcus faecium* of

Table 1.3: Carbohydrate utilization in the genus *Enterococcus*.

Metabolized	**Not metabolized**
N-acetyl glucosamine	D-arabinose
Amygdalin	Eythritol
Arbutin	Fucose
Cellobiose	Glycogen
D-fructose	Alfa-methyl-D-xyloside
Galactose	Pullulan
Beta-gentiobiose	L-xylose
Glucose	
Lactose	
Maltose	
D-mannose	
Methyl-beta-D-glucopyranoside	
Ribose	
Salicin	
Trehalose	

Compilation of data from [56].

animal origin cluster apart from the nosocomial isolates. However, this genus possesses an impressive capacity to exchange genetic material by horizontal gene transfer. Thus, it is possible that the generation of multi-resistance in the environment could be transferred to clinical isolates.

1.2.3 Epidemiology

As we mentioned before, various *Enterococcus* species were isolated from patients but the two mains ones are *Enterococcus faecalis* and *Enterococcus faecium*, described here. Enterococci are intrinsically resistant to a broad range of antimicrobial agents including cephalosporins, sulphonamides and low concentrations of aminoglycosides. Moreover, through mobile elements, they have the ability to acquire additional resistance, which severely limits the number of treatment options, especially in the case of the vancomycin resistance.

In the late 1970s, *Enterococcus faecalis* accounted for 90–95 % of clinical enterococcal isolates in United states and represented also the majority of isolates identified in Europe [23]. Associated with the increased use of vancomycin and broad-spectrum antibiotics, the prevalence of *Enterococcus faecium*, which is much more frequently resistant to vancomycin and ampicillin than *E. faecalis*, drastically increased in clinical enterococcal isolates [23]. Indeed, vancomycin resistance in *Enterococcus faecium* was first reported in France as early as 1988 and in England the year afer [61, 62], but showed the most dramatic increase in the United States where it was attributed to the widespread use of vancomycin in hospitals [23].

This change in species is of paramount clinical importance, as *E. faecium* tends to acquire resistance to a higher number of antibiotics and, thus, is by far more difficult to treat. For example, in the United States, the percentage of *E. faecium* isolates that were resistant to vancomycin rose from 0 % before the mid 1980s to more than 80 % by 2007; by contrast, only 5 % of *E. faecalis* isolates were vancomycin resistant [23]. Regarding the current situation in Europe, both enterococci display a high-level resistance to aminoglycoside, accounting to 28.8 % of the isolates according to the last European report from 2014 [30]. The situation in respect to the vancomycin resistance is not so problematic as in the United States. The prevalence remained low in the case of *E. faecalis* and ranged from 0 to 45.1% for *E. faecium* in the European countries in 2014 [30]. Nevertheless, there was an increasing trend in the period 2011-2014 as the mean percentage for vancomycin resistance rose from 6.2 % in 2011 to 7.9 % in 2014 [30]. Vancomycin-resistant *E. faecium* is an increasing threat in Europe because of the limited number of existing therapeutic options and its capacity to rapidly acquire new resistance genes. As I will describe, there are already documented cases of infection by isolates resistant to the newest antibiotics.

1.2.4 Genomic characteristics

In order to design an efficient strategy to control the increasing prevalence of infections by vancomycin-resistant *E. faecium*, it is necessary to understand the main characteristics of the genus allowing its expansion in the hospital environment.

Several studies have demonstrated the separation of *Enterococcus faecium* into two subpopulations, one containing primarily commensal/community-associated (CA) strains and one that contains most clinical or hospital-associated (HA) strains [63–66]. Thus, the clinical-associated strains present specific characteristics responsible for their success in the hospital environment. It can be highlighted that animal isolates cluster apart, but are more similar to HA strains than to CA strains (Figure 1.2) [67, 68].

One important difference between both human-associated clusters is the expansion of the accessory genome, generally acquired through horizontal gene transfer (HGT) [64, 65]. Indeed, the HA subpopulation more frequently has insertion sequence (IS) elements, transposons, pathogenicity island(s), and plasmids or genes associated with antibiotic resistance, colonization, and/or virulence [65]. Thus, the primal characteristic that drove the separation of both clusters seems to be a difference in the capacity to acquire foreign DNA.

The acquisition of foreign DNA is a vital characteristic of prokaryotes that allow them to adapt to new environments. However, the HGT can also be detrimental, allowing parasitism. To control the possible invasion by foreign DNA, the host cell possesses two main post-transfer barriers : restriction-modification (RM) systems, and clustered, regularly interspaced short palindromic repeats (CRISPR). The RM system is unspecific, recognizing all the sequence that do not share the host methylation pattern while CRISPRs are defense systems that provide a type of acquired immunity against specific sequences [69].

As mobile elements constitute a significant part of the genome of *Enterococcus faecium* nosocomial isolates, Palmer and Gilmore [70] assessed the presence of the CRISPR-cas system in these isolates as well as in CA strains to check whether the highest acquisition of foreign DNA in HA strains was related to a compromised bacterial immunity. They

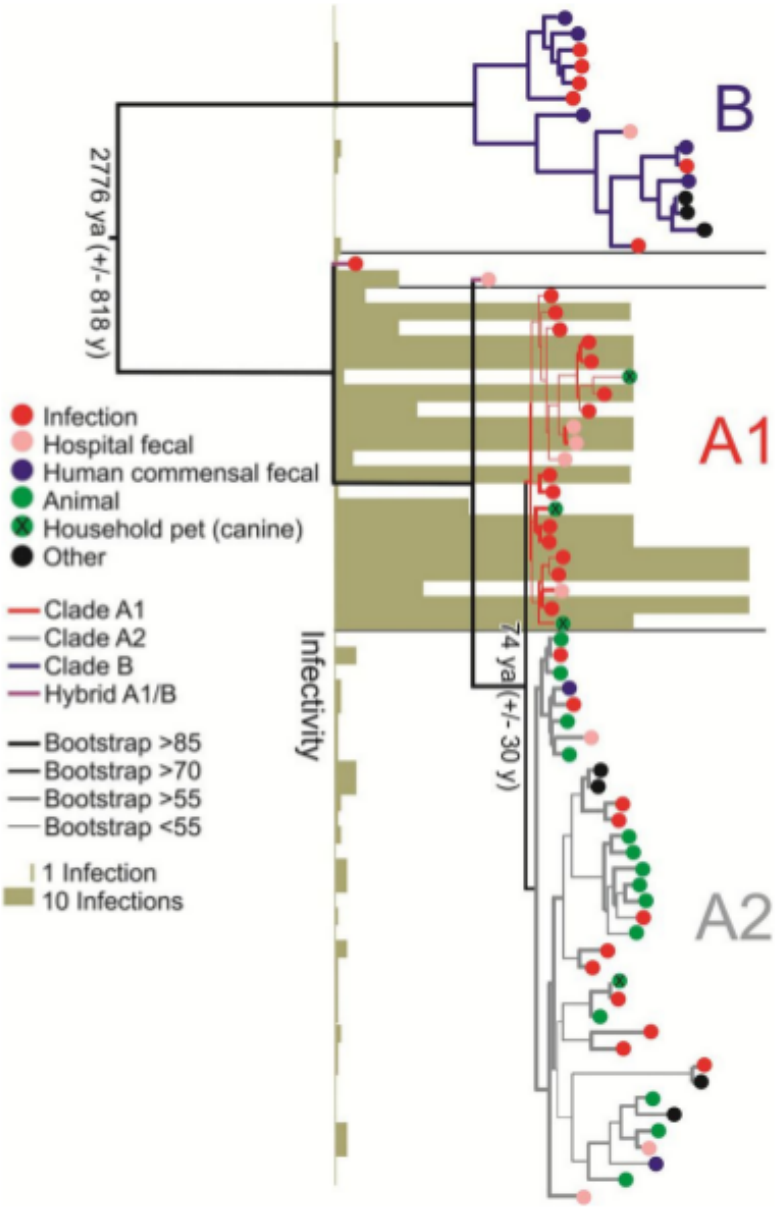


Figure 1.2: Phylogenetic reconstruction of *E. faecium* RAxML SNP-based tree based on the concatenated alignments of DNA sequences of 1,344 single-copy core genes in 73 *E. faecium genomes*. Bootstrapping was performed with 1,000 replicates. The origins of the strains are indicated. The dates for the split between the clades, estimated by a BEAST analysis, are indicated (ya, years ago). The infectivity score reflects the number of strains of a particular ST isolated from infection. The clades are color coded as follows: clade B (similar to the CA strains) in dark blue, clade A1 in red (similar to the HA strains), and clade A2 (principally animal associated strains) in gray. Reproduced from [67].

found a highly significant correlation between the absence of a CRISPR-cas locus and the presence of acquired antibiotic resistance in both *E. faecalis* and *E. faecium* [70]. Thus, the absence of CRISPR system in clinical isolates seems essential to allow the acquisition of resistance genes.

The same group also checked that the presence of a functional RM and CRISPR-cas system, both individually and collectively, significantly diminish the capacity of conjugative plasmids to be transfered into *Enterococcus faecalis* [71].

Taken together, it seems that the nosocomial isolates have a compromised genome defense and consequently a higher capacity to acquire antibiotic resistance and virulence genes through HGT. Probably, the antibiotic use in the hospital setting selects for enterococcal strains with this higher capacity to acquire antibiotic resistance.

According to this observation, HA isolates display a greater accessory genome compared to CA isolates. However, the differences between both clades are not only defined by their accessory genome. The analysis of a subset of 100 core genes of *Enterococcus faecium* showed a significant separation between isolates from the CA and HA clades, suggesting that there is no population admixture between both groups [66].

Possibly, the diversification of *E. faecium* into a clade adapted to the nosocomial environment could be due to the absence of RM and CRISPR-cas systems in the HA isolates. In the absence of defensive systems, IS elements could integrate inside non-essential genes for the subsistence of the bacteria in such environment, leading to their deletion. These evolutionary patterns have been detected in genomes of *E. faecalis* and *E. faecium*, where the expansion of IS elements volatilized regions of a pathogenicity island (and possibly other regions of the chromosome), leading to an extremely high rate of pseudogene [52, 64].

The antibiotic usage in the hospital setting alters the gut microbiota and thus, the genes required for survival of the multiresistant *Enterococcus* in this specific conditions are different than those required for the survival in an untreated human. In these conditions, some genes, usually essential for *Enterococcus* survival, are not required anymore and can be lost, possibly leading to the diversification of *E. faecium* into a cluster whose chromosome is specifically adapted to the nosocomial environment.

The difference in the HA core genome could affect the virulence of the strains in case of loss of functions implicated in the interaction with the host. This phenomenon was described in *Bordetella pertussis* where, through the proliferation of an IS resulting in the loss of determined functions, has lost the ability to colonize the host in a non-pathogenic manner [52, 72]

Despite the description of differences in the core genomes, based on the current knowledge, the success of *Enterococcus faecium* as a nosocomial opportunistic pathogen is related to its capacity to acquire antibiotic resistance and virulence genes, which we will describe further.

Resistance to antibiotics The resistance to antibiotics is an essential characteristic that enables the spread of *Enterococcus* in the hospital setting. As previously mentioned, this genus can acquire numerous genes of resistance by HGT and, moreover, it is intrinsically resistant to a broad range of antimicrobial agents.

First, *E. faecium* is resistant to β-lactams and cephalosporins that inhibit the wall cell synthesis by binding to the enzyme, named penicillin binding proteins (PBPs), responsible

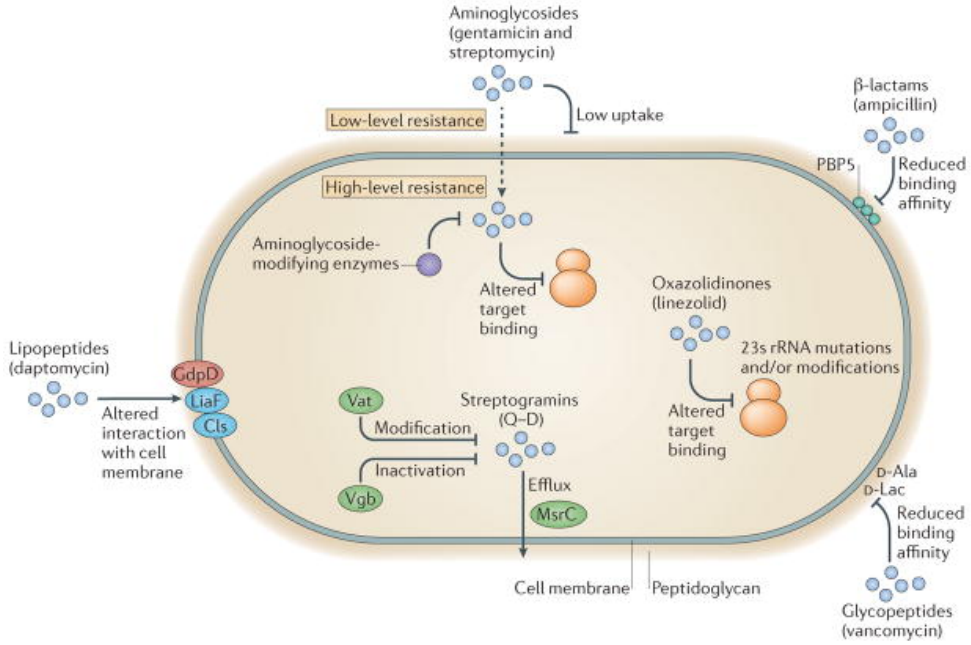


Figure 1.3: Main mechanisms of enterococcal antibiotic resistance. Enterococci are intrinsically resistant to several antibiotics and can acquire mutations and exogenous genes that confer resistance to additional drugs. The main mechanisms of antibiotic resistance are shown. *Enterococcus faecium* exhibit intrinsic low-level resistance to aminoglycosides such as streptomycin or gentamicin owing to low uptake of these highly polar molecules. High-level resistance results from the acquisition of aminoglycoside-modifying enzymes (acetylation) or, for streptomycin, can result from ribosomal mutations that result in altered target binding (methylation of the 16S rRNA encoded by the gene efmM). In *Enterococcus faecium*, resistance to ampicillin occurs through the production of penicillin-binding protein 5 (PBP5), which has low affinity for β-lactams. Although unusual, some strain can produce a β-lactamase. Resistance to the glycopeptide vancomycin occurs through a well-characterized mechanism of reduced vancomycin-binding affinity, involving alterations in the peptidoglycan synthesis pathway (The pentapeptide involved in the peptidoglycan structure, normally ending in a D-Ala-D-Ala motif can be modified to D-Ala-D-Lac or D-Ala-D-Ser). Resistance of *Enterococcus* spp. to the streptogramin quinupristin–dalfopristin (Q–D) involves several pathways, including drug modification (by virginiamycin acetyltransferase (Vat)), drug inactivation (through virginiamycin B lysase (Vgb))and drug efflux (via MsrC and VgaD ABC-efflux pumps) [73]. Resistance to the oxazolidinone linezolid is rare, but the most common pathway involves mutation in the 23S ribosomal RNA ribosome-binding site. Resistance of *E. faecalis* and *E. faecium* to the lipopetide daptomycin has been shown to involve altered interactions with the cell membrane and requires the membrane protein LiaF and enzymes involved in phospholipid metabolism, such as a member of the glycerophosphoryl diester phosphodiesterase family (GdpD) and cardiolipin synthase (Cls). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology ([23]), copyright (2012).

for the cross-linkage of pentapeptide precursor molecules into a peptidoglycan cell wall. *Enterococcus* are intrinsically resistant to these antibiotics due to the production of a low-affinity PBPs (PBP5 in *E. faecium*, PBP4 in *E. faecalis*) that bind weakly to β-lactam antibiotics [74]. Moreover, different levels of expression of the gene coding for PBP5 in various *E. faecium* clades correspond to different levels of resistance [75].

Second, due to the inability of the antibiotic to enter the cell, both *E. faecium* and *E. faecalis* are intrinsically resistant to low concentrations of aminoglycosides, a class of antibiotics that act by inhibiting ribosomal protein synthesis [73]. Besides, they can acquire a high resistance to this antibiotic through the inactivation of the antibiotic or the modification of the 16S rRNA, resulting in a lower affinity of the ribosome targeted by the amynoglycosides [76, 76].

In the case of the lincosamides and streptogramins, antibiotics that inhibit the protein synthesis by targeting the ribosome, *E. faecalis* are intrinsically resistant to clindamycin (lincosamide), quinupristin (streptogramin B class) and dalfopristin (streptogramin A class) through the production of an efflux pump [77, 78]. Fortunately, *E. faecium* has a distinct efflux pump, which provides only a low level resistance to streptogramin B class [79]. The *E. faecium* strains that have not acquired resistance to streptogramin A class can still be targeted by the synergistic action of quinupristin-dalfopristin.

The association trimethoprim-sulfamethoxazole targeted the de novo folate synthesis, necessary in most bacteria to produce nucleic acids. *Enterococcus* can absorb folic acid from the environment, thus this antibiotic association is ineffective [73].

Apart from the intrinsic resistances, *Enterococcus* can acquire a broad spectrum of resistance through sporadic mutations or through the acquisition of foreign genetic material. So far, the Horizontal transfer among enterococci has been described via conjugation or bacteriophage promoted transduction [80].

Due to the transfer of mobile elements, resistance to tetracycline and erythromycin is widespread, as well as *Enterococcus faecium* resistant to aminoglycosides [26, 76, 81]. Resistance to aminopenicillins and ureidopenicillins has emerged in *E. faecium* through mutations of PBP5, yielding strains resistant to all β-lactam antibiotics and highly resistant to the cephalosporins [82]. Although infrequent in enterococci, they can also reach a high β-lactam resistance through the production of β-lactamases [83].

Resistance to glycopeptides, such as vancomycin, once optimistically assumed to be unachievable, is now widespread in *E. faecium* in the USA and is increasing in Europe. These antibiotics inhibit the bacterial cell wall synthesis by binding with high affinity to the pentapeptide precursors, preventing their incorporation into the peptidoglycan cell wall [83]. Currently, eight phenotypic variants of acquired glycopeptide resistance in enterococci have been described, two of which have clinical relevance. The first and most prevalent one, VanA, confers a high-level resistance to vancomycin and a variable level of resistance to teicoplanin whereas the second one, VanB, confers variable levels of resistance to vancomycin [83]. Both VanA and VanB are located in transposons, initially identified in plasmids of the pMG1 family, and can be transferred both by plasmids and through conjugative transposition [30, 84]. This resistance is specially worrying as vancomycin is the main option to treat *Enterococcus* infections.

As a result of its intrinsic resistance to various antibiotics and its capacity to acquire resistance to most of the antibiotics habitually used, *Enterococcus faecium* is fully adapted to the hospital environment. Indeed, although they have been reported only occasionally

for now, it has also developed resistance to some newer antibiotics, such as linezolid, tigecycline, and daptomycin, that are used to treat vancomycin-resistant enterococci (Figure 1.3) [68].

Factors of virulence Besides its stunning broad range of antibiotic resistance, *Enterococcus* can also acquire numerous factors of virulence, that can be transferred intra- and interspecifically. A large number of mobile elements have been described in *Enterococcus faecalis*, as summarized in [23, 52, 85]. In the present work we will uniquely refer to those *Enterococcus faecium* mobile elements with a possible implication in virulence, mainly in relation to the capacity of adhesion and the usage of carbohydrates.

Factors of virulence related with the adhesion. *E. faecium* nosocomial isolates are enriched in various functional adhesins, which have been shown to intervene in biofilm formation as well as in in-vivo pathogenicity. The *esp* gene, integrated in a pathogenicity island (PAI), encodes a surface protein with a signal sequence for transport and a LPxTG-like motif for cell wall anchoring. This gene contributes to biofilm formation [68, 86]. Esp does not seem to intervene in gastrointestinal colonization [87] but it has been shown to contribute to urinary tract infection [88] and endocarditis [89] in animal models.

Besides, three collagen binding proteins of the MSCRAMM family (i.e. Acm, Scm and EcbA) have been identified and their activity demonstrated [90–92]. They bind to different subunits of the collagen and fibrinogen. Acm have been demonstrated to intervene in the *Enterococcus* attachment to the heart valve in a rat endocarditis model [93]. In addition, Hendrickx *et al.* [92] uncovered a nidogen-binding LPXTG surface adhesin, SgrA, that binds to the fibrinogen. Moreover, it was demonstrated in vitro that SgrA promotes *E. faecium* adhesion to intestinal epithelial cells although it is not essential [92].

Other factors of adhesion are known in *E. faecium*. Based on the premise that a virulence factor will be regulated by host specific characteristics, Guzmán Prieto *et al.* [94] identified proteins that were thermo-regulated, higher produced at mammal temperature of 37°C. Following this approach, they identified a peptidoglycan-anchored surface protein, PrpA (proline-rich protein A), that (i) binds to the extracellular matrix proteins fibrinogen and fibronectin and (ii) interacted with both non-activated and activated platelets [94].

Finally, in 2010, Sillanpaa*et al.* [95] described the operon ebpABCfm, that encodes a pili implicated in the ability of *Enterococcus faecium* to form biofilm and to cause infection in an ascending UTI model [95].

Thus, none of the factors implicated in adherence have been demonstrated to play a role in intestinal colonization. Their virulence is associated with the biofilm formation and related infections (catheter related infections such as UTI and cardiac valve infections).

Capacity to metabolize carbohydrates as a factor of virulence. In the case of *Enterococcus*, its capacity to metabolize carbohydrates seems related with the virulence. Indeed, recent studies have highlighted that clinical *Enterococcus faecium* strains carry a increased number of genes associated with the usage of amino sugars (e.g., galactosamine), which occur on epithelial cell surfaces and in mucin [96].

The capacity to metabolize carbohydrates can also be acquired through HGT. For example, the PAI where *esp* is located can alternatively carry either elements that encode

a phosphotransferase system (PTS) and a glycoside hydrolase or a gene cluster putatively encoding a pathway for the metabolism of inositol [65]. On a second genomic island, also specifically enriched in clinical strains, *Enterococcus faecium* can display elements putatively implicated in the uptake and metabolism of complex carbohydrate substrates [97].

In addition to the high prevalence of genes associated with the carbohydrate usage in clinical strains, Zhang *et al.* [98] demonstrated that the possibility to use specific carbohydrates influences the capacity of intestinal colonization during antibiotic treatment. Through a fitness competition experiment, they highlighted that a mutant defective in a PTS specifically associated with clinical strains was outcompeted by the wild type. Consequently, they demonstrated the importance of this PTS for the gut colonization in the condition tested, that is to say after an antibiotic treatment.

Finally, the megaplasmid pLG1, specifically enriched in *Enterococcus faecium* clinical isolates, is known to intervene in gastrointestinal colonization under antibiotic pressure and to increase the lethality of *E. faecium* in a mouse peritonitis model [99–101]. However, it is not clear which elements of the pLG1 are responsible for the virulence. The plasmid pLG1 encodes for the pilA/fms21-fms20 gene cluster, that encodes for a pilus structure, and several genes involved in carbon uptake and metabolism, as well as genes that confer resistance to heavy metals and antibiotics [99, 102].

Note that, first, it was thought that the pLG1 mediated virulence was due to the hyl gene. Initially erroneously classified as a hyaluronidase, this gene is likely to encode a family 84 glycosyl hydrolase, which may function as a β-N-acetylglucosaminidase [103, 104]. It was demonstrated that the specific deletion of the hyl gene did not lead to lower virulence in a mouse peritonitis model, which indicates that other genes encoded by the plasmid pLG1 contribute to virulence [104].

In summary, we have seen that *E. faecium* clinical isolates differ from the commensal ones both considering their core and accessory genome. One important difference is the absence of CRISPR-cas system in the clinical isolates, which confers a higher capacity to acquire mobile genetic elements (MGE). These mobile elements are enriched in genes contributing to the antibiotic resistance and virulence. This resistance to several antibiotics allows the survival of *Enterococcus* in the hospital environment, specially of *Enterococcus faecium* which has the highest prevalence of vancomycin resistance, an antibiotic commonly used to threat *Enterococcus* infections. Besides the antibiotic resistance, MGE carry other virulence factors mainly implicated, on the one hand, in the adhesion and biofilm formation and, on the other hand, in the metabolism of carbohydrates. Importantly, the enhanced metabolism of carbohydrates has been demonstrated to influence the capacity of intestinal colonization during an antibiotic treatment. As we will discuss along this work, antibiotics treatments alter the gut microbiota composition. Thus, the virulence factors acquired by multiresistant *Enterococcus* provide the opportunistic bacteria with a competitive advantage in this altered environment.

1.2.5 Infection mechanisms

Enterococcus is normally present in low abundance in the gastro-intestinal tract. In case of being a resistant strain, this opportunistic pathogen can expand upon antibiotic treatment, prescribed for an unrelated disease. It has been described that antibiotics,

especially the broad spectrum ones, alter the microbiota and disrupt its protective capacity against the colonization of external bacteria (this capacity is known as "colonization resistance") [105, 106]. Thus, the antibiotic treatment enables the expansion of VRE to high loads in the intestine and, as it is resistant to harsh environmental conditions, it can spread through fecal contamination of the hospital environment, including inanimate objects and the hands of health-care workers and visitors. Indeed, the application of the standard hospital hygiene protocols does not totally prevent a cross-contamination between patients (Figure 1.4A) [23].

Starting from a heavily intestinal colonized patient, VRE can colonize extra-intestinal sites, such as catheters, from the same patient or others, causing an infection (Figure 1.4B) [23]. Thus, it is important to understand the factors that can promote the VRE intestinal expansion, such as the alterations of the microbiota, in order to prevent its spread to the hospital environment and avoid infections in susceptible patients. Indeed, this is one of the main objective of my thesis, as I will explain later.

In the event that the intestinal expansion of VRE could not be prevented, the bacteria can contaminate the skin or form biofilms on catheters. As we have mentioned in the previous section, the VRE clinical isolates are indeed enriched in various virulence factors implicated in the adhesion and the formation of biofilms. Then, the enterococci can spread from the infected catheter, producing various types of enterococcal infection, the most common one being the urinary tract infection [107]. Starting from this focal point, the bacteria can disseminate to the blood, causing a bacteriemia.

Nosocomial enterococcal bacteremias are considered to be mainly acquired from intravascular or urinary catheters [83]. In the US, this pathogen is responsible for 18% of all central line-associated bloodstream infections (CLABSIs), ranking second overall [83]. In addition to CLABSIs, other paths could lead to bloodstream infection (i.e. direct dissemination from the gut to the bloodstream).

Kamboj *et al.* [108] showed that VRE can be detected in blood without being detected in the catheters of patients with neutropenia due to cytotoxic chemotherapy [108].

The chemotherapy used in these patients, such as cyclophosphamide, causes both neutropenia and damage the intestinal mucosa [109, 110]. Thus, the pathogen could translocate directly from the damaged intestine to the blood.

Indeed, Miyazaki *et al.* demonstrated that the concomitant administration of an antibiotic mix and of cyclophosphamide increased the translocation of VRE to MLN, blood, liver and spleen of VRE orally infected mice. This result was not observed when only antibiotics or cyclophosphamide were administered. Thus, the cumulative effect of the antibiotic-driven increase of intestinal VRE load and chemotherapy administration were necessary to cause a translocation of VRE from the gut to the systemic level [111]. Similarly, the intestinal domination by VRE precedes the bloodstream infection in patients treated with a cytotoxic chemotherapy [112, 113].

In summary, VRE can reach high intestinal levels upon an antibiotic treatment. This high VRE level can lead to a bacteremia through fecal contamination of catheters or directly by translocation from the intestine in patients undergoing a cytotoxic chemotherapy. Finally, bacteremia by *Enterococcus* can lead to endocarditis, one of the most serious enterococcal infections [83, 107] .

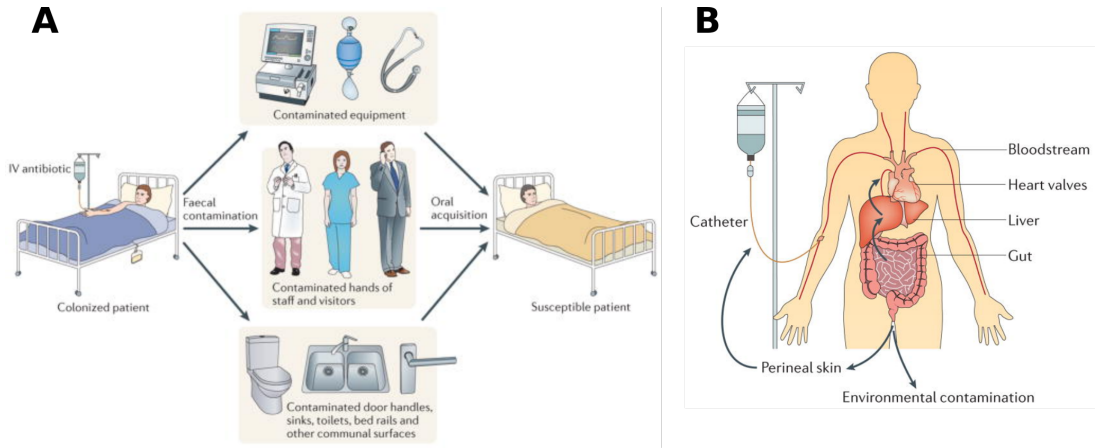


Figure 1.4: Major routes of nosocomial VRE transmission and infection. (A)The main risk for colonization and subsequent nosocomial infection with vancomycin-resistant enterococci (VRE) include close physical proximity to patients who are infected or colonized with VRE (or to the rooms of these patients); a long period of hospitalization; hospitalization in long-term facilities, surgical units or intensive-care units; the presence of a urinary catheter; and the administration of multiple courses of antibiotics. Antibiotic treatments increase the density of VRE organisms in the gastrointestinal tract, which, in turn, facilitates the spread of these organisms through fecal contamination of the hospital environment, including inanimate objects and the hands of health-care workers and visitors. Enterococci can survive for long periods on environmental surfaces, including medical equipment, toilets, bed rails and doorknobs, and are tolerant to heat, chlorine and some alcohol preparations. (B)Enterococci from the gastrointestinal tract can access the bloodstream by moving across the intestinal lining and passing through the liver. In the bloodstream, these organisms can reach the heart and then potentially cause infective endocarditis. Fecal contamination of the environment (which can then be a source for colonization of other patients) and of the patient's skin (the main source of infections of the urinary tract and of intravenous catheters) frequently occurs. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [23], copyright (2012).

1.2.6 Treatments

The nowadays guidelines on pharmacological treatment for VRE recommend the use of ampicillin monotherapy for any ampicillin-susceptible VRE infection that does not require bactericidal activity, inclusive for bacteremia. If bactericidal activity is required for the treatment of an endovascular infection, a synergistic combination of a β-lactam with an aminoglycoside (gentamicin or streptomycin) is recommended.

In case of serious ampicillin and vancomycin-resistant enterococcal infections with high-level resistance to aminoglycosides, the treatment options are very limited. The only treatment left are daptomycin, linezolid, tigecycline, the combination of quinupristin/dalfopristin or, to treat urinary tract infection, nitrofurantoin and fosfomycin.

Worryingly, resistance to these antibiotics, used to treat VRE, has already been reported [68, 73]. Thus, another option is to target specific mechanisms of VRE necessary for the colonization of the host. For example, Flores-Mireles *et al.* [114] produced an

antibody specific for a pili of *Enterococcus faecalis* limiting the formation of biofilms in catheters.

Considering that the first step of the VRE infection is the gut colonization, another approximation would be to reduce the intestinal expansion responsible for the latter transmission and infection.

As we have already commented, the VRE intestinal growth relies on a previous disruption of the microbiota. So, to tackle this problem different approaches are being considered. In one hand, new drugs, more selective for a specific pathogen, are being designed [115, 116]. On the other hand, it should be possible to restore the colonization resistance normally proportionated by the healthy microbiota. Indeed, some bacteria, mainly from the genus *Lactobacillus*, have been tested as non-antibiotic strategies against vancomycin-resistant enterococci in mammals [117, 118].

In the present work, we take advantage of the recent advances in the techniques and knowledges concerning the microbiota to analyze how antibiotic treatments affect the gut microbiota and which commensal bacterial taxa could effectively inhibit VRE colonization after an antibiotic treatment. A main part of my thesis will be the identification of these taxa as well as the mechanisms of inhibition implicated.

1.3 Microbiota

1.3.1 Description

The microbiota is the collection of microorganisms, including bacteria, viruses, fungi and single-cell eukaryotes that inhabit a particular habitat, such as our Gastrointestinal tract. In the present work, we will study the most abundant fraction of the gut microbiota, the bacteria, later referred also as "microbiota".

The microbiota composition has been thoroughly studied across the whole body, including the gut, stool, several locations of oral cavity, skin, airway and the vagina [119–122]. Importantly, alterations in its composition (dysbiosis) are associated with an ample panel of diseases such as obesity [123, 124], tumorigenesis in genetically predisposed individuals [125], psoriatic arthritis [126], inflammatory bowel disease (IBD) [127, 128], diabetes type 2 [129], alteration of mental health [130, 131] or periodontitis [132].

The gut microbiota, the most densely populated part of our body, has some specific characteristics. First, it is a site of interaction with the external environment through the diet. In this regard, the microbiota is essential because the complete genomic composition of this microbial community (microbiome) encodes metabolic functions that we do not express on our own, improving our ability to extract energy and nutrients from our diet [133]. Consequently, the kind of diet and microbiota we have will determine the metabolites that our organism absorb through the gut and can have systematic repercussions through the modulation of the immune system. For example, gut microbiota metabolism of dietary fiber has been demonstrated to influence the allergic inflammation in lung [134, 135]. As we will later discuss, numerous factors can deeply influence the microbiota composition and affect the host.

1.3.2 Healthy state

As we mentioned in the section 1.3.1, a dysbiotic gut microbiota has been related to several diseases. Thus, it is important to identify what is actually a healthy microbiota.

This is a complicated question to deal with. Indeed, the microbiota composition exhibits a relatively high intra-individual stability over wide scale period but the inter-individual variability is very important [136]. At a broad scale, the phyla Bacteroidetes and Firmicutes usually dominate the microbiota of adults, whereas Actinobacteria, Proteobacteria and Verrucomicrobia, although found in many people, are generally minor constituents. Methanogenic archaea (mainly *Methanobrevibacter smithii*), eukaryotes (mainly yeasts) and viruses (mainly phages) are also present [137] .

In a first attempt to define a core set of species in the adult microbiota, it was determined that *Faecalibacterium prausnitzii, Roseburia intestinalis* and *Bacteroides uniformis* were present in almost all the individuals, but in some individuals even these represented less than 0.5% of the microbiota [121, 137–140]. Given the huge variability in the species present, and their relative proportions across individuals, besides variations at the strain level [140], the existence of a phylogenetic core community in human gut is very unlikely.

The second approach to determine a few clearly distinguishable stable states in the healthy human population, was obtained with the definitions of the enterotypes. Analyzing 39 fecal samples, the authors stratified the human microbiome into three distinct host–microbial symbiotic states driven by groups of co-occurring species and genera, which are characterized by a relatively high representation of *Bacteroides*, *Prevotella* or *Ruminococcus*. They were able to stratify the subjects included in the study in three specific groups although being very variable both regarding their nationality as well as their physiological condition (i.e. healthy, obese or elderly and two subjects had IBD), thus the results could be extrapolated to the population [141]. However, a subsequent study performed with a higher number of healthy adults failed to show the same pattern [136]. Now, it is accepted that the enterotypes are not defined states but a gradient of variation between different types of composition. It seems that gut bacterial community structure is driven by greater abundance and high variability in populations of *Prevotella* and *Bacteroides* against a diverse background assemblage of Firmicutes. Particularly, *Prevotella* and *Bacteroides* are nearly mutually exclusive in communities dominated by Bacteroidetes [139] (Figure 1.5).

Another important feature of a healthy microbiota could be the absence of specific bacteria known to be detrimental to the host. Taking as unwanted the bacteria listed by the National Institute of Allergy and Infectious Disease (NIAID), the Human Microbiome Project Consortium [136] confirmed the absence of NIAID class A–C pathogens above 0.1% abundance (aside from *S. aureus* and *E. coli*) from the healthy microbiome of 242 adults. They also discarded, for the level of sensitivity of the study, the presence of canonical pathogens including *Vibrio cholerae, Mycobacterium avium, Campylobacter jejuni*, and *Salmonella enterica*. Nonetheless, they detected 56 out of the 327 opportunistic pathogens defined in PATRIC database (at >1% prevalence of >0.1% abundance) [136,143,144]. According to these results, a healthy microbiota can be defined by the absence of particularly detrimental microbes [136].

This study also demonstrated that, despite the highly divergent composition of gut microbiota across individuals, the functional gene profiles are quite similar [121,136]. Given

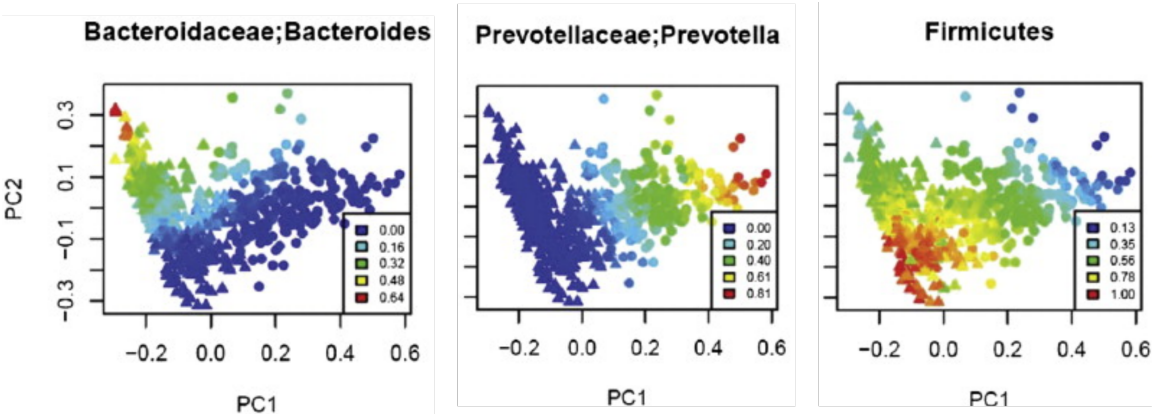


Figure 1.5: Enterotypes represent a continuum with regard to the relative abundance of dominant taxa. Each circle represents a single individual, and the data in each panel are identical but have been color-coded based on the relative abundance of *Bacteroides* (phylum Bacteroidetes) (left panel), *Prevotella* (phylum Bacteroidetes) (middle panel), or Firmicutes (right panel). From this analysis, it is apparent that the subjects do not cluster into three distinct enterotypes but instead represent a continuum with regard to the relative abundance of dominant taxa. Reprinted from Cell Host & Microbe, 12/5, Fredrik Bäckhed,Claire M. Fraser,Yehuda Ringel,Mary Ellen Sanders, R. Balfour Sartor,Philip M. Sherman,James Versalovic,Vincent Young,B. Brett Finlay, Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications, 611-22, Copyright (2012), with permission from Elsevier. This figure was already an adaptation of [142].

that individuals share genes encoding specific metabolic functions, or clusters of orthologous groups, possibly a healthy microbiota could be in reality defined by a functional core microbiome or core microbiome-encoded gene set [139, 140, 145]. Both a dysbiotic and a healthy microbiota should express some basic functions, namely house-keeping functions necessary for individual microbial life (i.e. transcription, translation, energy production, and structural components) and the functions specific to microbes' niches in the human ecosystem such as adhesion to host cell surfaces. Unlike a dysbiotic microbiota, a healthy "functional core" should also express the genes necessary for the production of compounds implicated in host–microbe interaction (including essential vitamins, such as vitamin K, and immunostimulatory compounds) [140].

As it was already described that the microbiota displays an important variability across the human body-site habitats related with different environmental characteristics [136], it is logical that different body habitats each have their own specialized core functions [146]. For example, in the gut, core functions include glycosaminoglycan biodegradation, the production of several short-chain fatty acids, enrichment for specific lipopolysaccharides, and the production of vitamins and essential amino acids [140].

Thus, if we recapitulate the characteristics of a healthy microbiota, it does present a huge phylogenetic variability in the population and a relatively conserved functional core. At the individual level, a healthy microbiota is usually very stable. The microbiota composition changes with the modification of the diet and host conditions, but the range of this modifications is reduced in the absence of an important disruptive factor. Indeed,

the uniqueness of each individual's microbial community appears to be stable over time relative to the population as a whole [136, 139]. To adapt to the changing conditions and, in the same time, maintain its stability over time, the microbiota must (i) have the capacity to come back to an initial state upon a perturbation and (ii) be able to resist to colonization by external bacteria [137, 139, 140] These important properties of the microbiota are known as the resilience and the resistance.

The resilience of the microbiota rely upon the functional response diversity, that is to say the degree to which species that contribute to the same function in an ecosystem vary in their sensitivity to ecosystem changes. As we have seen, the human microbiota displays a huge phylogenetic diversity in its composition whereas the functional core is more conserved. This implies that phylogenetically disparate microbes, with different resistance to antibiotics and other factors susceptible to affect the microbiota, often perform similar metabolic functions. In consequence, the functional core will be preserved upon a partial alteration of the microbiota composition, maintaining the existing metabolic network. Thus, a high diversity is an important feature of a healthy microbiota as it is necessary for its resilience.

On the other hand, species-rich communities are less susceptible to invasion because they use limiting resources more efficiently [137]. Thus, the richness would also be a characteristic of a healthy microbiota. Indeed, a low richness is associated with various diseases, such as obesity, IBD (inflammatory bowel disease) and the loss of colonization resistance upon an antibiotic treatment, which we will study further in the present work [147–149].

So far, we have highlighted several important characteristics of a healthy microbiota. Nevertheless, the definition of such a state is difficult. Indeed, the microbiota can be made up of bacteria normally present according to our definition and still cause a disease in the host. The bacteria implicated in such a relation, the pathobionts, are commensal bacteria that, due to the specific condition of the host or to the abnormal proportion they reached, drive disease through the disregulation of the mucosal immunity and the disruption of the mucosal barrier [150].

To sum up, a healthy microbiota could be defined by a site-specific functional core which implies a high richness and diversity that contribute to the stability of the ecosystem. The absence of specific pathogens is key, as well as the adequate interaction of the microbial community with the host.

This observation has several implications. In the absence of a taxonomic defined core microbiota, and given that the healthy state of the microbiota relies on its adequate interaction with the host, a microbiota can be defined as healthy only in relation with the specific background of its host. Thus, the transplant of the complete microbiota from a healthy donor, as done to treat *Clostridium difficile* infection, could result deleterious for the receptor. The analysis of the microbiota changes totally our way to apprehend several diseases and the possibility to manipulate it broadens our treatment options. However, it is primordial to study the mechanisms and act cautiously as an alteration of the microbiota can have important consequences for the host.

1.3.2.1 Differences in the gut microbiota by sites

When considering the gut microbiota, we have to take into account that it is constituted by different ecosystems.

The host absorbs nutrients in the small intestine. For this reason, the host has developed several mechanisms to limit the growth of bacteria that would compete for the usage of these nutrients. For example, the small intestine has a higher concentration of oxygen, a low pH and secrete several antimicrobial agents, including the bile, that inhibits the bacterial growth. Moreover, the transit time is shorter than in the colon and therefore, bacteria need to express factors that allow their adherence to the epithelium or mucus to colonize this part of the intestine. In these conditions, the bacterial load is smaller in the small intestine and mainly include fast-growing facultative anaerobes (i.e certain *Clostridium* spp. and Proteobacteria in the human whereas in mice Proteobacteria and Lactobacillaceae are enriched) [120].

In the cecum and the colon, the reduced availability of simple carbon sources facilitates the growth of fermentative polysaccharide-degrading anaerobes. Humans have a small pouch-like cecum where the appendix is attached, whereas the mice have a bigger one. Due to their relative isolation and protection to disruptive factors, the appendix, as well as the crypts of the colon, have been hypothesized to play a possible effect in re-seeding bacteria [120].

Both the cecum and the colon harbour strictly anaerobic fermentative bacteria and, although very similar, the cecum is enriched in species of the families Ruminococcaceae and Lachnospiraceae whereas the colon is enriched in members of the Bacteroidaceae and Prevotellaceae (Figure 1.6) [120, 151].

As well as the variation in microbial community composition longitudinally within the gut, various host factors drive community differences over the cross-sectional axis of the gut. Throughout the small intestine and colon, specialized epithelial cells called goblet cells secrete a mucus layer of varying thickness, being organized as a single layer in the small intestine and in two layers in the colon. In the large intestine, the external mucus layer serves as nutrient source for certain bacteria and, in comparison with the central lumenal compartment, the colonic mucus is enriched in mucin degrader bacteria, such as various *Bacteroides* species or *Akkermansia muciniphila*. The inner layer, denser, serves as a physical barrier for microorganisms. This effect is reinforced by the secretion of antimicrobial molecules and oxygen from the epithelium. Moreover, the mucus is continuously secreted and the outer layer is constantly renewed and eliminated in the feces. As a result, the inner mucus layer present almost no bacteria. However, some symbionts capable both to modulate the immune system and use specific host-derived nutrients, such as *B. fragilis*, have already been described entering crypts of the proximal colon of healthy mice [120].

In the present work we analyze fecal samples to determine the compositional alteration of the gut microbiota. The microbiota composition of feces is much more similar to that of the colon than to the one of the small intestine. The analysis of fecal samples gives us an approximation of the alterations that occur in the cecum and colon without the necessity to sacrifice the mice. As I will describe in chapter 2, this condition is essential in our experimental design because it allows us to analyze the microbiota composition and to latter infect the same mouse with a pathogen. Second, one of the main objectives

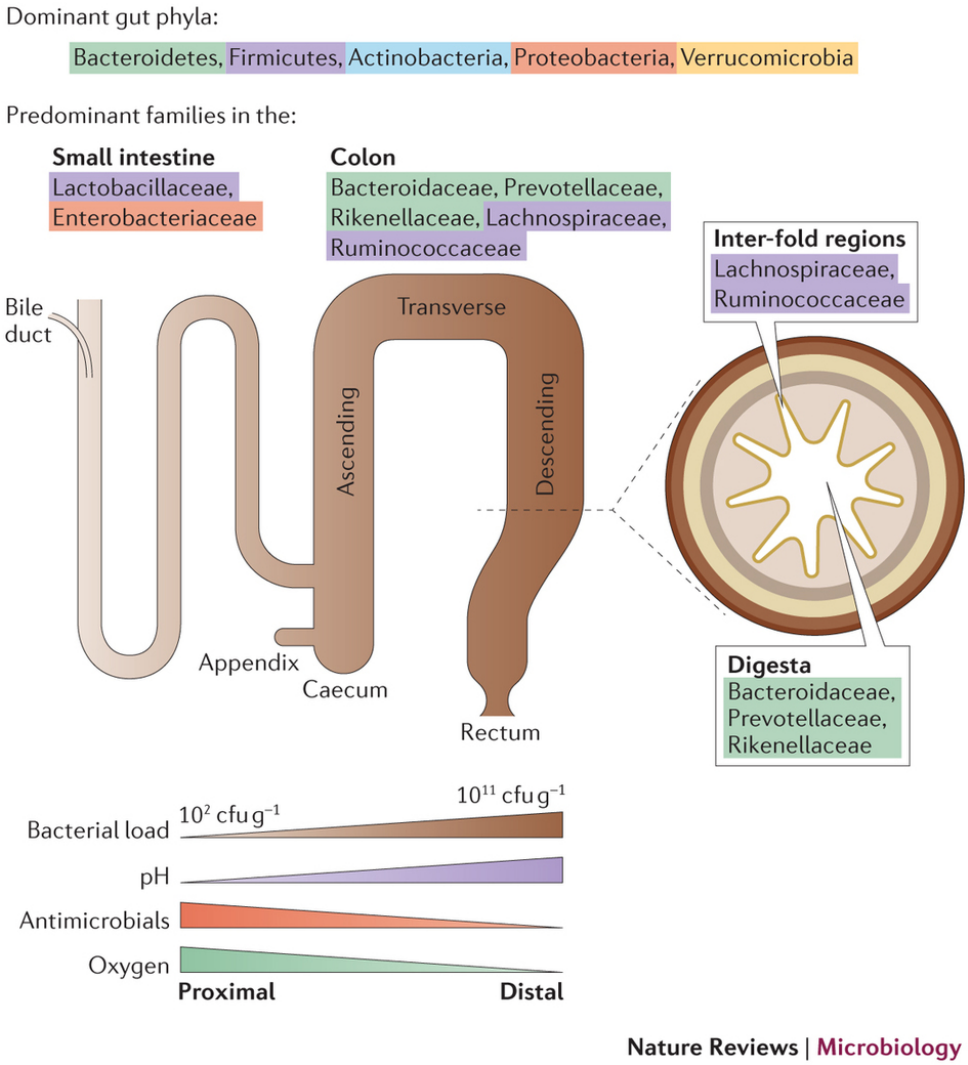


Figure 1.6: Microbial habitats in the human lower gastrointestinal tract. The dominant bacterial phyla in the gut are the Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the gut. For example, a gradient of oxygen, antimicrobial peptides (including bile acids, secreted by the bile duct), and pH limits the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. In the small intestine, Lactobacillaceae and Enterobacteriaceae dominate, whereas the colon is characterized by the presence of Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae. A cross-section of the colon shows the digesta – which is dominated by Bacteroidaceae, Prevotellaceae and Rikenellaceae – and the inter-fold regions of the lumen – which are dominated by Lachnospiraceae and Ruminococcaceae. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [120], copyright (2015)

of the present work is the identification of bacteria that inhibit the intestinal colonization by VRE to prevent the contamination of the hospital environment. Thus it is logical to analyze the intestinal compartments that have the highest bacterial load and the highest probability of interaction between bacteria.

Finally, we used cecal samples to determine the mechanisms of inhibition against VRE. Both the cecum and the colon exhibit a similar composition but the murine cecal content is greater than the one from the colon. Indeed, the cecum contains the critical amount of sample necessary to perform both a metabolic and meta-transcriptomic study analysis, that were also performed in this thesis.

1.3.3 Functions of the microbiota

The microbiota affects the host by the production of specific metabolites and by the presentation of microbial-associated molecular patterns (MAMPs), such as lipopolysaccharides (LPS), to the immune system [152–154]

For example, Short-Chain Fatty-Acids (SCFAs), derived from the bacterial fermentation of fibers, feed enterocytes, modulate immune functions and influence the host metabolism [155, 156]. Commensal bacteria also transform primary bile acids into secondary bile acids and produce vitamins of the B and K groups [155]. Overall, the microbiota influences substantially the metabolites landscape and its composition can have systemic consequences.

The systemic effects of the microbiota are illustrated by its influence on autoimmune encephalomyelitis, sclerosis multiple, crohn disease, respiratory diseases or metabolic diseases, as we have just mentioned. [127, 131, 135, 157]

Part of the effects of the microbiota are due to its interaction with the immune system. The microbiota is at the interface with the immune system and has been demonstrated to participate in its maturation. It is now known that during pregnancy, the mother's microbiota already participate in shaping the fetus gut mucosal immune system through the transfer of molecular signals through antibodies transport [158]. After birth, in case of breast-feeding, the transfer of IgA, bacterially loaded dendritic cells and several metabolites have been proposed to contribute to neonatal immune imprinting [159]. It is also evident from germ-free mouse models that normal immune development is dependent of the presence of commensal microbiota, particularly in the gastrointestinal tract. For example, germ-free mice show an altered immune phenotype, with deficits in both innate and adaptive immune components of the gut mucosa [160, 161].

The microbiota participates in the maturation of the immune system and also interacts with it on a daily basis. To maintain the homeostasis at the gut level, it is important to segregate the bacteria from the host. This is achieved first by the production of a thick mucus layer by the goblet cells as well as the secretion of antimicrobial peptides by the epithelial cells and expression of IgA specifics against the commensals by B cells [159]. The presence of the microbiota is necessary for the generation of these defenses. For example, the lipopolysaccharides (LPS), constituents of the cell wall of gram-negative bacteria, induce the production of antimicrobial peptides by the epithelial cells [162]. The homoeostasis is a dynamic state that requires constant adaptation to the fluctuations of the gut environment. Thus, the host has developed several mechanisms to regulate its interaction with the microbiota. Indeed, the commensals bacteria are in constant contact

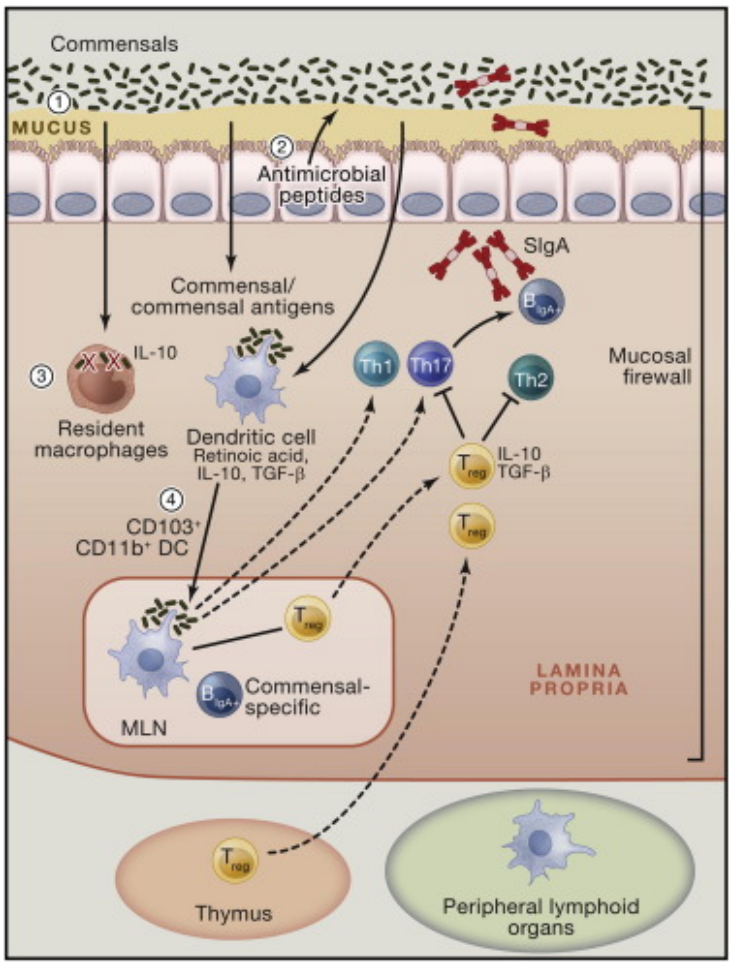


Figure 1.7: The mucosal firewall. (1) The mucus represents the primary barrier limiting contact between the microbiota and host tissue preventing microbial translocation. (2) Epithelial cells produce antimicrobial peptides that also play a significant role in limiting exposure to the commensal microbiota. (3) Translocating commensals are rapidly eliminated by tissue-resident macrophages. (4) Commensals or commensal antigens can also be captured by CD103+ CD11b+ DCs that traffic to the mLN from the lamina propria but do not penetrate further. Presentation of commensal antigens by these DCs leads to the differentiation of commensal-specific regulatory cells (Treg), Th17 cells, and IgA-producing B cells. Commensal-specific lymphocytes traffic to the lamina propria and Peyer's patches. In the Peyer's patches, Treg can further promote class switching and IgA generation against commensals. The combination of the epithelial barrier, mucus layer, IgA, and DCs and T cells comprises the "mucosal firewall," which limits the passage and exposure of commensals to the gut-associated lymphoid tissue, preventing untoward activation and pathology. Reprinted from Cell, 157/1, Yasmine Belkaid,Timothy W. Hand, Role of the Microbiota in Immunity and Inflammation, Pages No. 121-141 , Copyright (2014), with permission from Elsevier.

with the immune system of the host through the translocation of antigens or complete bacteria to the lamina propria. Next, these antigens can be directly eliminated by resident macrophages or sampled by dendritic cells and transferred to the mesenteric lymph nodes where they will trigger (i) the differentiation of B cells for the production of IgA specific against commensals and (ii) the proliferation of Treg cells as well as Th17 cells. Treg cells are important producer of IL-10, a powerful anti-inflammatory cytokine and Th17 cells produce IL-17 and IL-22. IL-22 is especially important for the gut homeostasis as it does promote the regeneration of epithelial cells as well as it induces the production of antimicrobial peptides and mucus (figure 1.7) [159].

Thus, the commensal microbiota interacts with the host immune system and promotes its own containment, which prevents the development of an excessive inflammatory response by the host [163].

Due to the importance of the microbiota and its possible alteration by numerous factors, it was suggested, as a continuity of the "hygiene hypothesis", that the increase in the prevalence of metabolic diseases such as diabetes type 2, IBD, allergy, etc. in the western countries could be due to the type of diet consumed and the administration of numerous antibiotic treatments which promote a low microbial diversity in these populations. It was named the “disappearing microbiome hypothesis” [140].

1.3.4 Factors that influence the gut microbiota composition

As we mentioned during the description of a healthy microbiota, its composition is subjected to a huge inter-human variability. Numerous factors intervene to shape the microbiota. First, the delivery mode will influence the early colonizers of the new-born gut, vaginally delivered newborns having a microbiota similar to their mother vaginal microbiota and cesarean delivered ones will have a microbiota more similar to the mother's skin microbiota [164]. Independently of this first colonization, it seems that by 6 weeks the infant adopt a site-specific microbiota and that the delivery mode does not allow to segregate both groups anymore [165].

Then, the feeding method will shape the development of the microbiota. On the one hand, the breast-feeding allow the transfer of antibodies from the mother, including Proteobacteria-specific IgAs that have been proven to regulate the maturation of the intestinal microbiota [166]. On the other hand, milk oligosaccharides cannot be used by newborns for nutrition and also serve to guide the composition of the developing infant gut microbiota (reviewed in [167]). For this reason, the preponderance of *Bifidobacterium* species during the first year of life is explained by their capability to break down milk oligosaccharides [167].

Clearly, the genetics of the host influence also the construction of the microbiota community through a cross-talk between the immune system and the microbiota. Like so, it is described that genetically modified mice defective in different parts of the immune system present an altered microbiota [168].

The diet is probably one of the most important and most studied factors that normally shape our microbiota. It can promote rapid shifts in the microbiota, but a recuperation is achievable by a return to the old diet [169]. The influence of the high fat/high sugar western diet on the microbiota composition and its relation with various diseases has been especially studied. It has been seen, for example, that the combination of both the

dysbiosis related to a western diet and a susceptible phenotype exert a cumulative effect that alters host barrier function favouring adherent-invasive *Escherichia coli* colonization. This phenomenon triggers an inflammation response in the host that could intervene in the onset of inflammatory bowel diseases [170]. Similarly concerning, the interaction between high-fat-diet induced dysbiosis and a susceptible phenotype were also demonstrated to promote intestinal carcinogenesis [125].

All these factors act on a relatively long time-scale. On the opposite, antibiotic administration, as reviewed by multiple authors [155,171,172], modify drastically the microbiota, overcoming its resilience capacity and rapidly promoting a dysbiosis with possible metabolic consequences and lost of colonization resistance.

The tremendous effect of antibiotics on the gut microbiota is illustrated by one of the earliest studies in which high throughput sequencing was applied. They explored the impact of a short-course ciprofloxacin treatment on the gut microbiota from three healthy adults [173]. After administration of a standard posology for the treatment of urinary tract infections, the authors detected a dramatic, albeit differing, impact of ciprofloxacin treatment on the relative abundance of 30% of taxa in the gut.

Other studies compared the effect of antimicrobial agents of different spectrum of action on the composition of the microbiota. Rea *et al.* [174] saw that vancomycin and metronidazol provoked a diminution in the richness and the diversity in a human distal colon model. Both treatments resulted in a decrease of the phyla Firmicutes and Bacteroidetes, with a corresponding increase in Proteobacteria, although in distinct proportions. Compared to the relative abundance present in untreated mice, the Firmicutes decreased from 67.7% to 28%(vancomycin) and 20%(metronidazol), whereas the corresponding shift in Bacteroidetes was from 19.6% to 4.2% (vancomycin) and 0.17% (metronidazol). Proteobacteria assignments increased from just 5.1% in the control to 74%(metronidazol) and 55% (vancomycin). Overall, these results demonstrate that antibiotics of different spectrum cause different alterations on the microbiota.

The antibiotic-driven alterations of the microbiota have several consequences, the most direct one being the loss of the colonization resistance as we will see in more details in another section of this thesis. To give an example, *C. difficile* infection are treated by the administration of vancomycin or metronidazol. This antibiotic treatment present secondary effects itself as the patient can undergo a recurrence of the *C. difficile* infection or an infection by another bacteria, such as VRE, the opportunistic pathogen studied in the present work. In this case too, the treatment by vancomycin or metronidazol impacts differently the gut microbiota. Lewis *et al.* found that mice treated with metronidazol alone experienced relatively transient disruptions in their fecal microbial communities, and returned to a state similar to pretreatment composition by 1–2 weeks after stopping the treatment. In contrast, animals treated with vancomycin or metronidazol plus vancomycin experienced a profound shift in their microbiota composition, with taxa such as Bacteroidales falling permanently below the limit of detection. In parallel, bacterial species present at low or undetectable levels before antibiotics expanded greatly, including members of the genus *Enterococcus*, unclassified Proteobacteria, and members of *Lactobacillus* and *Clostridium* genus [175]. As a consequence of the difference in the alteration of the gut microbiota, metronidazol and vancomycin affected differently the colonization ability of vancomycin-resistant *Enterococcus*, carbapenem-resistant *Klebsiella pneumoniae*, and *Escherichia coli*. Mice recovered the colonization resistance against these pathogen seven

days after the withdrawal of metronidazol whereas the pathogens still highly colonized vancomycin-treated mice, as long as 14 days after completing the treatment.

The drastic perturbations of the microbiota caused by antibiotics treatment can also have systematic consequences on the host. As described in the section 1.3.3, the commensal microbiota promote the maturation of the host immune system. In consequence, perinatal antibiotic exposure can also have long-lasting consequences and is indeed related with exacerbated allergic syndromes [176]. On the other hand, in a previous section we also described the influence of the microbiota on the metabolic landscape of the host, including the production of short-chain fatty acids (SCFAs) that modulate immune functions [155]. Alteration of the microbiota that lead to a diminution of SCFAs-producers, as seen during vancomycin treatment, disrupt the microbiota-host relationship. It was indeed demonstrated that treatment with antibiotics leads to a subtle elevation of some inflammatory markers in the intestinal mucosa [172].

Similarly, it has been described that an antibiotic treatment alters the metabo-lic profile of fecal samples, such as the bile acid metabolism, the metabolism of steroid hormones and the eicosanoid synthesis pathway. These alterations can have systemic consequences as steroids and eicosanoids are important inflammatory mediators and have been implicated in immunological responses to infection [177].

As mentioned, antibiotic treatments cause drastic alterations on the microbiota composition and, moreover, these alterations can be long-lasting. For example, one study reported that healthy volunteers treated with clindamycin experimented alteration of the gut microbiota lasting as long as 6 months to 2 years after the treatment. The alteration included a dramatic loss in diversity as well as in representation of specific taxa, apparition of antibiotic-resistant strains, and upregulation of antibiotic resistance genes [178]. Similarly, antibiotic treatment in mice recapitulates the impact and long-term shifts in human gut communities. For example, a single dose of clindamycin has been shown to induce profound changes in mouse microbiota composition and, consequently, to confer long-lasting susceptibility to *C. difficile* infection [179].

Most of the antibiotics used nowadays are broad-spectrum. Thus, they alter the microbiota community to such an extend that they exceed its resilience capacity. Another potential explanation for the magnitude and duration of antibiotic effects in vivo is the interdependence of different bacterial taxa. That would explain the observation that gram-negative commensals can be depleted by vancomycin, which is a gram-positive targeting drug [112].

In summary, antibiotic treatments affect the composition of the microbiota. Theses alterations change the metabolites landscape of the intestine, with important consequences for the host physiology. In addition, antibiotic treatment can cause long-lasting effects on the intestinal microbiota, far beyond the actual period of administration of the antibiotic.

In the present work, we are specially interested in the alterations caused by vancomycin as this antibiotic is associated with VRE infections. Nowadays, most studies performed in humans included patients that already received a previous treatment, thus, the specific effect of vancomycin cannot be inferred. In addition, the long-lasting effects of this antibiotics are not known. Consequently, one of our objectives in the present work will be to explore on a long scale time the alterations caused by vancomycin treatment in humans that had not received previous antibiotic treatment.

1.3.5 Relevance for infectious diseases

The microbiota, besides its preponderant role in the metabolism of the host, also protects it from infectious diseases. The capacity to prevent the colonization by canonical or opportunistic pathogens is known as the colonization resistance.

The microbiota exerts that colonization resistance by direct interaction with the bacteria present in the same ecosystem or by stimulation of the host immune defenses.

1.3.5.1 immune independent

The immune independent mechanisms rely on the direct interaction of the microbiota with the pathogenic bacteria. The cecum is a highly competitive environment, there is an important pool of nutrients but also an important community of bacteria with very varied functional capacity. Thus, one mechanism by which the microbiota inhibit the colonization of external bacteria is the nutritional competition.

For example, *E. coli* O157:H7 infections can be prevented in mice that have been pre-colonized with two commensal *E. coli* strains (HS and Nissle 1917) that compete for the same sugars [180]. Each of these commensal strains can utilize some but not all of the five most important sugars consumed by the pathogen in the gut. Thus, if only one strain is present, some sugars are still available and the pathogen can grow but in case of the concomitant administration of both commensal strains, all five sugars are depleted, preventing gut colonization by *E. coli* O157:H7. This example is specially interesting for us, as it illustrates the possible necessity to combine several bacteria to achieve the inhibition of colonization through a nutritional competition mechanism.

Consistent with the effect that the microbiota has on resistance to infection through nutrient depletion, antibiotic clearance of commensal bacteria can lead to pathogen expansion through liberation of nutrient sources. This mechanism was demonstrated in the case of *Clostridium difficile* infection. Upon an antibiotic treatment, commensals that liberate sialic acid from the mucus survived while numerous bacteria that were using it disappear. In this condition, *C. difficile* take advantage of the newly available nutrient source to expand [181].

In the case of *Enterococcus*, it is known that in normal conditions the commensal strains outcompete the hospital specialized one (VRE) [182]. Indeed, VRE is unable to colonize an undisturbed gut and it relies on the alteration of the microbiota, for example due to an antibiotic treatment, to reach high intestinal levels [112].

As it is known that VRE is not able to ferment complex polysaccharides but can use various monosaccharides, Pultz et al. [183] investigated if VRE could, similarly to *Clostridium difficile*, use simples sugars liberated by commensals and made available upon an antibiotic treatment. Using the enzymatic extract of *Ruminococcus torques* or of human fecal sample to digest mucus and other complex polysaccharides that could be found in the intestine, they determined that VRE was able to grow on the carbohydrate fragments liberated. In this case, however, it is not known if the antibiotic treatment will increase the availability of these carbonhydrates, subsequently promoting VRE growth.

Another mechanism by which the microbiota confers protection is through the direct antibiosis. Commensal strains can produce and secrete small molecules with bacteriostatic or bactericidal activity, such as bacteriocins or microcins produced by gram-positive and

gram-negative Enterobacteriaceae species, respectively [184,185]. For example, a commensal *Enterococccus faecalis* strain is capable of clearing vancomycin-resistant *Enterococcus* (VRE) from the intestinal tract of mice through the expression of a plasmid-encoded bacteriocin [184]. In the case of gram-negative bacteria, *Escherichia coli* strain Nissle 1917 produce microcins that reduces intestinal colonization by *Salmonella enterica* serovar Typhimurium [185].

On the other hand, the intestinal microbiota may also confer resistance through the production of short-chain fatty acids (SCFA) which, in vitro, markedly suppress the growth of the pathogenic *E. coli* strain O157:H7 [186]. The SCFA, as well as some bacterial products such as the quorum-sensing (QS) signal AI-2, can also influence the colonization capacity of pathogens by altering the expression of virulence genes [187, 188]. For example, the concentration of different SCFAs regulate the expression of *S. typhimurium* pathogenicity island 1 (SP1), required by this pathogen for invasion of intestinal epithelial cells (IECs) [188].

In addition to bacterial-synthesized molecules, host-derived molecules can be metabolized by commensals, resulting in the production of secondary metabolites that play a crucial role in defense against pathogens. For example, the bile acids, synthesized in the liver and secreted as primary bile acids into the intestinal tract, are converted into secondary bile acids by specific commensals. These secondary bile acids inhibit the growth of *Clostridium difficile* while high concentrations of untransformed primary bile acids promote the germination of its spores [189, 190].

1.3.5.2 immune dependent

Innate-immune derived mechanisms of protection. The layer of mucus that covers the intestinal tract epithelium can be considered the first line of host defense against pathogens as it segregates the microbiota from the intestinal epithelium situated underneath. The epithelium, which is composed of different cell types, such as enterocytes, paneth cells and goblet cells is especially important for its secretion of the mucus and the production of antimicrobial peptides. Both phenomena are driven by the microbiota. For example, administration of an antimicrobial therapy can diminish the level of microbiota-derived lipopolysaccharide (LPS). This molecule stimulates the production by the enterocytes of RegIII-γ, a bactericidal C-type lectin effective against gram-positive bacteria. As a consequence of the antimicrobial therapy and subsequent diminution of LPS and RegIII-γ level in the gut, mice were rendered susceptible to vancomycin-resistant enterococcus (VRE) infection [162]. Beneath the epithelium, several specialized innate immune cells are necessary to create an adequate response against intestinal pathogens. Within these cells, the recently identified innate lymphoid cells (ILCs) have been described protecting the host against different pathogens [191]. These cells depend of the microbiota stimulation to produces several cytokines, such as IL-22, which induces the production of RegIIIγ by the enterocytes [191–193].

On the other hand, myeloid cells are also influenced by the microbiota as a study demonstrated that GFM possess lower numbers of splenic macrophages, monocytes and neutrophils. Importantly, this alteration of the innate immune system undermined protection against systemic *L. monocytogenes* infection [194]

Adaptive-immune derived mechanisms of protection Microbiota induction of the adaptive arm of the immune system, including B cells and T cells, plays a central role in the defense against intestinal pathogens in the gastrointestinal tract [195].

Within the intestinal tract, two major T-cell subsets with very different functions are greatly influenced by commensal microbes: Th17s, which synthesize IL-17 and IL-22 cytokines and play a major role in pathogen protection but also in autoimmune disorders due to their pro-inflammatory potential; and Tregs, which synthesize immunosuppressive cytokines such as IL-10, TGF-β or IL-35, and play a key role in controlling inflammation to avoid excessive tissue damage upon infection [195].

Th17 cells, through the production of IL-22, promotes the secretion of antimicrobial components by the enterocytes. They may also confer protection against infection by increasing the translocation of IgA into the intestinal lumen [196].

Intestinal Tregs play an important role in diminishing the tissue damage caused by the immune response against pathogenic bacteria. Importantly, Treg cells also promote the synthesis of IgA by B cells. These immunoglobulins intervene in the control of specific pathogens. Moreover, IgA are essential to regulate the interaction between commensals and the host [197–199]. Indeed, most of the IgA generated target commensals that could otherwise translocate and cause inflammation in the lamina basal [159, 200, 201].

Tregs development and functionality in the intestinal tract depend on the presence of specific commensal microbes, such as Clostridia [202, 203]. Among other mechanisms, these commensals influence the differentiation of Tregs through the production of SCFAs [203].

In summary, most of the immune-mediated mechanism of defense against the intestinal colonization by pathogens rely on an adequate relation between the microbiota an the host. Thus, disruption of the microbiota, as seen upon antibiotic treatment, diminishes the immune protection of the host at multiple levels.

1.3.6 Microbiota-based therapy to eliminate VRE

Enterococcus is a commensal host of the human and, as such, can acquire a detrimental behavior only in determined circumstances. For example, when expanding in the intestinal tract upon antibiotic treatment. In the hospital environment, VRE is especially associated with treatment of *C. difficile* infections [204, 205]. Indeed, the recommended pharmacological treatment of *Clostridium difficile* is metronidazol or vancomycin in case of a first episode [206] and alteration of the microbiota caused by both antibiotics have been demonstrated to highly promote VRE colonization.

Due to the difficulty to treat this increasing multi-resistant bacteria and knowing that the normal microbiota inhibit its growth, a number of laboratories are investigating the mechanisms that mediate this colonization resistance against VRE. Indeed, recently acquired knowledge on the microbiota and the mechanism of interaction with pathogens, as described in the section 1.3.5, allow the design of news therapeutic approaches to treat VRE infections. For example, it was proposed to use a commensal *Enterococcus* expressing a plasmid encoding a bacteriocin specific against VRE [184].

On the other hand, several strategies have been designed to restore the levels of RegIIIγ, an antimicrobial peptide that can kill VRE. Whereas antibiotic treatment markedly reduces the expression of Reg3γ in the small intestine of mice, oral administration of LPS

to stimulate TLR4 or systemic administration of flagellin to stimulate TLR5 can restore murine Reg3γ expression and enhance resistance to dense colonization by VRE [162, 207]. Recently, it was also demonstrated that oral administration of TLR7 agonist indirectly activate the ILCs with the subsequent production of IL-22 and restoration of Reg3γ production [208]. However, the risks of innate immune receptor stimulation are not negligible and include potentially inducing acute or chronic inflammation and enhancing existing inflammatory diseases.

Another solution to restore the colonization resistance after an antibiotic treatment would be to transfer a complete bacterial community. The fecal transplant (the transfer of a healthy microbiota to another person) has already been applied to cure numerous cases of recurrent *Clostridium difficile* infection [209]. It is indeed becoming so popular that a European consensus conference on fecal microbiota transplantation in clinical practice was published [210]. It can be highlighted that they do not recommend the fecal transplant for other applications than the treatment of recurrent *Clostridium difficile* infection.

It is also important to highlight possible safety issues. Indeed, the inability to fully define the composition of feces raises concerns in patients with compromised immune defenses. In order to achieve the safest practice possible and discard the transfer of infectious agents, they recommend checking numerous conditions for the donor (through a questionnaire and extensive blood and stool analysis) [210]. Furthermore, recently discovered associations of fecal microbiota composition with a range of metabolic and inflammatory diseases raise concerns about the long-term impact of fecal transplantation from heterologous donors.

Similarly to the fecal transplant administration that has resulted highly efficient in order to clear *Clostridium difficile* from multi-recidivist patients, it was demonstrated that the transfer of a healthy microbiota to ampicillin treated mice was sufficient to clear VRE [211]. These authors also achieved the clearance of VRE through the administration of a strictly anaerobic culture of a fecal sample. Thus, the complete microbiota is not necessary to achieve the colonization resistance and the bacteria implicated can be cultivated [211]. Moreover, investigating the relation between the microbiota composition on the VRE colonization capacity, they identified *Barnesiella*, a Bacteroidetes, as being the most negatively correlated with VRE load, suggesting a possible implication of this bacterium in the colonization resistance observed [211].

Therefore, the administration of defined bacterial populations after an antibiotic treatment may facilitate the reestablishment of colonization resistance, overcoming the difficulties associated with a fecal transplant.

So far, a relatively low number of potential probiotics have been tested as non-antibiotic strategies against VRE in mammals [117]. *Lactobacillus rhamnosus* was successfully tested in mice and human although no mechanism was proposed. In mice, *Lactococcus lactis* and *Pediococcus acidilactis* were shown to be effective through the production of a bacteriocin whereas no mechanism was proposed for the action of *Bacillus coagulans*.

In the present work, we aim to identify the key commensal bacteria that confers resistance against VRE using a mouse model. Also, we aim to identify mechanisms by which this commensal bacteria could confer protection. The results obtained in this study could therefore lead to novel therapeutic approaches to combat infections by VRE.

Objectives

The general objective of the present work is to understand the role of the intestinal microbiota in the defense against intestinal colonization by vancomycin-resistant Enterococcus. We will (i) study changes in the microbiota composition derived from antibiotic treatment that promote intestinal colonization by this pathogen, (ii) identify the commensal bacterial species that are key for conferring protection and (iii) study mechanisms by which these commensal bacteria may confer protection against the pathogen.

The specific objectives are:

(I) To investigate the alterations of the human gut microbiota associated with oral administration of vancomycin, an antibiotic that promotes intestinal colonization by VRE. Subsequently, we will assess, using a mouse model, the effect of the microbiota changes induced by vancomycin on the intestinal capability of VRE to colonize the gut.

(II) To understand, using a mouse model, how other antibiotics of different spectrum including ampicillin, ciprofloxacin, clindamycin, ceftriaxone and neomycin, promote changes in the gut microbiota composition.

(III) To study, using a mouse model, how alterations of the gut microbiota caused by antibiotics of different spectrum impact the ability of VRE to colonize the intestinal tract, which will allow us to identify commensal bacteria, associated with protection against the intestinal colonization by VRE.

(IV) To obtain a collection of murine commensal bacterial isolates in order to test their capabilities of restoring colonization resistance against VRE intestinal colonization after antibiotic treatment.

(V) To restore resistance against VRE intestinal colonization, lost after antibiotic treatment, through the administration of the murine isolated commensal bacteria, found to be associated with protection against VRE (objective III). This objective will be studied using a mouse model.

(VI) To study, in mice, changes in the transcriptome and metabolome upon the administration of the identified protective bacteria against VRE intestinal colonization. This objective will allow us to identify bacterial functions and changes in metabolites that may be relevant to confer protection against VRE colonization. This

objective will suggest possible mechanisms of microbiota-derived protection against VRE intestinal colonization.

(VII) To study, using a mouse model, genes expressed by VRE in vivo and to identify nutrients utilized by VRE using in vitro culture arrays under anaerobic conditions. This last objective will enhance our understanding of the nutritional and genetic requirements for VRE intestinal colonization. Results from this objective will also enhance our understanding of how commensal bacteria could be providing protection against VRE through competition for specific nutrients.

2. Material and methods

2.1 Vancomycin effect on the human fecal microbiota: rationale and subjects

To analyze the impact of the oral vancomycin on the human intestinal microbiota, we analyzed fecal samples collected during a clinical study that was performed in patients with rheumatoid arthritis (RA) [212].

This study was performed to investigate the possible positive effect of vancomycin on the inflammatory status of RA patients. The rationale behind the use of vancomycin in these patients was based on pre-clinical observations in animal models (K/BxN). It was observed that this antibiotic led to a dramatic reduction of segmented filamentous bacteria (SFB), a decrease in Th17 cells activation and proliferation and the abrogation of inflammatory arthritis when treating mice with vancomycin. Of note, this study was initiated several months prior to the realization that SFB was not a normal inhabitant of the human intestinal microbiota.

After consent was obtained, we randomly divided new-onset RA (see definition below) patients into 2 arms groups. The first group (the vancomycin-treated group) received vancomycin orally (250 mg four times a day) for 2 weeks, followed by treatment with methotrexate (treatment for RA) starting 6 weeks after discontinuation of antibiotic therapy. The second group (control group) received methotrexate from the beginning of the study and did not receive vancomycin. This control group was included to identify changes in the microbiota due to methotrexate administration. Importantly, as described below, no alterations in the gut microbiota composition were observed at any studied time-point in the control group.

Although vancomycin treated group did not display an improvement of RA symptoms in comparison with the control group, this study enabled us to investigate the effect of vancomycin on patients that had not previously received antibiotic treatments and in the absence of any pathogen.

This study was approved by the Institutional Review Board of New York University School of Medicine, protocol number 09-0658. Further details are published in www.ClinicalTrials.gov website, Identifier NCT01198509. Among criteria of inclusion, persons of both sex between 18 and 70 years old were accepted. Patients met American College of Rheumatology (ACR)/European League Against Rheumatism 2010 classification criteria for RA [213]. Subjects included in this study had new-onset RA which was defined as disease duration of a minimum of 6 weeks and up to 6 months since diagnosis, and absence of any treatment with disease-modifying anti-rheumatic drugs (DMARDs),

biologic therapy or steroids (ever). To select a group of patients homogeneous respect to the activity of the disease, only patients that had a Disease Activity Score 28 (DAS28) greater than or equal to 5 were incorporated. The DAS28 accounts for the number of swollen or tender joints out of 28 ones, the erythrocyte sedimentation rate (ESR) or C reactive protein (CRP) and takes into account the patient perception of his own global health state. A DAS28 score of 5 or above indicates the RA is highly active. As all the patients presented a DAS28 score into the highest range of clinical activity, we did not assess differences in the baseline microbiota due to different states of disease activity. Exclusion criteria were as follows: recent (<3 months prior) use of any antibiotic therapy, current extreme diet (e.g., parenteral nutrition or macrobiotic diet), current consumption of probiotics or proton pump inhibitors, known inflammatory bowel disease, known history of malignancy, any gastrointestinal tract surgery leaving permanent residua (e.g., gastrectomy, bariatric surgery, colectomy), or significant liver, renal, or peptic ulcer disease. All patients included in the study fulfilled the exclusion criteria until study completion.

2.2 Mice experiments

All mouse procedures were performed in accordance with institutional protocol guidelines at the "Servei Central de Suport a la Investigació Experimental" at the University of Valencia. Mice were maintained accordingly to the National guidelines (RD 53/2013), under protocols approved by University of Valencia Animal Care Committee describing experiments specific for this study. Experiments were done with 7-week-old C57BL/6J purchased from Charles River laboratories and housed with autoclave-sterilize food and autoclave-sterilize water.

2.2.1 Mice experiments: antibiotic treatments

To investigate the effect of different antibiotics on the murine gut microbiota and their relationship with VRE colonization capacity, mice were treated for 1 week with oral vancomycin (0.5g/l, Alfa Aesar), ampicillin (0.5g/l, AppliChem), neomycin (1g/l, Calbiochem) or streptomycin (0.45g/l, Fluka) in their drinking water. Treatment with ceftriaxone (2.4mg/ml; 500µl SC, Sigma), clindamycin (1.4mg/ml; 500µl SC, Fluka) and ciprofloxacin (0.6mg/ml; 150µl IG, Fluka) were administered twice daily for one week. In the model without recovery, after one week of antibiotic treatment, mice were orally challenged with 10E6 CFUs of VRE (stock no. 700221; ATCC). A fecal sample was retrieved before the infection and conserved at -80^{o}C in order to determine the microbiota composition (see section 2.3). Two days after infection, VRE counts were determined by plating serial dilutions of collected fecal samples in Gelose BEA plates (Biokar) containing 8 µg/ml of vancomycin (Alfa,Aesar) and 10 µg/ml of ampicillin (AppliChem). We will refer to this medium as BEA AV plates along the present work. Alternatively, in the model with recovery, we assessed VRE colonization capacity after letting the mouse recover their microbiota for two weeks. In this case, mice received the treatment for one week then it was withdrawn to allow the microbiota to recover. Two weeks after the antibiotic withdrawal, a fecal sample was retrieved and conserved at -80^{o}C in order to determine the microbiota composition (see section 2.3). Next, mice were orally challenged with

10E6 CFUs of VRE and the level of VRE was assessed as previously described two days post-infection (figure 2.1).

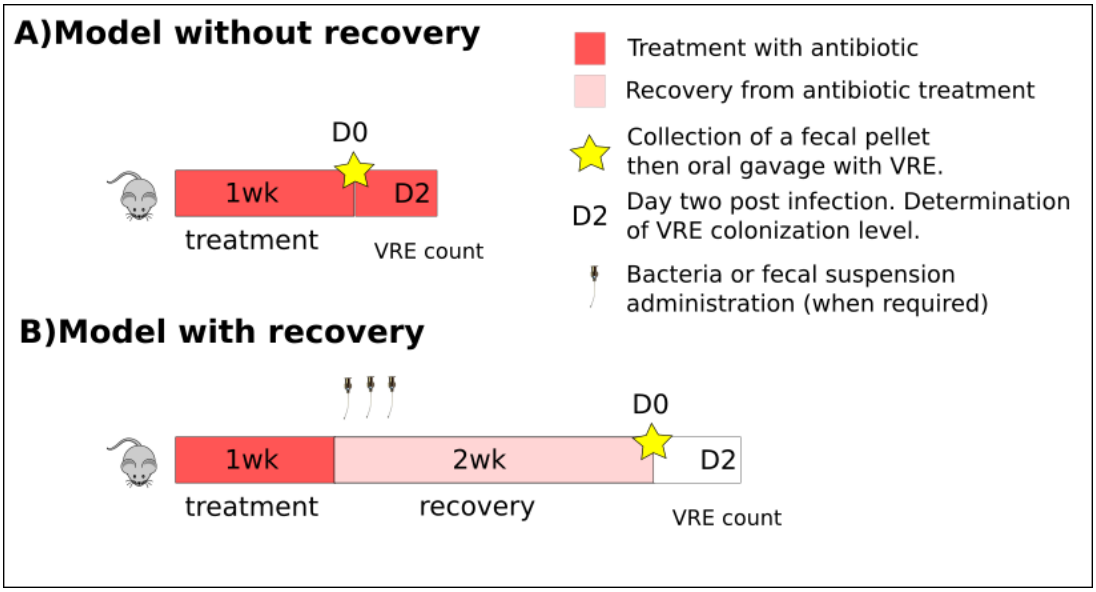


Figure 2.1: Schematic representation of the performed mouse experiments to study the effect of antibiotic treatment on microbiota composition and VRE colonization as well as testing the protective action of specific bacteria. Mice received an antibiotic treatment for 1 week, then, according to the model (A) the treatment was maintained, a fecal sample was collected to determine the microbiota composition and mice were orally challenged with 10E6 VRE CFUs, (B) after the withdrawal of the treatment, the microbiota was allowed to recover for two weeks, (in some experiments, as we will indicate in the text, specific bacteria or fecal pellets were administered orally during the first three days after the antibiotic withdrawal). After two weeks of recovery, a fecal sample was collected to determine the microbiota composition and mice were orally challenged with 10E6 VRE CFUs. In both models, cages were changed the day of the infection and the next day to avoid the re-infection of the mice by coprophagy. The levels of VRE were assessed by plating fecal samples on selective media two days after the infection.

To check that no bacteria present in the microbiota of the mice was able to grow in this medium and that there was no mice contamination with VRE previous to the infection, we plated on the BEA AV medium fecal samples collected before the infection. As expected, no colony was detected on BEA AV plates when fecal samples obtained before infection were grown .

A summary of antibiotics and posology used can be found in the table 2.1, as well as an overview of their spectrum of action in figure 2.2. These antibiotics were selected to target the different fractions of the microbiota (gram positive, gram negative and anaerobes).

Table 2.1: Antibiotics tested in the mouse model

antibiotics	Concentration/daily dose	administration route
ampicillin	0.5g/l	drinking water
vancomycin	0.5g/l	drinking water
ciprofloxacin	0.6mg/ml;300µl	Intra-gastric, IG
ceftriaxone	2.4mg/ml;1 ml	subcutaneous, SC
clindamycin	1.4mg/ml;1 ml	subcutaneous, SC
neomycin	1g/l	drinking water
streptomycin	0.45g/l	drinking water

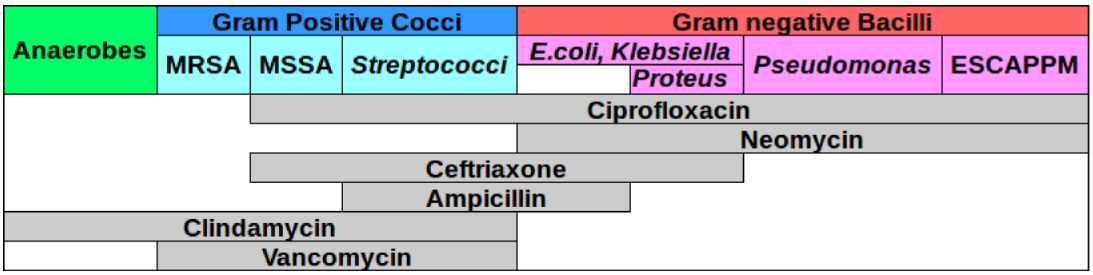


Figure 2.2: Antibiotics spectrum. Adapted from the Wellingtonicu.com drug manual. MRSA : Methicillin Resistant *Staphylococcus aureus*, MSSA : Methicillin Sensitive *Staphylococcus aureus*, ESCAPPM : bacteria with β-lactamase activity: *Enterobacter* spp., *Serratia* spp., *Citrobacter freundii*, *Aeromonas* spp., *Proteus* spp., *Providencia* spp., *Morganella morganii*

2.2.2 Mice experiments : fecal transplant

To verify that the restoration of a normal microbiota do reestablish the colonization resistance against VRE, we performed a fecal transplant in antibiotic-treated mice and allowed them to recover for two weeks. Subsequently, mice were challenged with VRE as described in section 2.2.1. The fecal solution administered consisted of fecal pellets from untreated mice resuspended in phosphate-buffered saline (PBS) (3 fecal pellets/1 ml of PBS). For each experiment, several fecal pellets from different untreated mice were resuspended together in PBS. A total of 100 µl of the resuspended pool fecal material was given by oral gavage to antibiotic-treated mice over 3 consecutive days, starting 1 day after antibiotic treatment was stopped. Two weeks after stopping the antibiotic treatment, a fecal sample was retrieved from these mice and conserved at -80°C in order to determine the microbiota composition (see section 2.3). Immediately after the collection of the fecal sample, mice were challenged with VRE as previously described.

2.2.3 Mice experiments : probiotics administration

To assess the capacity of specific bacteria to restore the colonization resistance against VRE, several mice were treated with 0.5g/l of vancomycin in water for one week and the bacteria to be tested were orally administered over 3 consecutive days, starting one day after the antibiotic withdrawal.

The bacteria were selected as explained in the section 3.2.5 and they were isolated as described in the section 2.6. To preserve the viability of anaerobic bacteria, manipulations were performed inside an anaerobic chamber (Whitley DG250 Anaerobic Workstation, Don Whitley Scientific Limited), supplied with a 10% CO2, 10% H2 and 80% N2 compressed gas mixture (Linde AG®). The material used for bacterial growth, solid or liquid media, was let to reduce overnight (O/N) inside the anaerobic chamber previously to its use.

Bacterial isolates were grown under anaerobic conditions at 37°C on Columbia Blood Agar plates (VWR) for 3 days (*Alistipes* (CU159), unclassified ruminococacceae (CU153), *Barnesiella* (Cu46)), for 6 days (*Oscillibacter* (Cu176)) or on schaedler agar plates (Conda) for 3 days (*Allobaculum* (Cu185)) (Table 2.2). The grown bacterial colonies were resuspended in PBS complemented with glycerol (20% v/v) and cysteine (0.1% w/v). These bacterial suspensions were used individually or combined according to the bacteria we wanted to test, maintaining an individual absorbance of 0.5 (OD600). In order to diminish the contact of these anaerobic bacteria with the atmosphere (containing oxigen) during their administration, individual mixes (one per cage) were prepared to be unfrozen and directly administered.

Table 2.2: Bacteria isolated and tested in a mouse model.

ID	Genus	Growth medium	Growth time (days)
Cu159	*Alistipes*	CBA	3
Cu185	*Allobaculum*	Sch	3
Cu46	*Barnesiella*	CBA	3
Cu176	*Oscillibacter*	CBA	6
Cu153	un. Ruminococcaceae	CBA	3

CBA: Columbia blood agar. Sch: Schaedler agar. un: unclassified.

To test the bacteria, we used the model with recovery after antibiotic treatment as described in 2.2.1. Briefly, mice were treated with vancomycin (0.5g/l in the drinking water) for one week and, starting next day after the antibiotic withdrawal, the bacterial isolates were administered by oral gavage for three consecutive days. Then, two weeks after the antibiotic withdrawal, a fecal sample was retrieved and conserved at -80ºC in order to determine the microbiota composition (see section 2.3). Next, mice were orally challenged with 10E6 CFUs of VRE and the level of VRE were assessed two days after VRE inoculation as previously described in the section 2.2.1(Figure 2.1).

To control that a bacteria that should not affect VRE colonization capacity does not diminish VRE levels in our model, we administered to another group of mice *Klebsiella pneumoniae* (KPN) (clinical isolate obtained from a bacteremia in the Memorial Sloan Kettering Cancer Center, New York), instead of the commensal bacteria isolated. We selected this bacteria because it is not negatively correlated with VRE levels (see result section 3.2.5) and it was demonstrated to occupy a distinct niche in the intestine and not to compete with VRE [214].

KPN was grown aerobically in LB agar (Pronadisa) for one day and resuspended in PBS to achieve the absorbance of 0.5 (OD600). The mouse model was identical as the one used to test the protective activity of the bacteria isolated. In order to identify the levels

of KPN after the inoculation, fecal pellets from mice were grown on LB agar (Pronadisa) plates containing 100 µg/ml of ampicillin (AppliChem) and 50 µg/ml of neomycin sulphate (Calbiochem). In order to identify the levels of colonization of the others isolates that were administered, 16s rDNA high-throughput sequencing was performed as indicated in section 2.5.7.

In these experiments, two mice were co-housed during the recovery period and separated just before the infection with VRE. This design allowed the sacrifice of one uninfected mouse to retrieve cecal samples (for metabolomic and metatranscriptomic study) whereas the second mouse can be infected to assess VRE levels.

2.3 DNA extraction

For human fecal samples, bacterial DNA was extracted using the MoBio power soil DNA extraction kit, based on cell membrane disruption by high-speed shaking in the presence of beads [212]. In this case, the DNA extraction was performed by the group of Jose Scher, New York University School of Medicine, New York, United States.

For mice fecal samples analyzed to determine the effects of various antibiotic treatments and to check the effect of bacterial administration on VRE levels, bacterial DNA was extracted using the QIAamp® DNA Fast Stool Mini kit (QIAGEN, Spain, ref 50951604).Extractions were performed according to manufacturer instructions with introduction of a previous mechanic disruption step with beads beating. Basically, samples resuspended in the first buffer of the extraction kit were shaken on a Vortex-Genie 2 equipped with a Vortex Adapters (Mobio, ref 13000-V1-24) at maximum speed for 5 minutes in the presence of 500µl of glass micro-beads (acid-washed glass beads 150-212 µm, Sigma®). DNA was eluted in 50 µl of milliQ water and was quantified using Qubit 3.0 Fluorometer.

2.4 16s rRNA gene high-throughput sequencing

The bacterial gene 16S rRNA is broadly used to determine the taxonomy of bacteria. This gene is present in all the bacteria and contains nine hyper-variable regions (V1–V9) that allow the phylogenetic classification of bacteria, to the species level if the complete gene is sequenced.

Using second-generation sequencing, it is possible to sequence the 16S rRNA gene of the different bacteria present in an environment to determine its microbiota composition. However, these high-throughput techniques can produce only a partial sequence of the 16S rRNA gene (100–500 bp), thus it is possible to give a taxonomic assignation only to the genus level. Nevertheless, subsequent non-taxonomic analysis that cluster sequences by similarity can define Operational Taxonomic Units (OTUs), which can aid to the study of the microbial diversity to a level similar to that of the species taxonomic level.

For human samples, V1-V2 regions of the 16s rRNA gene was amplified and sequenced on a 454 GS FLX Titanium platform (454 Life Sciences, Branford, CT, USA) to a depth of at least 2,600 reads per subject. For each sample, 3 replicate 25-µl PCRs were performed, each containing 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 1.25 U Platinum Taq DNA polymerase, 2.5 µl of 10x PCR buffer, and 0.2 µM of the modified primer 8F and 338R designed to amplify the V1-V2 region (Table 2.3). Cycling conditions were

94°C for 3 minutes, followed by 25 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute. Replicate PCRs were pooled, and amplicons were purified using the Qiaquick PCR Purification Kit (Qiagen) A library of the PCR products was prepared using emPCR Kit Lib-L (adapters were added to the amplicons to allow their sequencing by the high-throughput technique) and sequenced on a 454 GS FLX Titanium platform following the 454 Roche recommended procedures. Sequences are accessible in the NCBI Sequence Read Archive under the accession number SRP023463.

Fecal samples collected from the experiments performed in mice in order to identify changes in the microbiota induced by antibiotic treatments (section 3.2 of results) were sequenced on a 454 platform. For each sample, 2 replicate 25-µl PCRs were performed, with each containing 20 ng of purified DNA, 0.25mM deoxynucleoside triphosphates (dNTPs), 0.6U of Taq DNA polymerase (Thermoscientific), 2.5 µl of 10X PCR buffer, and 0.2mM of the modified primer 8F and 534R, designed to amplify the V1-V3 region (Table 2.3).

Table 2.3: Primers used to sequence the 16s rRNA gene using the 454 platform

Primer	**Sequence (5'-3')**
modified primer 8F	CCTATCCCCTGTGTGCCTTGGCAGTCTC*AGAGAGTTTGATCCTGGCTCAG*
modified primer 338R	GCCTCCCTCGCGCCATCAGNNNNNNN *NTGCTGCCTCCCGTAGGAGT*
modified primer 534R	CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNN *ATTACCGCGGCTGCTGG*

underlined: 454 Lib-L primer (B in case of 8F and A for 338R and 534R). italics: universal bacterial primer. NNNNNNN: a unique barcode.

Cycling conditions were 94ºC for 5 min, and 22 cycles of 94ºC for 30 sec, 56ºC for 30 sec and 68ºC for 30 sec, and a final elongation cycle at 68ºC for 5 min. Replicate PCRs were pooled, and amplicons were purified using the ExcelaPure 96-Well UF PCR Purification Kit (EdgeBio, REF: 95674). A library of the PCR products was prepared using emPCR Kit Lib-L and sequenced using GS FLX Titanium Sequencing Kit XL+ on a 454 GS FLX Titanium platform following the 454 Roche recommended procedures.

Due to the discontinuity of 454 platform and possibility to obtain a higher number of sequences by pyrosequencing, samples proceeding from antibiotics experiments were re-sequenced on a MiSeq platform (Illumina). Mice samples derived from experiments in which specific bacterial isolates were administered to mice to test their protective effect against VRE, were directly sequenced on this platform. In this case, the V3-V5 region of the 16s rRNA gen was amplified (Kapa HiFi HotStart Ready Mix), indexed with Nextera® XT Index Kit (96 indexes, 384 samples) and sequenced as described in the manual for "16S Metagenomic Sequencing Library Preparation" of the MiSeq platform (Illumina) using Miseq Reagent Kit V3.

2.5 High-throughput sequencing analysis of the 16s rRNA gene

Sequence data were compiled and processed using MOTHUR V1.34.4 and V1.36. [215]. We specify in the next section the parameters used for the analysis of the different types of data.

Due to the high variety of data analyzed and the use of two high-throughput techniques, we divided this section in several subsections.

First, we describe the parameters used in the pretreatment of the data sequenced by 454 and by illumina. This section includes the removal of remaining sequence of the primers from the obtained sequences and the control of quality. Both sequencing platforms present different outputs and for this reason it is necessary to adapt the parameters of the pretreatment to each of the sequencing platforms. Moreover, the size of the subsampling has to be adapted to each sequencing analysis (in each analysis we analyze for all the samples the same amount of sequences which are equal to the lowest number of sequences present in one of the samples to be analyzed in that particular analysis). Therefore, it is necessary to specify the parameters selected for each analysis.

Afterwards, we describe the analysis step performed on the cleaned sequences that are similar to the different type of data processed. This section includes the removing of chimeric sequences, the taxonomic assignation and the clustering of sequences in OTUs (Operational taxonomic unit, which are groups of sequences that present a minimal similarity of 97%). In the sequencing by high-throughput technique, the size of the fragments obtained reach between 200bp and around 500bp. We do not sequence the complete 16s rDNA, thus it is not possible to assign a taxonomy to the species level using these techniques. As we have previously indicated, to be able to discriminate the diversity present in a bacterial community to a deeper level than the genus, OTUs are generated as an intra-experiment approximation of bacterial species.

Finally, we describe additional analysis performed on each experiment. The purpose of the different experiments were different and thus specific analysis were performed for each particular experiment.

2.5.1 Pretreatment of 454 sequencing data

2.5.1.1 Vancomycin effect on human gut microbiota data

Sequences were converted to standard FASTA format. Sequences shorter than 200 bp, containing undetermined bases or homopolymer stretches longer than 8 bp, with no exact match to the forward primer or a barcode, or that did not align with the appropriate 16S rRNA variable region were not included in the analysis. Using the 454 base quality scores, which range from 0–40 (0 being an ambiguous base), sequences were trimmed using a sliding-window technique, such that the minimum average quality score over a window of 50 bases never dropped below 30. Sequences were trimmed from the 3′-end until this criterion was met.

2.5.1.2 Antibiotics effect on the murine gut microbiota determined by 454 sequencing data

The analysis was done as previously described except that the minimum sequence length was 250 bp and that the average quality score over a window of 50 bases never dropped below 25.

The parameters of this analysis differ from the ones applied to the human samples because the primers used are different, thus we were able to obtain larger amplicons. Regarding the quality parameter, the data were sequenced on a distinct platform and it

was necessary to diminish a bit the threshold of quality to obtain a higher number of sequences to follow the analysis.

Because of the low number of sequences obtained with this technique, we did not perform a subset to the number of sequences in the sample with the lowest deep of sequencing. Nevertheless, the analysis comparing the different groups were performed normalizing the data by the number of sequences obtained per sample.

2.5.2 Pretreatment of illumina Miseq sequencing data

The sequencing data obtained thanks to the 454 Roche platform or by Miseq illumina sequencing are quite different. The 454 sequencing output consist of unique reads (the amplicons are sequenced from one extreme) whereas the output of illumina sequencing by pair-end consists of pair-reads for each amplicon (2 sequences per amplicon). The amplicon is sequenced by both extremes and the resulting reads must be assembled on the overlapping region. For this reason, the pretreatment differs between both techniques.

For each sample a forward and reverse pair-end sequences are retrieved. Quality assessment of sequences was performed using printseq-lite program [216]. Sequences were trimmed using the sliding-window technique, such that the minimum average quality score over a window of 20 bases never dropped below 30. Sequences were trimmed from the 3'-end until this criterion was met. Then, trimmed forward and reverse pair-end sequences were assembled using fastq-join from ea-tools suite [217] applying default parameters (maximum 8 percent of difference and minimum overlap of 6bp). Assembled pair-end sequences larger than 400 bp were kept for the subsequent analysis.

Since different number of sequences per sample could lead to a different diversity, in order to compare the diversity of different fecal samples, we rarefied all samples to the number of sequences obtained in the sample with the lowest number of sequences. The number of sequences subsampled for each analysis can be consulted in the table 2.4.

Table 2.4: Subsampling size for the different sequencing analysis performed.

Analysis	**Nºsequences by sample**
Effects of oral vancomycin on the human intestinal microbiota	2883
Effect of antibiotics on the gut microbiota	29667
Study of the bacterial diversity in different media	10000
Study of the microbiota composition upon bacterial isolate administration to mice	19947
Selection of the samples included in the metatranscriptomic study	22904

2.5.3 Identification of OTUs and taxonomy performed on cleaned sequencing data obtained from the 454 and illumina Miseq platforms

Sequences were aligned to the 16S rRNA gene, using as a template the SILVA reference alignment [218], and the Needleman-Wunsch algorithm with the default scoring options. Potentially chimeric sequences were removed using the ChimeraSlayer [219] option for 454 data and Uchime [220] for illumina data in MOTHUR. To minimize the effect of pyrosequencing errors in overestimating microbial diversity [221], rare abundance sequences

that differ in 1% from a high abundance sequence were merged to the high abundance sequence using the pre.cluster option in MOTHUR. Sequences were grouped into OTUs using cluster.split with the average neighbor algorithm method for 454 data and the faster implementation of Vsearch [222], cluster, with the abundance based agc method, for the Miseq data. Sequences with distance-based similarity of 97% or greater were assigned to the same OTU. It is known that 454 platform produce an error on homopolymers (indel) while substitution type miscalls are the major source of errors for Illumina sequencing [223]. As a result, the 454 sequences present less variability due to the sequencing process than the Miseq data (the pretreatment filtering include a parameter of filtering on homopolymers). For this reason, we decided to remove the rare OTUs (singletons) for the analysis of Miseq data. Then, we subsample the data without singleton to obtain again the same number of sequences by sample. OTU-based microbial diversity was estimated by calculating the Shannon diversity index [224]. Phylogenetic classification was performed for each sequence using the Bayesian classifier algorithm described by Wang and colleagues with the bootstrap cutoff 60% [225]. Classification was assigned to the genus level when possible; otherwise the closest level of classification to the genus level was given, preceded by "unclassified".

2.5.4 Sequencing analysis performed to study the effects of oral vancomycin on the human intestinal microbiota.

Sequences were processed as described in 2.5.1.1 and 2.5.3. In order to compare the overall microbiota similarity between different fecal samples (Figure 3.2 and 3.9), we calculated for every pair of samples the unweighted UniFrac phylogenetic distance [226]. This distance is calculated by generating a phylogenetic tree containing all the 16s rRNA sequences identified in the samples under comparison. Subsequently, the UniFrac distance is calculated as the fraction of the total branch length of the tree which is not shared between the two samples under comparison. UniFrac distance values range from 0 to 1, being 1 the distance obtained between a pair of samples that do not share any branch of the phylogenetic tree (their microbiota is totally different and do not share any bacterial lineage), and 0 the distance obtained between a pair of samples that have exactly the same microbiota. To perform UniFrac analysis we first inferred a phylogenetic tree using clearcut [227], on the 16s rRNA sequence alignment generated by MOTHUR. Unweighted UniFrac, which tabulates the presence or absence, but not the proportion, of different bacterial lineages within the tree, was run using MOTHUR. We then applied the Unweighted Pair Group Method with Arithmetic mean (UPGMA) hierarchical clustering to the matrix of UniFrac distances generated in order to cluster samples by microbiota similarity (Figure 3.2A). The Shannon index was calculated for each sample using MOTHUR.

To compare human and mouse microbiota changes after vancomycin administration (Figure 3.9A and B), only the shared part of the 16s rRNA, the V1-V2 regions, were taken into account. Sequences were analyzed essentially as described above except that 800 sequences (minimum number of sequences found in one of the mouse samples) were used from each sample to calculate the number of OTUs shown in Figure 3.9B.

To calculate the correlation of microbiota recovery upon vancomycin cessation with VRE intestinal colonization levels (Figure 3.9D), sequences from murine fecal samples were obtained using the MiSeq platform. For this reason, sequencing analysis was performed

essentially as described in the section 2.5.2 except for the minimum average quality score over a window of 20 bases never dropped below 20. Sequences were trimmed from the 3'-end until this criterion was met. 13078 sequences of assembled, non-chimeric sequences were used from each sample in order to define the OTUs and calculate the UniFrac distances to untreated mice.

2.5.5 Sequencing analysis performed to study the effects of antibiotics on the gut microbiota and its influence on VRE colonization

For the section 3.2.1, we calculated in MOTHUR the number of OTUs and shannon index on the data without singleton OTUs (data obtained by illumina sequencing). The biomass was directly calculated from the concentration of DNA extracted from the sample (ng of DNA/g of feces) as previously described [228].

For the section 3.2.2 we performed the analysis as explained below:

Non Metric Multidimensional Scaling (NMDS) To verify that the sample are grouped according to the treatment given, we performed a non metric multidimensional scaling (NMDS) in two dimensions on the genus abundance matrix using function metaMDS with the Braycurtis distance [229]. This analysis present the advantage of combinating all the information on two dimensions whereas the PCA generates as many axis as the number of variables (taxa) present in the matrix. In a PCA, the axis one allows the separation of the most dissimilar samples. However, a high number of axis, not easily visualized, could be necessary to explain the separation of an important number of samples with a very distinct microbiota (as obtained administering antibiotics of different spectrum). Moreover, the NMDS analysis support matrix with a high number of values equal to zero (which is not the case of PCA). This property is important because the administration of antibiotics can eliminate specific bacteria. Then, we determined the taxa that best explain this separation using an adaptation of the bioenv function [230]. Basically, a new matrix of distance is calculated on the table of abundance of the specific bacterial combination tested. Then, this table of distance is correlated with the complete one. Only combinations of taxa that give a minimal correlation are kept. In our case, aleatory combinations of a maximum of 16 bacteria were tested. Starting from a single bacteria, the correlation of the distance matrix is assessed. In case that the bacteria or the combination gave a rho value of 0.95, it is selected. In the opposite case, a bacteria is added and the new combination is tested (with a maximum of 16 bacteria tested). Then, a backward analysis is conduced on the combination of bacteria that give a rho value of 0.95 or on the mix of 16 bacteria. All the combinations with bacteria removed are tested and the best one is selected. The complete process is performed 10 times to avoid the selection of a local optimum and the best combination is selected. We finally mapped the abundance of these bacteria on the NMDS plot using envfit.

In figure 3.21, we calculated and plotted the Braycurtis distance of each group (by antibiotic, with recovery or not) to the untreated mice. For each sample, we used the mean of the distance from this sample to the five untreated mice. To determine the Braycurtis distance between the microbiota of recovered mice and of mice under treatment, we

calculated the mean distance of each recovered sample to the samples under treatment. In the case of untreated mice, we calculated the mean distance of each sample respect to the four others untreated mice, thus we could obtain the basal microbiota Braycurtis distance among untreated mice.

Heatmap of fold changes at the genus and phylum level. To determine the taxa significantly altered by each antibiotic treatment, we performed a wilcoxon test comparing the level of individual taxa in untreated mice and in mice that received ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin or vancomycin. The p-value obtained were adjusted for multiple testing thanks to the false discovery rate (FDR) method [231]. We selected the taxa with an adjusted p value$<$ 0.05 and with a fold change (increase or decrease) superior to 2 in order to distinguish the major alterations associated with each treatment. The analysis was conduced both at the phylum and the genus level.

Barplot of the mean abundance by treatment. Numerous low abundant bacteria are significantly altered upon antibiotic administration. Thus, with the heatmap of fold changes only, we cannot appreciate well which are the changes in the most abundant populations of the microbiota. To obtain an overall view of the microbiota composition resulting from each treatment, we represented in the figures 3.14 and 3.22 the genus whose mean abundance reach 5% in untreated mice or upon administration of an antibiotic. The other genus were grouped in "other bacteria".

Determination of putative protective bacteria against VRE colonization Using the information obtained with the 454 platform we started the isolation of the commensal bacteria identified to be associated with the protection against VRE. Subsequently, we tested using a mouse model the role in the protection of the isolated bacteria. For this reason, I will hold on the 454 data to describe the selection of bacteria as potential VRE colonization inhibitors. In this case, an average of 2652 sequences/sample were obtained. We used the data of different antibiotic driven gut dysbiosis in relation with VRE load two days post infection to identify commensal bacteria associated with resistance against VRE. In order to identify commensal bacteria that associates with VRE colonization levels, we performed a correlation between the levels of each commensal bacteria and the levels of VRE with the spearman method using the R functions cor and cor.test [232]. Due to the high number of bacteria tested, it is necessary to adjust the p-value obtained to avoid the selection of false positives. In the present case, the p-value obtained were adjusted for multiple testing using the false discovery rate (FDR) method [231] implemented with p.adjust. Adjusted p values $<$0.05 were considered significant.

Within those commensal bacteria that had a significant negative correlation with VRE levels, we decided to isolate and test their protective effect against VRE. Those commensal bacteria that were highly prevalent in untreated mice were selected. A taxa was considered present if at least 2 sequences were detected. Only the taxa detected in the five untreated mice were selected. In the case of the bacteria positively correlated with VRE load, we selected all the taxa with an adjusted spearman p.value $< 0,05$. In this case we did not apply any criteria of minimal prevalence as we do not expect these bacteria to be abundant in untreated mice (these bacteria were basically found in antibiotic-treated mice, and absent or very low-abundant in untreated-mice).

In order to confirm that a specific commensal bacteria was negatively associated with the VRE levels, we separated the samples into two groups with high and low VRE level (Figure 3.26). Most of the mice presented levels of VRE colonization lower than $< 10E4$ ufc/g or higher than $> 10E6$ ufc/g. Thus, these thresholds of VRE colonization were used to define the two groups of mice (highly susceptible or resistant to VRE colonization). Both groups were constituted by a similar number of mice (N=23 for the mice with low VRE level and N=25 for the mice with high VRE level).

Subsequently, we used LEfSe (Linear discriminant analysis effect size) [233] a tool developed by Huttenhower's group to define the major microbiota differences that discriminated two groups of samples. Briefly, LEfSe pairwise compares abundances of all taxa (e.g., bacterial genus) between groups using the Kruskal-Wallis test, requiring all such tests to be statistically significant. Vectors resulting from the comparison of abundances between groups (e.g., unclassified Ruminococcaceae relative abundance) are used as input to linear discriminant analysis (LDA), which calculates an effect size. The main utility of LEfSe over traditional statistical tests is that, besides the evaluation of the significancy thanks to the p or q value, an effect size is calculated which allows the identification of those taxa that mostly contribute to the differences observed among groups.

The LEfSe analysis was performed online using the galaxy interface with the default parameters. (https://huttenhower.sph.harvard.edu/galaxy/).

2.5.6 Sequencing analysis performed to study the bacterial diversity upon growth of a cecal samples in different media.

As we will mention in the section 2.6, we cultived a mouse cecal sample in various agar media to determine which media allowed the growth of the bacteria of interest that we wanted to isolate. The overall bacteria that grew in each media was identified using high-throughput sequencing of the 16s rRNA gene with the Miseq platform. For this purpose we utilized the pretreatment parameters described in 2.5.2 and the general step of sequencing analysis described in the section 2.5.3. We calculated in MOTHUR the shannon index to determine which medium presented the highest diversity. In addition, we also defined the taxonomic groups that were able to grow in each media using as a template the SILVA reference alignment [218], and the Needleman-Wunsch algorithm with the default scoring options as described in the previous section.

2.5.7 Sequencing analysis performed to study the microbiota composition and bacterial colonization upon the administration of specific bacterial isolates to mice

After determining the putative protective bacteria against VRE colonization, we tested their protective effect using a mouse model. The isolation of the bacteria and the sequencing of their 16s rRNA are described in the sections 2.6, 2.7 and 2.8. Here we describe how we determine their capacity to colonize the intestinal tract using the 16s rRNA high-throughput sequencing approach. For this purpose, we determined which OTU of the murine microbiota correspond to each bacteria administered. To do this, we first created a Blast database using the 16s rRNA sequences obtained of the administered bacteria.

Subsequently, we obtained a representative sequence from each OTU detected in the mouse microbiota. Then, the representative sequences from the OTUs of the mice microbiota were compared to the database of the 16s rRNA from the bacteria administered with blastall. The OTUs of the microbiota that gave a best match with the administered bacterium (>99% of similarity and >99% alignment length) were considered to be the administered bacterium.

2.6 Intestinal bacteria growth and isolation

To be able to test the protective effect of the bacteria negatively correlated with VRE, we needed to isolate them from the murine gut.

Since all the bacteria that we wanted to isolate were anaerobic, in order to preserve the viability of anaerobic bacteria, manipulations were performed inside an anaerobic chamber (Whitley DG250 Anaerobic Workstation, Don Whitley Scientific Limited), supplied with a 10% CO2, 10% H2 and 80% N2 compressed gas mixture (Linde AG®). The material used for bacterial growth, solid or liquid media, was deoxygenated overnight inside the anaerobic chamber previously to its use.

In order to grow and isolate intestinal anaerobic commensal bacteria from the mice, we collected the cecum content of 7-week-old female mice SPF-C57BL/6J purchased from Charles River laboratory. The cecum content was immediately resuspended in PBS/0.1% cysteine previously reduced and transported to the laboratory in an anaerobic jar. Back in the laboratory, working in the anaerobic working station Whitley DG250, glycerol/cystein 0.1% was added to the suspension in order to obtain 7 ml PBS/glycerol 20%/ cystein 0.1%. The suspension was frozen at -80ºC in aliquots of 150 µL. One aliquot was plated on the culture media Columbia Blood Agar(VWR), Schaedler(Pronadisa), Reinforced Clostridium(Oxoid) and Brucella(Pronadisa) agar (dilution from 10E-2 to 10E-6). Plates were incubated in the anaerobic chamber for 6 days at 37ºC (Columbia Blood Agar was also incubated at 28ºC). Subsequently, we collected the bacteria growing in each medium and sequenced them by Illumina Miseq. As a control we also sequenced an uncultivated cecum sample. Briefly, DNA was extracted using QIAamp® DNA Stool Mini kit (QIAGEN, Spain, ref 51504) as mentioned in the section 2.3, the 16s rRNA gene was PCR amplified as described in protocol "16S Metagenomic Sequencing Library Preparation". Obtained amplicons were sequenced on a MiSeq platform (Illumina). Sequences were analyzed as described in section 2.4, with filtering quality parameters set to minlength=200, qaverage=25, maxambig=0 and maxhomop=8. To standardize the number of reads, samples were downscaled to 10000 reads. The analysis performed allow us to identify which bacteria grows in each media as well as their relative abundance and indicate the most appropriate media to isolate specific bacteria. After selection of the medium, we re-saw a cecal aliquot in the selected medium (CBA, see result section 3.3) at an appropriate dilution to obtain individual colonies. Colonies were resuspended in 30µl of PBS, 5µl being used to determine their taxonomy (see section 2.7), 1µl was used to grow them on a new plate and subsequently store in the case the identified bacteria was of interest, and the rest was frozen in case we had to repeat the PCR.

As will be explained in the results section 3.3, *Lactobacillus*, *Parasutterella*, *Escherichia* and *Bacteroides* were the most abundant bacteria in the medium selected.

Since the bacteria that we were more interested in were strictly anaerobic (see results section 3.2.5) we decided to add a filtering step to discard the facultative anaerobes (i.e.*Lactobacillus*, *Parasutterella* and *Escherichia*). For this purpose, we also re-saw the colonies identified in CBA agar in the media Schaedler and LB under aerobic conditions. Only bacteria that present no growth in aerobic condition were further analyzed as explain in the next section.

2.7 Bacterial taxonomic identification through 16s sanger sequencing

In order to characterize taxonomically the colonies grown in culture media, we sequenced their 16s rRNA gene. In a first step, we amplify this gene through PCR using universal primers F27 and R553. Later, we used the universal reverse primer R803 or R1492 in combination with the primer F27 to obtain a larger sequence.

Table 2.5: Sequence of primers used to verify the identity of bacteria

Primer	**Sequence (5'-3')**
F27	ACGAAGCATCAGAGTTTGATCMTGGCTCAG
R553	TTACCGCGGCKGCTGGCACG
R803	CTACCAGGGTATCTAATCC
R1492	CGGTTACCTTGTTACGACTT
Ruminococcaceae F16	AACGCCGCGTGARGGAAGAM
Ruminococcaceae R310	CCGCTACTCHVGGAATTCCG

Primers in the lower part were designed using PRIMROSE to facilitate the isolation low-abundant taxa, as explained in the section 2.8.

Each PCR reaction was done in a total volume of 25µL, using Thermopol® Reaction Buffer 10x (2,5 µL), dNTPs 10 mM (0,625 µL), primer forward 10 µM (0,5 µL), primer reverse 10 mM (0,5 µL), Taq-polymerase (0,5 µL), 1 or 5 µL of bacteria resuspended in PBS as DNA source (according to the concentration of the bacterial suspension) and ultrapure water to adjust the volume to a total of 25µL. The parameters of PCR reaction were : initial denaturation (5 min, 94ºC); 35 cycle of denaturation (30 sec, 94ºC), hybridization (30 sec, 56ºC), elongation (30 sec, 68ºC). After the 35 cycles, the reaction finalizes with an elongation (5 min, 72ºC).

To check that the PCR amplification did work, we performed an electrophoresis in agarose gels. PCR products mixed with the loading buffer (LB-6X) in proportion 4:1 were loaded on agarose gel (1,4%) (Sigma-Aldrich®) with 1µL RedSafe™ Nucleic Acid Staining Solution (20x). Electrophoresis was performed during 30 min at 120V. Separated PCR amplified products were visualized using an Ultra Violet Light Chamber (Bio rad). To assess the size of the fragments, we used the molecular weight marker GeneRuler 1 kb DNA Ladder (Thermo Scientific™) mixed with LB-6X in the same proportion as the PCR products. LB-6X used was commercialized bought (Thermo Scientific™ 6X Orange Loading Dye).

After confirmation that the PCR amplification did work, PCR products were purified using purification plates ExcelaPure™ 96-well Ultrafiltration plate (EdgeBio). According to manufacturer guide, ultra-pure water was added to the PCRs products to obtain a final volume of 100 µL. After 5 minutes of incubation at room temperature, centrifugation (3500 x g in a Hermle Z400 centrifuge) was applied for 10 minutes. The flow-through was discarded, samples were resuspended with 100 µL of ultra-pure water and the incubation and centrifugation process was repeated. The DNA retained in the well filter was resuspended with 50 µL of ultra-pure water. Subsequently, DNA was incubated at RT for 5 min before resuspending carefully the purified samples and transferring them to new tubes. If necessary, samples were conserved at -20ºC.

The purified amplicons were used as a template to perform the dye-terminator reaction in order to obtain their sequences. To verify the identity of the bacterial isolate, a unique reaction with the primer 27F was performed.

The dye-terminator reaction was performed using for each sample : ultra-pure water (4 µL), BigDye® Terminator v3.1 Sequencing Buffer 1,5x (1,6 µL), BigDye® Terminator v3.1 (0,4 µL), purified PCR products (1 µL), primer 27F (1 µL, 5µM). Reaction conditions were 99 cycles of : 94ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes. The resulting product was sent to the "Servicio Central de Soporte a la Investigación Experimental (SCSIE)" where the purification and capillary electrophoresis were performed . Quality of retrieved sequences in ab.1 format was manually checked using Trev program from package Staden (avaible at http://staden.sourceforge.net/) and sequenced were kept in fasta format. Phylogenetic classification of the sequences was done using MOTHUR [215].

For those bacteria that we selected to test in the mouse model, we also obtained the complete sequence of their 16S rDNA gene, since this would allow us to identify and characterize them better. To obtain the complete 16S rDNA sequence, a bacteria suspension was PCR amplified and purified as described previously, except that we used the primers F27 and R1492. Next, the purified amplicons were used as a template in three dye-terminator reactions, with one primer per reaction which was either 27F, 533F or 1100F. The resulting product was also sent to the "Servicio Central de Soporte a la Investigación Experimental (SCSIE)" and, after the quality check, the three overlapping fragments were assembled with Geneious software to obtain the complete sequence of the 16S rDNA gene. As before, the phylogenetic classification of the sequences was done using MOTHUR [215].

2.8 Isolation of low-abundant bacteria

As described in the results (see section 3.3), the strategy used to isolate the commensal bacteria described in the section 2.6 allowed us to isolate most of the bacteria we were interested in, except *Oscillibacter*, a member of the family Ruminococcaceae.

To isolate these very low abundant taxa, we designed specific primers of the family Ruminococacceae that could allow us to differenciate bacteria belonging to this family from the rest of the bacteria grown in the Columbia blood agar medium directly by performing a PCR from a bacterial colony. Subsequently, we could confirm the taxonomy of positive clones through 16s rRNA sequencing. This strategy could facilitate this task since it will avoid us the need of sequencing thousands of colonies.

Design of oligonucleotides specific for the family Ruminococcaceae was done using PRIMROSE Version 2.17 [234]. It was not possible to design primers specific for *Oscillibacter* since other genera from the same family Ruminococcaceae share a very similar 16s sequence. PRIMROSE program uses a rRNA 16S sequence database and generates the complementary oligonucleotides for the selected sequences of the database (e.g. Ruminococcaceae) while optimizing differences with the other sequences included in the database. To construct this database, we used the aligned sequences of the OTUs detected in Columbia blood agar culture (37ºC) (see section 2.6), where *Oscillibacter* was in higher abundance respect to the other media investigated.

For the construction of the specific oligonucleotides, we selected the OTUs classified within the family Ruminococcaceae (*Pseudoflavonifractor*, *Clostridium IV* and *Oscillibacter*). In the parameter to generate the oligonucleotides, we selected a mean length of 20 nucleotides, which ensures a temperature of hybridization high enough. We selected forward-reverse pair of nucleotides with a similar Tm and a minimal distance of 200 bp to ensure the generation of a PCR product long enough to separate it, in an agarose gel, from any primer dimer generated during the PCR reaction. We selected the primer-pairs that had the highest number of mismatches respected to the other OTUs present in the media (not belonging to the family Ruminococcaceae), with a preference for mismatches in region 3' end (hybridization in this position is important to ensure the capacity of the polymerase to start elongation of the DNA during the PCR). The sequences of the identified primers are indicated in table 2.5.

The isolation of *Oscillibacter* was done as described in the section 2.6 except that the first PCR was performed both with the universal primers and the newly designed one (the hybridization temperature was 61ºC for these specific primers). We selected the bacteria for which we obtained an amplification product in the PCR reaction with the specific primers. The PCR reaction with the universal primers served as a control that the amplification did work and was subsequently sequenced to verify that the colony isolated was in fact *Oscillibacter*.

2.9 Genome sequencing and analysis

In order to determine the protective mechanism by which the bacteria isolated prevent the colonization by VRE and the contribution of each of the bacteria present in the mix, we needed to know which functions they encode. For that, we sequenced their complete genome, including also the genome of the VRE strain used in the mouse experiments. We also performed a meta-transcriptomic analysis to determine the functions implied in the protection (see results 3.5). In this case, by mapping the meta-transcriptome to the genome of each bacteria administered, we can determine, emphin vivo, what functions are expressing each of the bacteria administered.

The bacteria of interest were cultured in anaerobic conditions on Columbia blood agar or Schaedler agar according to their requirements (see section 2.2.3). *Enterococcus faecium* was cultivated in aerobic condition on BHI agar (Pronadisa). Bacteria were resuspended in PBS and DNA was extracted using QIAamp® DNA Fast Stool Mini kit (QIAGEN, Spain, ref 50951604) as described in 2.3. Subsequently, a genomic DNA library was elaborated with the kit Nextera® XT DNA Sample Preparation Kit according to manufacturer guide.

The samples were indexed with Nextera® XT Index Kit and the genome sequencing was performed with an Illumina MiSeq® System, using Miseq Reagent Kit V3 (paired-end).

An average of 1,114,877 paired-end reads per isolate were obtained. Remaining adaptor sequences were removed from the raw data using Cutadapt v. 1.10 [235]. On average, 4% of the reads contained adaptor sequences and were trimmed accordingly. Cleaned sequences were then filtered by quality using UrQt v.1.0.18 (last update September 2016) [236]. UrQt trims low quality read extremes, to avoid data loss. Nevertheless, to avoid possible misclassification of the short reads, only reads with a size of 75 or higher were further processed. In total, 8.5% of the bases were trimmed, and 1,65% of the reads were removed.

Cleaned genomic data was assembled using SPAdes v. 3.7.1 using the "careful" algorithm to improve the contig reconstruction [237]. A multi-kmer Bruijn graph reconstruction was followed as it has been suggested to improve the assembly outcome. We used 6 different kmer lengths (21, 33, 55, 77, 99, 127), as it is the best kmer combination for estimated read sizes over 250bp. SPAdes resulted in an average draft genome assembly of 258 contigs with a N50 of 101,450bp (Given a set of contigs, each with its own length, the N50 length is defined as the shortest sequence length at 50% of the genome. Thus, summing the contigs starting from the biggest one, the smallest contig included to cover 50% of the genome is 101,450bp long in this assembly). The average basepair coverage was 27.17X. Open-reading frames (ORFs) were identified and annotated using PROKKA, resulting in an average of 2486,6 ORFs per genome [238]. To maximize the annotation, ORFs were translated into amino-acids and queried against three independent databases: Pfam v.27.0 [239], EggNOG v.4.5 [240] and KEGG (last accessed October 2014) [241]. Annotation was performed using HMMer v.3.1.2 [242] with the following parameters: only Hits with an e-value lower than 0.05 and a minimum coverage of 0.50 were kept as significant results. For each ORF, only the best hit per database was kept.

2.10 Acquisition of meta-transcriptomic and metabolomic samples

In order to determine the protective mechanism by which the bacteria isolated prevent the colonization by VRE, we decided to detect the metabolites and the transcripts that are differentially expressed upon the administration of the protective bacteria.

By comparing the metabolome of cecal samples from antibiotic-treated mice that received the protective bacteria or not, we aim to determine how the cecal environment and the availability of nutrients (i.e. carbonhydrates,..) is altered. These differences, detected before the challenge with VRE, can influence its growth capacity.

We also sequenced the meta-transcriptome of the same samples. This will allow us to determine which functions drive the alterations detected at the metabolome level and it can allow the identification of other mechanisms, such as the synthesis of bacteriocins. Moreover, mapping the meta-transcriptomic data against the genome of the bacteria administered (see 2.9), it is even possible to determine the individual action of the different bacteria administered.

Samples for metabolomic and meta-transcriptomic study were obtained from the same mice. As described in section 2.2.3, mice were co-housed (2 mice by cage) for 2 weeks

after removal of antibiotic treatment. The control group, which received only suspension media (PBS/glycerol 20%/cystein 0.1%) instead of the bacterial suspension, were co-housed similarly. The same day as we performed the VRE infection, mice were separated in individual cages and one mouse was orally infected with 10E6 cfu VRE/200µl whereas the other mouse was sacrificed in order to collect the cecum for the metabolomic and the meta-transcriptomic analysis.

Importantly, we already demostrated that co-housed mice present similar microbiota and VRE colonization levels when challenged (see Results section 3.5.1). Thus, by co-housing two mice, we can sacrifice one to obtain the samples pre-infection whereas the second one is challenged with VRE to determine the colonization load associated with this microbiota composition.

The cecal content of each sacrificed mouse was resuspended in twice its volume of phosphate buffer (100 mM Na2HPO4 pH 7.4) respect to weight (considering 100 mg as 100 µl), homogenized and centrifuged (13 200g, 1 min). The supernatant was separated and frozen using a dry ice bath whereas the pellet was resuspended in 1 ml RNAlater (Ambion) and kept at 4ºC for 24h before preservation at -80ºC. Next two days after infection, VRE counts were determined in the infected mice by plating serial dilutions of collected fecal samples in Bile Esculin agar plates containing 8 µg/ml of vancomycin (Alfa,Aesar) and 10 µg/ml of ampicillin (AppliChem). As control, fecal samples from every mouse were plated before infection in VRE selective plates. No colonies were identified in the fecal samples plated before infection in VRE selective plates.

2.10.1 Verification of the microbiota likeness in co-housed mice

As described in the previous section 2.10, to obtain the samples for metabolomic and metatranscriptomic studies, we use mice that were co-housed. We obtained the sample necessary for the metabolomic and meta-transcriptomic studies from one mouse whereas the other one reports the VRE colonization levels after orally challenging it. For this purpose, we first performed an experiment to verify that co-housed mice indeed present a similar microbiota composition and VRE loads when challenged. Mice were treated with antibiotics, separated two by cage (co-housed) and allowed to recover for two-weeks. Next, a fecal sample of each mouse was retrieved to assess the gut microbiota composition and they were infected with VRE after separation in individual cages. The VRE levels were assessed by plating fecal samples two days after the infection.

The consistency and conformity of VRE colonization levels in co-housed mice were checked by calculating the intraclass correlation coefficient (ICC) on fecal VRE levels detected at day two post-infection in co-housed mice. The function used, ICCest from ICC package, is similar to a one-way analysis of variance (ANOVA), where the total variance is split into within-subject and between-subject variability.

To check that co-housed mice also shared a similar microbiota, we sequenced and analyzed the 16s rRNA (as described in 2.3, 2.4 and 2.5). If co-housed mice share a similar microbiota, the unweighted unifrac distance between two co-housed mice should be smaller than the mean distance between the different samples of the same group. To test this, we calculated the unweighted unifrac distance on the data excluding the singleton OTUs using MOTHUR V1.36. Then, we compared, using Wilcoxon test, the mean distance between co-housed mice with the mean distance between all the samples.

2.11 Metabolomic study

In order to determine the protective mechanism by which the bacteria isolated prevent the colonization by VRE, we compared the metabolome of the cecum from treated mice that received a mix of protective bacteria or not. This analysis can highlight differences in concentration of metabolites, such as nutrients, between the group that received the protective bacteria or that did not received the mix.

This analysis was performed in collaboration with Leonor Puchades, in the laboratory of Antonio Pineda-Lucena, part of the nuclear magnetic resonance service of the Research Center Principe Felipe (CIPF), Valencia, Spain.

2.11.1 H-NMR spectroscopy measurement protocols

Supernatants obtained as described in section 2.10 were diluted with cold buffer (75 mM Na2HPO4 pH 7.4, 100% D2O) to obtain a dilution 1:30 from the initial cecal sample. After homogenization, 1 ml of the sample was centrifuged (11000g, 4ºC, 20 min), filtered with a cellular filter (pore size 100 µm, 732-275VWR) through centrifugation (1000 rpm, 5 min). The resulting filtrate was centrifuged (11000 g, 4ºC, 15 min) and the supernatant was filtered (pore size 0.2 µm, 25mm, Acrodisc 4612). 500µl of this last filtrate was mixed with 100µl of RMN buffer (100 mM Na2HPO4 pH 7.4, 4.6 mM TSP, 100% D2O) for acquisition of data.

All spectra were recorded using a Avance III spectrometer equipped with a 5mm TXI probe and operated at a 500 MHz 1H resonance frequency. All spectra were recorded jointly and in a random order for the different sample groups included in the study. The temperature was set to 300 K and a representative sample was used for NMR probe tuning and matching, determination of the transmitter offset value for water pulse presaturation (25 Hz). These parameters were kept for all the samples of the experiment. The impedance of the probe was adjusted automatically for each sample using the wobble curve and a pulse adjustment of 90º.

A representative sample of each group was used to determine the required number of repetitions to obtain a good relation signal/noise (S/N). All spectra were acquired and performed using the TopSpin software package version 3.1 (Bruker Biospin, Rheinstetten, Germany).

For every sample included in the study, a 1D-NOESY experiment was run and for determined samples a second experiment, 1H-1H TOCSY, was run.

2.11.2 Data reduction and metabolomics analysis

Phase correction and baseline correction were carefully performed, and the 1H chemical shifts referred to the doublet signal of alanine (δ 1.47). The corrected NMR spectra corresponding to the chemical shift range between δ 0.2–10.0 were imported into AMIX 3.9.5 (Bruker Biospin, Rheinstetten, Germany), and all of the spectra were reduced into integral regions with equal lengths of 0.005 ppm.The spectra was integrated in two regions from both sides of the resonance from residual water (δ 0.5–4.5 and δ 5.0-8.5). To reduce the concentration differences between samples, the data was normalized to the total spectral area (100%).

The datasets were analyzed by pattern recognition methods using the software packages Simca-P, version 11.5 (UmetricsAB, Umea, Sweden), and MetaboAnalyst 3.0. To make the skewed distributions more symmetric, log transformations were used for nonlinear conversions of the data. To explain the maximum variation between samples, a PCA bilinear decomposition method was used to view the clusters within the multivariate data. To eliminate the effect of inter-subject variability among the participants and identify endogenous metabolites that significantly contributed to the classification, OPLS-DA was applied to remove linear combinations of variable X orthogonal to the Y vector. The specific metabolites between classes were interpreted using VIP (variable importance in the projection), considering as important all the variables with a VIP above 1.

2.12 RNA extraction and metatranscriptome sequencing

In order to determine the protective mechanism by which the bacteria isolated prevent the colonization by VRE, we studied the meta-transcriptome of the cecum from treated mice that received a mix of protective bacteria or received PBS instead. This analysis can highlight differences in transcript expression between the group that received the bacterial suspension and the one that received the PBS. This study can serve to confirm results obtained through the metabolomic approach and can also highlight other mechanisms such as the expression of bacteriocins.

The samples were obtained as described in the section 2.10. Samples conserved in RNAlater were unfrozen on ice and a volume corresponding to 100-250 mg of cecal sample was transferred to a weighted 2.0 ml eppendorf. Each subsample was diluted with a volume of ice cold DEPC water equal to its weight and centrifuged for 5 minutes at 13400 rpm, 4ºC. The samples conserved in RNAlater were diluted 1:2 with ice cold DEPC water before their centrifugation to diminish the viscosity and to be able to retrieve the pellet.

The supernatant was discarded and the resulting pellet was weighted. RNA was extracted from the obtained pellets using the kit Power Microbiome RNA isolation kit (reference 26000-50, Mobio) according to manufacturer protocol with slight modifications. First, to improve the process, the first step was performed combining a phenol-chloroform extraction (pH5) and bead disruption, as suggested in the kit protocol and already documented [243]. Second, to improve the elimination of the DNA, the DNAse treatment step was increased to 45 min at 37ºC instead of 15 min at room temperature . Third, in the final step, the RNA was eluted with 100µl of PM8 for 5 min (instead of 1 min). After the extraction, the purity of RNA was checked performing a qPCR with bacterial 16s rRNA universal primer 27F (sequence:AGAGTTTGATCMTGGCTCAG) and 338R (sequence:TGCTGCCTCCCGTAGGAGT). If the PCR is positive, it means that DNA remains in the sample. In case of amplification, the sample was treated with Baseline-ZERO Dnase (Epicentre) to completely remove the remaining DNA. A standard ethanol precipitation protocol was used to precipitate RNA and wash out the Stop solution that could interfere with the following steps. The RNA was precipitated by mixing 22µl of sample, 2.2µl NaAc 3M pH5.2 and 72.6µl ethanol absolute ice cold and letting the mix overnight at 4ºC. Next day, the eppendorf was centrifuged for 15 min, 13200 rpm, and

the ethanol was removed. Subsequently, the pellet was washed with ethanol 70%, dried at 65ºC for about 10 min and finally resuspended in DEPC water.

Because we were interested in identifying the functions express by protective bacteria, we were mainly interested in the mRNA. However, most of the RNA present in a cell is rRNA, thus we needed to remove it before performing the sequencing. To remove the rRNA, every sample was treated with Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina). The library for sequencing was constructed using the ScriptSeq Complete Kit Bacteria according to the manufacturer protocol. Libraries were sequenced on a Nextseq high (150bp, pair-end) according to the manufacturer protocol.

2.13 Metatranscriptome analysis

To obtain a high coverage, we limited the number of samples to be inluded in the run. We included one sample to determine VRE *in vivo* transcriptome and 6 samples of each group (untreated mice, treated mice that received PBS and treated mice that received the protective bacterial mix) from the experiment conduced to determine the metatranscriptomic alterations related with the administration of the bacterial mix. We selected the most representative samples for each group, that is to say the nearest one to the log10 median level of VRE at day two post infection.

2.13.1 Metatranscriptomics sequencing data processing

A total of 19 metatranscriptomics samples were sequenced with Illumina MiSeq using paired-end technology. An average of 4.67*10E6 paired-end reads was obtained for each sample. Adaptor sequences and low-quality reads were removed using Cutadapt and UrQt (see 2.9). Although the RNA extraction method utilized contained a step to remove the ribosomal RNA, this step does not remove the 100% of the ribosomal RNA. For this reason, cleaned metatranscriptomic data was mapped against a Short Ribosomal Subunit database (including 16S and 18S rRNA reference data) from SILVA [244] using bowtie v.2.2.9 [245], and discarded. The remaining reads were re-mapped against the Long Ribosomal Subunit database from SILVA and the Mouse reference genome v.38 from the NCBI reference repository [246]. Hits mapping any of both databases were discarded in further analyzes.

2.13.2 Assembly and Annotation of metatranscriptome NGS data

Cleaned genomic data was assembled using SPAdes v. 3.7.1 using the “rna” algorithm to improve the transcript reconstruction. A multi-kmer de Bruijn graph reconstruction was followed as it has been suggested to improve the assembly outcome. We used 6 different kmer lengths (21, 33, 55, 77, 99, 127), as it is the best kmer combination for estimated read sizes over 250bp. ORFs were identified and annotated using MetaGeneMark v.1.0.1 [247]. Only ORFs with a minimum size of 50aa and with at least one extreme complete were kept. To maximize the annotation, ORFs were translated into amino-acids and queried against three independent databases: Pfam v.27.0 [239], EggNOG v.4.5 [240]

and KEGG (last accessed October 2014) [241] Annotation was performed using HMMer v.3.1.2 [242] using the following parameters: only Hits with an e-value lower than 0.05 and a minimum coverage of 0.50 were kept as significant results. For each ORF, only the best hit per database was kept.

2.13.3 Metatranscriptomic data analysis

A catalogue of non-redundant ORFs (NR) was constructed. ORFs were clustered at 90% identity, as described in [248], using vsearch using the "cluster smallmem" algorithm [222]. Non-redundant ORFs were annotated using KEGG and Pfam databases using HMMSearch. To calculate the average coverage and the total number of mapped reads per ORF, the cleaned metatranscriptomic data was mapped against the formatted NR catalogue using bowtie2 [245]. Read counts were converted to transcript reads per million (TPM), by first dividing the number of read counts by the length of each ORF (RPK). Then we have divided the number of RPK per sample by 1,000,000 (resulting in a scaling factor) and finally dividing each RPK by the scaling factor. This normalization results in each sample summing up to 1,000,000 TPMs (transcript per million). Cleaned metatranscriptomic data was also mapped against the bacterial genomes obtained from the protective bacteria (see section 2.9)) using bowtie2, in order to identify which transcripts are expressed by the protective bacteria.

2.14 VRE *in vivo* transcriptome analysis

To analyze the transcriptome of VRE emphin vivo, we infected mice that were receiving vancomycin treatment with 10E6 VRE CFU (see section 2.2). During vancomycin treatment, VRE represents more than 1 percent of the microbiota and, by sequencing the metatranscriptome, we can obtain enough reads that map against VRE genome. Consequently, we can analyze the specific transcriptome of VRE.

RNA was extracted and sequenced as described in 2.12. Low quality sequences and ribosomal sequences were removed as described in section 2.13. Then, using bwa, the filtered reads were aligned against the VRE ATCC700221 genome sequenced. Next, using SAMtools we retrieved the number of reads that map on each gene [249]. Subsequently, the number of reads that map on each gene was normalized as TPM, as previously described. To control the specificity of the genes that map against the VRE genome, we mapped against the metatranscriptome of treated mice. We only found 29 ORFs from a total of 2718 ORFs detected to be expressed by VRE. Indicating that the great majority of the sequences that match the VRE genome, in mice colonised with VRE, where indeed genes expressed by VRE.

2.15 Phenotype Microarrays assays

To determine the capacity of VRE ATCC700221 to grow on different nutrients in anaerobic conditions, we used Biolog plates PM1 and PM2a (carbon sources) following the "PM Procedures for Anaerobic Bacteria (no dye)" protocol. The PM panels are 96 well plates where each well contains a different and unique source of carbon.

PM panels and inoculating fluid IF-0a GN/GP Base or IF-0a, were bought from Biolog, Inc. (Hayward, CA, USA). Other chemicals needed to perform the microarrays assays were purchased from Sigma-Aldrich except for the yeast extract (YE) that was purchased from Oxoid (UK).

To inoculate panels PM1 and PM2a, a mix of IF-0a with yeast extract was used (see table 2.6). Briefly, VRE was grown overnight on BHI plate in an anaerobic chamber (Whitlet DG250), at 37ºC with a gas atmosphere of 10% H2, 10% CO2, and 80% N2. The inoculation mix as well as 4 ml of IF-0a were reduced in the anaerobic chamber. PM panels were reduced in an oxoid anaerobic jar because some reagents are sensitive to the high relative humidity present in the anaerobic chamber.

Table 2.6: Biolog plates inoculation mix

	PM1,2 – for one plate (ml)
Cells in AN IF-0a (1.2x)	0.750
AN IF-0a (1.2x)	9.104
Glucose (1M, filtered sterile)	0.000
Yeast (20%, filtered sterile)	0.030
Sterile water	2.116
total	12.000

Inoculation of the panels was done rapidly in aerobic conditions. VRE was resuspended in reduced IF-0a and washed to remove possible nutritive contamination from the growth medium. After centrifugation, the supernatant was discarded and replaced by fresh reduced IF-0a. 750 µl of this suspension normalized to an absorbance of 0.3979 (corresponding to the transparency of 40%T specified in the manufacturer protocol) was added to the mix corresponding to the specific PM plate tested, obtaining a total volume of 12 ml. The PM plate was inoculated with 100 µl/well of this inoculation mix. We also plated several dilutions of this inoculation mix on BHI agar to determine the exact number of VRE CFUs that were used in each experiment.

The incubation and lecture were performed outside of the anaerobic chamber, thus, to maintain the anaerobic conditions, 30 µl of autoclaved mineral oil was added on the top of each well. The anaerobic condition was checked first growing a strictly anaerobic bacteria in the same conditions (see section 3.7.1) and using a redox marker, methylene green, that turn green in case of oxidation.

The lecture was performed in a Tecan Infinite F200 at a wavelength of 610 nm using the program Magellan (incubation for 24h at 37ºC). The absorbance was measured every 20 minutes. For analysis of the results, the background of each well at time 0 was subtracted.

The experiment was replicated for each type of panel, and only those nutrients with consistent growth of VRE in both replicas were considered to be important for VRE growth. In addition to the absorbance result, we confirmed the grow of VRE by plating each well of the replicated plate after the 24h growth. Finally, we considered that nutrients for which we observed a growth of 2 folds or more respect to the control were utilized by VRE as carbon source for its growth.

2.16 Statistical analysis

Statistical analysis was performed using R [232] or GraphPad Prism (GraphPad Software). To compare the VRE levels obtained by plating fecal samples, we used the Mann-Whitney-Wilcoxon test when the number of samples was too small to perform a test of normality and when the groups did not followed a gaussien distribution according to the D'Agostino and & Pearson omnibus normality test.

When all the values of one group are similar (for example, the level of VRE in untreated mice at day two post-infection is equal to 0), it is not possible to apply the non parametric Mann-Whitney-Wilcoxon test. In this case, we used an unpaired t-test with Welch correction to account for the unequal variance between groups.

When analysing the microbiota composition, we used a Wilcoxon test adjusted by FDR to determine the bacteria whose abundance vary between two groups. In this case, we perform an high number of comparisons thus it is necessary to adjust the p.value obtained through the FDR approach to avoid the selection of false positives. Other statistical tests used and parameters are described in each of the analysis performed, in the corresponding sections of the present Material and methods chapter or directly in the results.

3. Results

3.1 Short- and long-term effects of oral vancomycin on the human intestinal microbiota.

Vancomycin resistant *Enterococcus* (VRE) infections frequently occu after antibiotic treatment, mainly in hospitalized patients. In particular, VRE are highly prevalent in patients previously infected by *C.difficile* [205]. This might be due to the antibiotics used to treat *C.difficile* infections (CDI), metronidazol and vancomycin. Indeed, it has already been demonstrated in mice that vancomycin provokes a greater loss of microbiota-mediated colonization resistance (CR) to *C.difficile* infection as compared with metronidazol [175]. In this article, they studied in mice the effect of both antibiotic for a period of 21 days. By the end of the study, vancomycin treated mice had not recovered their microbiota and were still susceptible to *C.difficile* infection, albeit in lower level. Looking at the impact of the treatment on opportunistic infections, they also demonstrated that mice were susceptible to VRE infection as far as 14 days after antibiotic cessation.

Given the high impact of vancomycin, we decided to investigate the alterations induced by this treatment in the much more variable human gut microbiota. Unlike the publication mentioned, we studied the recovery on a long period (six months post-antibiotic cessation) to assess whether the microbiota could fully recover. Finally, we wanted to assess the relevance of the alterations observed in the microbiota on the capacity of VRE to colonize the intestinal tract.

We had the opportunity to study the effect of vancomycin in patients that had received no antibiotic treatment in the previous three months thanks to a clinical trial in patients with new-onset rheumatoid arthritis (RA) that received oral vancomycin. Thus, we could analyze in these patients the exclusive effect of vancomycin on the human microbiota, as opposed to other studies that had evaluate the effect of vancomycin on patients infected with *C. difficile*, which previously had been treated with other antibiotics.

The rationale behind the use of vancomycin in these patients was based on pre-clinical observations in animal models (K/BxN). It was observed that this antibiotic led to a dramatic reduction of segmented filamentous bacteria (SFB), a decrease in Th17 cells activation and proliferation and the abrogation of inflammatory arthritis when treating mice with vancomycin. Of note, this study was initiated several months prior to the realization that SFB was not a normal inhabitant of the human intestinal microbiota.

Independently of the outcomes of this clinical trial, we used the samples collected to explore the effect of vancomycin on an undisrupted microbiota. A total of 21 subjects were included in the study, 9 of which received vancomycin orally for 2 weeks while 12 of them

Table 3.1: Demographic and clinical data among control patients or patients treated with vancomycin.

Characteristic	Vanco (n=9)	Control (n=12)
Age, years, mean (median)	36 (32.5)	37.7 (36)
Female, %	75,00%	67,00%
Disease duration, months, mean (median)	7.5 (4)	6.7 (4)
Disease activity parameters		
ESR, mm/h, median	24,2	28,2
CRP, mg/l, median	12	10
DAS28, mean (median)	5.3 (5.0)	5.2 (5.5)
Patient VAS pain, mm, mean (median)	74.5 (80)	57.7 (50)
TJC-28, mean (median)	11 (11.5)	7.6 (6.0)
SJC-28, mean (median)	7.0 (7.0)	7.7 (7.0)
Medication use at baseline		
Antibiotics (previous 3 months), %	0,0%	0,0%
PPI, %	0,0%	0,0%
Methotrexate, %	0,0%	0,0%
Prednisone, %	0,0%	0,0%
Biological agent, %	0,0%	0,0%

Two sided t-test or Fischer's exact test was applied to study differences among groups. No significant differences were observed among groups in any of the analysed characteristics ($p>0.05$). Abbreviations:ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, Disease Activity Score with 28 joint count; VAS, visual analog scale; TJC-28, tender joint counts with 28 joint count; SJC-28, swollen joint counts with 28 joint count; PPI, proton pump inhibitors.

remained untreated. Importantly, no significant differences in clinical or demographic characteristics were observed between both groups of patients (Table 3.1).

Fecal samples were obtained immediately before the initiation of vancomycin treatment, the day the treatment was ended, and 2, 6, 14, and 22 weeks after antibiotic discontinuation. Samples from untreated patients were obtained at the same time points as controls. With only a few exceptions, samples for most time points were included in the study for all vancomycin-treated (4.9 ± 0.9 out of 6 time points; mean $\pm$ standard deviation), and untreated patients (4.2 ± 1.1) (Figure 3.1).

16s rRNA sequences from human samples were analyzed with mothur as described in 2.5. A total number of 1 434 274 sequences (average of 10393 sequences per sample) passed our quality thresholds. Samples with lower coverage (<2500 sequences) were removed from the analysis. In order to avoid any bias due to different sample coverage, 2883 sequences (minimum number of sequences found in a sample) were selected from each sample for subsequent analysis: taxonomic characterization and operational taxonomic units (OTUs) identification.

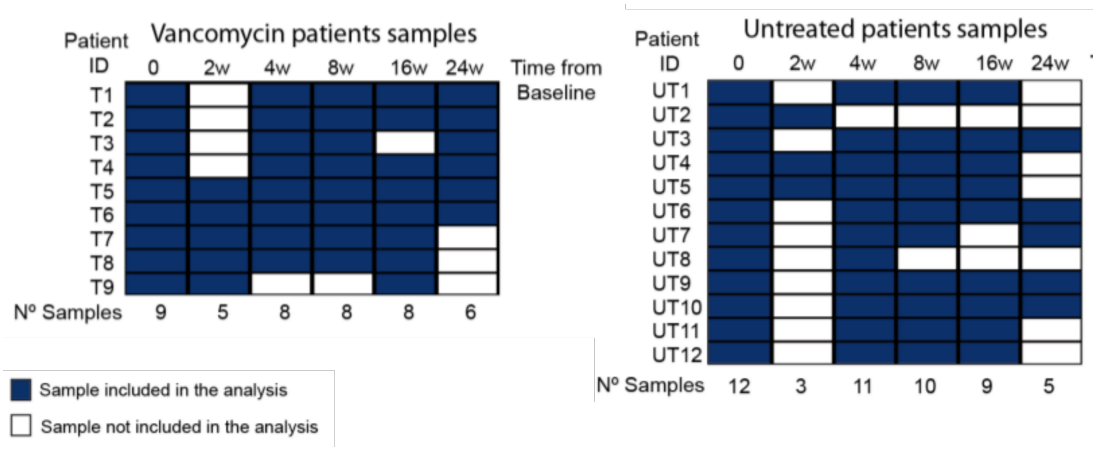


Figure 3.1: Samples included in the study. The fecal samples that were included for the microbiota analysis are shown in blue. The ID of the patient is indicated. Specific samples at specific time points were not analyzed because either the sample could not be collected at that specific time point or the number of obtained sequences was too low to be included it in the analysis. w: weeks.

3.1.1 Vancomycin induces persistent changes in the human microbiota structure.

First, to have a global view of the changes induced by vancomycin, we quantified microbiota similarities among fecal samples using unweighted UniFrac [226] and applied UPGMA hierarchical clustering to group samples by microbiota similarity as described in section 2.5.4 of materials and methods. Fecal samples were clustered into two major groups (Figure 3.2A): (1) baseline samples and prospective samples from untreated (UT) patients, and (2) samples obtained immediately after vancomycin treatment and most of the samples obtained after antibiotic cessation from the majority of vancomycin treated (T) patients. This result indicates that vancomycin induces a major shift in the overall structure of the intestinal microbiota, which persists in most patients even 22 weeks after antibiotic cessation. Interestingly, samples from cluster (2), could be further clustered into two major subgroups: (2a) which includes samples obtained immediately after vancomycin therapy, and (2b) which includes samples obtained weeks after antibiotic cessation. This result indicates that, irrespective of the baseline microbiota, vancomycin treatment promotes similar community perturbations. However, following antibiotic discontinuation, the microbiota alterations are of a different nature. In some cases, these changes lead to a completely new state of microbial composition that differs significantly from that of the baseline biologic community, even 22 weeks after antibiotic cessation (i.e. patients T2, T4, T3, T5). In other cases, the microbiota recovers to a greater extent (i.e. samples after antibiotic cessation from patients T6 and T8 cluster together with their respective baseline samples). As expected, samples from untreated patients clustered with their respective baseline samples, indicating that the microbiota of untreated patients is stable with inter-individual taxonomic differences maintained through the 6 months study period, even in the presence of methotrexate. These results were confirmed by applying Principal Coordinate of Analysis to the UniFrac distances (Figure 3.2B). The

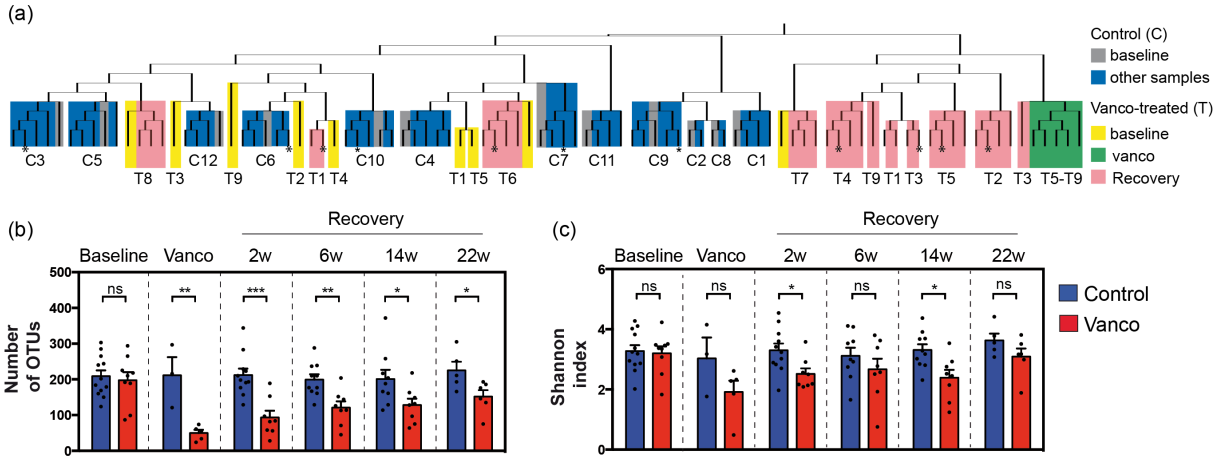


Figure 3.2: Vancomycin induces persistent changes in the human microbiota structure. Patients were treated with vancomycin for 2 weeks (T, treated) or left untreated (UT). Fecal samples were obtained immediately before treatment (baseline), the day of antibiotic cessation (vanco) and 2, 6, 14 and 22 weeks after antibiotic cessation (Vanco Recov). As control, fecal samples from untreated patients were obtained at similar time points. (A) UPGMA hierarchical clustering based on unweighted UniFrac distances among the fecal microbiota samples analyzed from vancomycin and untreated patients. Colors indicate the time frame where the fecal sample was obtained. Numbers indicate the patient ID. Those samples obtained at the last time point (22 weeks post-antibiotic withdrawal) are labelled with asterisks. (B) Values from the first principal coordinate of analysis obtained after applying PCoA to the unweighted UniFrac distance matrix generated from the comparison between the microbiota identified in fecal samples from treated and untreated patients. (C) UniFrac distances between samples obtained at a given time point and the corresponding baseline sample from the same patient. Boxes extent from the 25th to 75th percentiles. The line at the middle of the box represents the median. Whiskers extent from the minimum to the maximum values. **p<0.01, ***p<0.001, two-tailed t-test. N= 5-12 per group and time point except for the second time point of untreated patients were N=3.

first coordinate differentiated patients by treatment. Notably, following antibiotic cessation, the microbiota from some vancomycin-treated patients gradually returned to the baseline state, while in others remained significantly altered. Nevertheless, patients that developed a greater microbiota recovery after treatment, showed a greater dissimilarity from their baseline microbiota than did samples from untreated patients (Figure 3.2C). This result suggests that persistent changes in the microbiota composition occur in all vancomycin-treated patients, although to a different degree depending on the patient.

3.1.2 Vancomycin treatment diminishes the richness and diversity of the human microbiota.

Subsequently, we analyzed the impact of vancomycin treatment on microbiota richness, measured as the number of identified OTUs. The number of OTUs did not differ over time in patients that did not receive vancomycin (Figure 3.3A, Figure 3.4A).

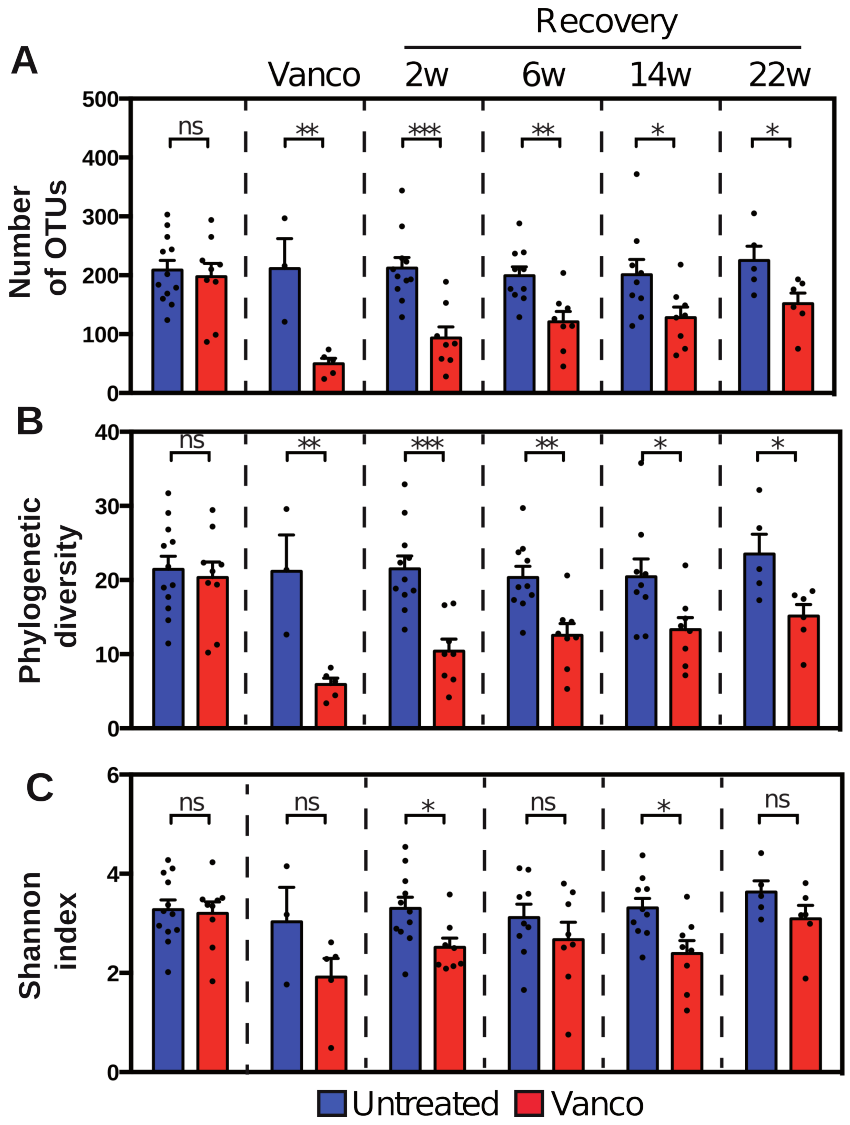


Figure 3.3: Vancomycin diminishes the human microbiota richness and diversity. (A) Number of OTUs, (B) phylodiversity index and (C) Shannon diversity index calculated from the microbiota of fecal samples obtained at baseline, immediately after treatment (vanco) and 2, 6, 14 and 22 weeks after the antibiotic cessation (Recovery). For comparison, the same indices were calculated from the fecal samples obtained at similar time points in untreated patients. Bargraphs represent mean ± SEM. * $p<0.05$, **$p<0.01$,***$p<0.001$, ns: not significant, two-tailed t-test. N= 5-12 per group and time point except for the second time point from untreated patients were N=3.

By contrast, microbiota richness was greatly reduced upon vancomycin administration. Following antibiotic cessation, microbiota richness gradually increased, although it never recovered baseline levels. A similar result was obtained when the phylodiversity index was calculated, which takes into consideration the number of OTUs and the phylogenetic relation among them (Figure 3.3B).

Similar results were obtained when the Shannon diversity index was calculated (Figure 3.3C, Figure 3.4B), which takes into account the number of OTUs and their relative proportion. A reduction in the Shannon index upon vancomycin treatment was observed for the majority of the patients, although it did not reach statistical significance. Up to 14 weeks after antibiotic cessation, the Shannon index remained lower compared to the baseline in most patients. However, baseline levels were recovered 22 weeks after antibiotic withdrawal.

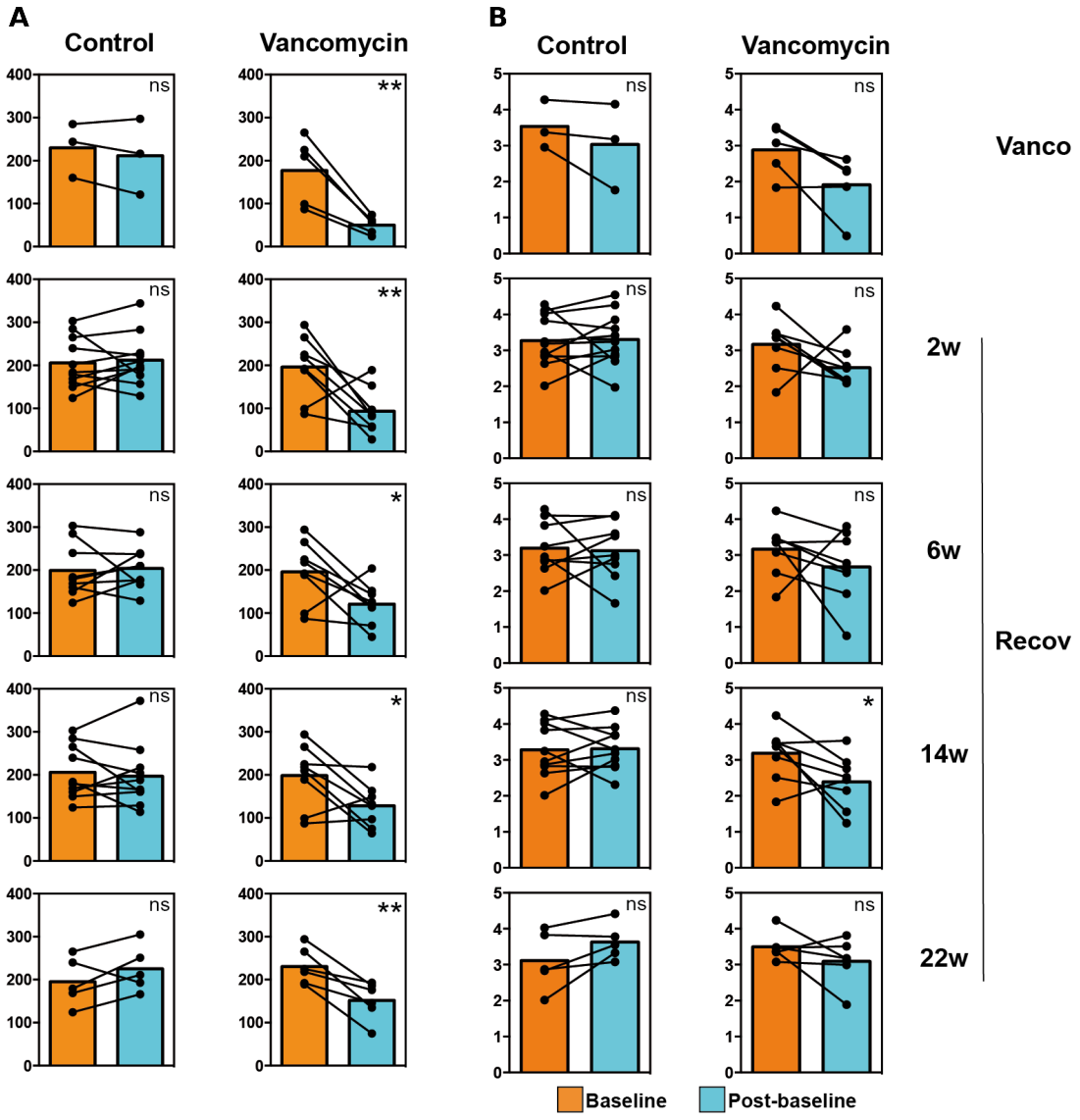


Figure 3.4: Individual variation of the human microbiota richness and diversity upon vancomycin treatment. The (A)Number of OTUs and (B)Shannon diversity index identified before vancomycin treatment (baseline) was compared with the number of OTUs or Shannon index identified in samples collected from the same patients treated with vancomycin for two weeks (vanco) or from fecal samples collected 2, 6, 14 and 22 weeks post antibiotic cessation (Recov). The number of OTUs or Shannon index was also calculated in vancomycin-untreated (control) patients at similar time points for comparison. Lines connect those samples that come from the same patient. * $p<0.05$, **$p<0.01$, ns: not significant, two-tailed t-test. N= 5-12 per group and time point except for the second time point from control patients were N=3.

3.1.3 The abundance of most intestinal bacterial taxa is altered during vancomycin treatment.

Considering the large effect of vancomycin on the overall microbiota structure and richness, we decided to examine its effects on specific taxa. We focused on those taxa that were present in at least 50% of the patients (either at baseline or after treatment). Vancomycin treatment highly reduced the levels of the Bacteroidetes phylum (Figure 3.5A). In contrast, the Proteobacteria and Fusobacteria phyla underwent a drastic expansion after vancomycin therapy. The abundance of most analyzed genera and OTUs was also significantly altered by vancomycin treatment (Figure 3.5B,C). Indeed, most genera and OTUs from the Bacteroidetes or Firmicutes phylum could not be detected in any of the patients after vancomycin treatment. By contrast, some Firmicutes increased after vancomycin treatment (i.e. *Megasphaera*, *Veillonella*, *Phascolarctobacterium*, *Lactobacillus*), likely explaining why, overall, the Firmicutes phylum did not diminish upon vancomycin treatment (Figure 3.5A). Importantly, several genera and/or OTUs belonging to the Proteobacteria phylum (i.e., *Escherichia/Shigella*, *Klebsiella*, *Kluyvera* and *Proteus*) or the Fusobacteria phylum (i.e. *Fusobacterium*), which are known pathogens, increased after antibiotic administration.

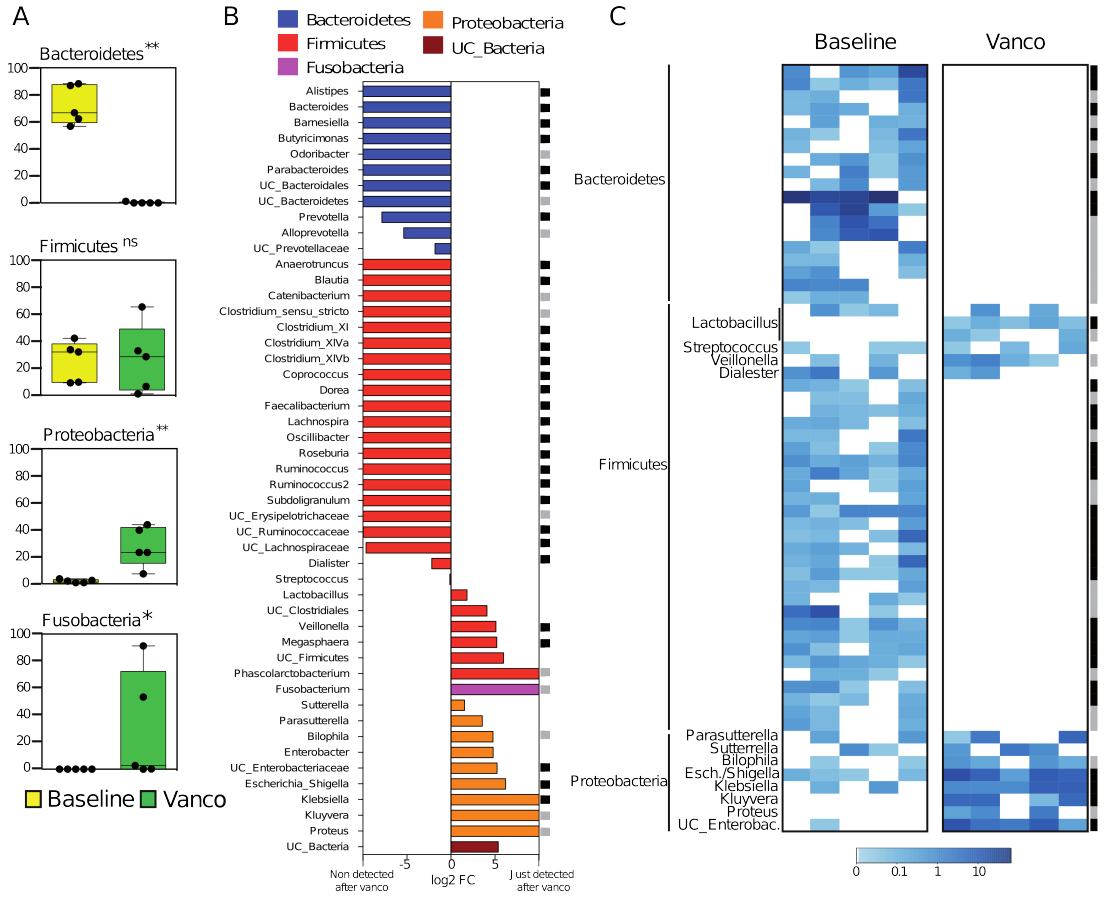


Figure 3.5: The abundance of the majority of taxa and OTUs is altered during vancomycin treatment. (A) Phyla relative abundance of patients treated with vancomycin at baseline or 2 weeks after treatment initiation. Only phyla that are present in at least 50% of the patients at baseline or after vancomycin treatment are shown. **p<0.01,*p<0.05, FDR<0.2, two-tailed wilcoxon test. ns: not significant. (B) Log2 average fold-change (FC) between the genera abundance from samples obtained immediately after vancomycin treatment compared with their respective baseline samples. Only genera that are present in at least 50% of the patients at baseline or after vancomycin treatment are shown. Genera are sorted by phyla, FC difference and then alphabetically. (C) Heatmap representing the relative abundance (%) of OTUs present at least in 50% of the patients at baseline or after vancomycin treatment. The genus taxonomy of OTUs that increased or were unaffected by the treatment is indicated. Both for B and C: black squares indicate significant changes (p<0.05, FDR<0.1); grey squares indicate close to significance changes (p<0.073, FDR<0.1); two-tailed wilcoxon test. N= 5. UC=unclassified.

3.1.4 Incomplete and individualized microbiota recovery after vancomycin withdrawal.

We next examined the capacity of the different commensal bacteria to recover after antibiotic cessation (Figure 3.6).

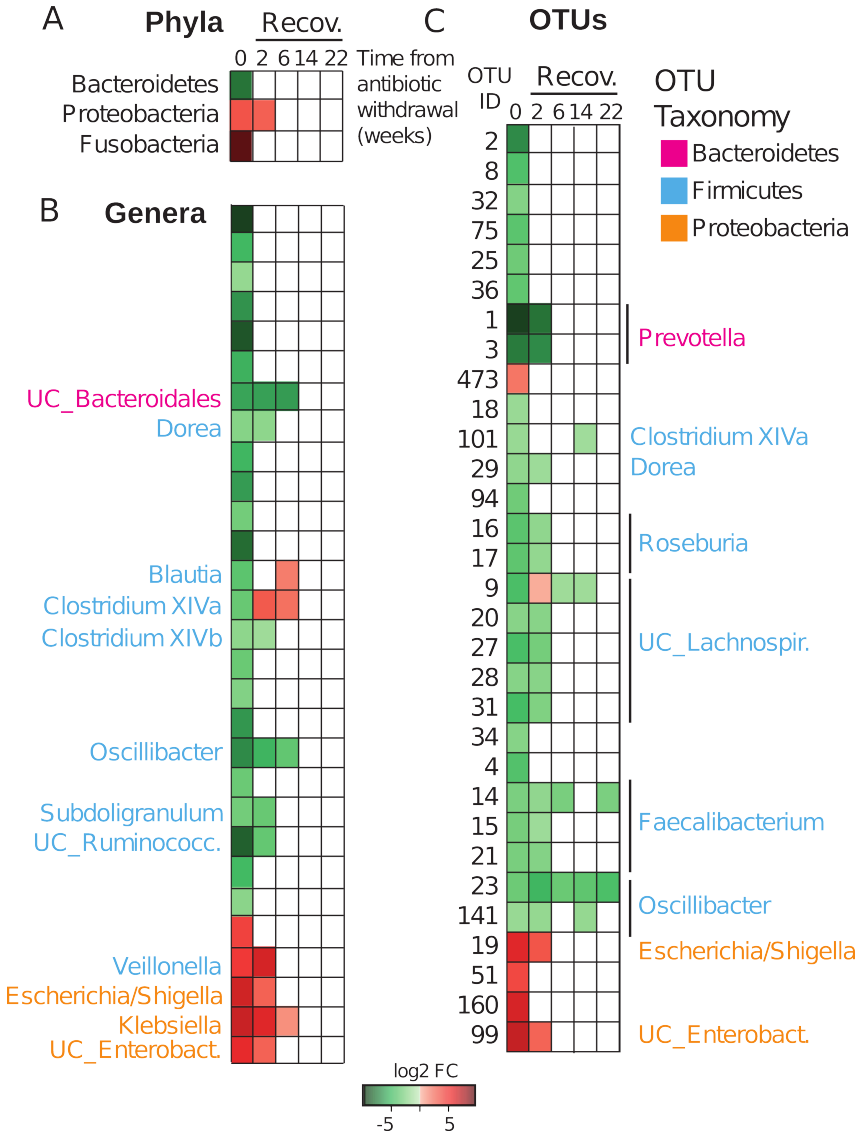


Figure 3.6: Changes in the human microbiota following vancomycin withdrawal. Heatmap representing the average fold change of (A) phyla, (B) genera, (C) OTUs that were significantly increased (red) or decreased (green) ($p<0.05$, FDR<0.2, two-tailed wilcoxon test) in a given time point as compared to the baseline. Time 0 represents the sample obtained the day of vancomycin withdrawal. N=6-9 per time point.

We focused on those commensals whose relative abundance significantly changed during vancomycin therapy. At the phylum level, Bacteroidetes and Fusobacteria recovered within 2 weeks post-vancomycin cessation. The abundance of the phylum Proteobacteria was increased 2 weeks after discontinuation, however, it returned to baseline levels 4 weeks later. At the genus level, most taxa recovered rapidly, within 2 weeks post-

cessation. However, the abundance of some genera including *Klebsiella* or *Clostridium XIVa*, continued to be increased 6 weeks post-antibiotic withdrawal, while *Oscillibacter* was still reduced at this time point. No differences at the genera level were observed 14 and 22 weeks post-antibiotic withdrawal as compared to baseline samples. A similar picture could be observed at the OTU level. In this case, however, the abundance of most of the OTUs that were altered by vancomycin 2 weeks post-antibiotic cessation remained significantly different as compared to baseline. In addition, one OTU from the genera *Oscillibacter* never recovered its baseline levels. None of these microbiota changes were observed at any time point in untreated patients.

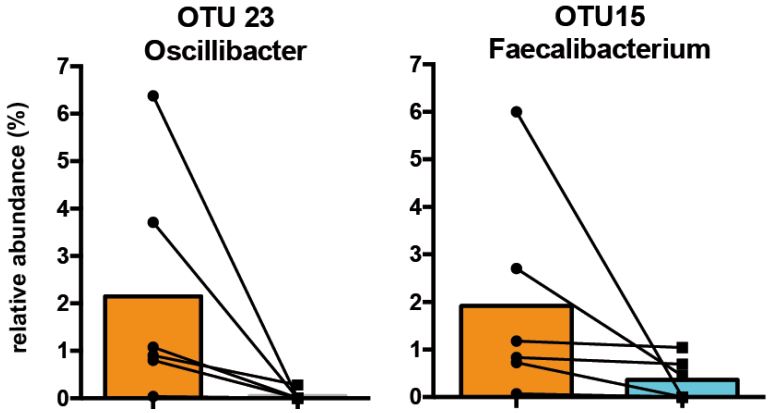


Figure 3.7: Different OTU recovery upon vancomycin cessation. The relative abundance of OTU23 and OTU15 in vancomycin treated patients at baseline or 22 weeks post-vancomycin cessation. Lines connect those samples that come from the same patient. N=6.

The performed analysis allowed us to identify perturbation patterns common to all patients after antibiotic cessation. For example, OTU23 abundance was significantly reduced 22 weeks post-antibiotic cessation since this OTU was not recovered in any of the patients analyzed (Figure 3.7). However, this type of analysis does not allow the identification of inter-individual differences in the microbiota recovery. For example, OTU15 abundance was diminished 22 weeks post-antibiotic cessation, but this change was not significant due to the variable recovery of this OTU among patients. Considering that previous analysis (Figure 3.2) suggested that major shifts in the microbiota of some patients still occurred 22 weeks post-antibiotic cessation, we decided to characterize the level of taxa recovery at the individual level for each patient0. To this end, we identified and plotted the most abundant OTUs present at baseline for each patient (>10 counts), and analyzed the recovery of those OTUs after antibiotic withdrawal (Figure 3.8). As expected, there was inter-individual variation in the recovery pattern. For example, patients T6 and T8, whose recovery samples clustered with their baseline samples (Figure 3.2), recovered the majority of their most abundant OTUs. Nevertheless, at the last time point (22 weeks) from these "high-recovery" patients, 42.1% and 15.4% of their most abundant OTUs still could not be detected. Among those patients whose recovery samples did not cluster with their baseline samples 3.2 A, a different degree of recovery was observed. In the most affected patient, T5, as few as 10.7% of its most abundant OTUs could be detected 22

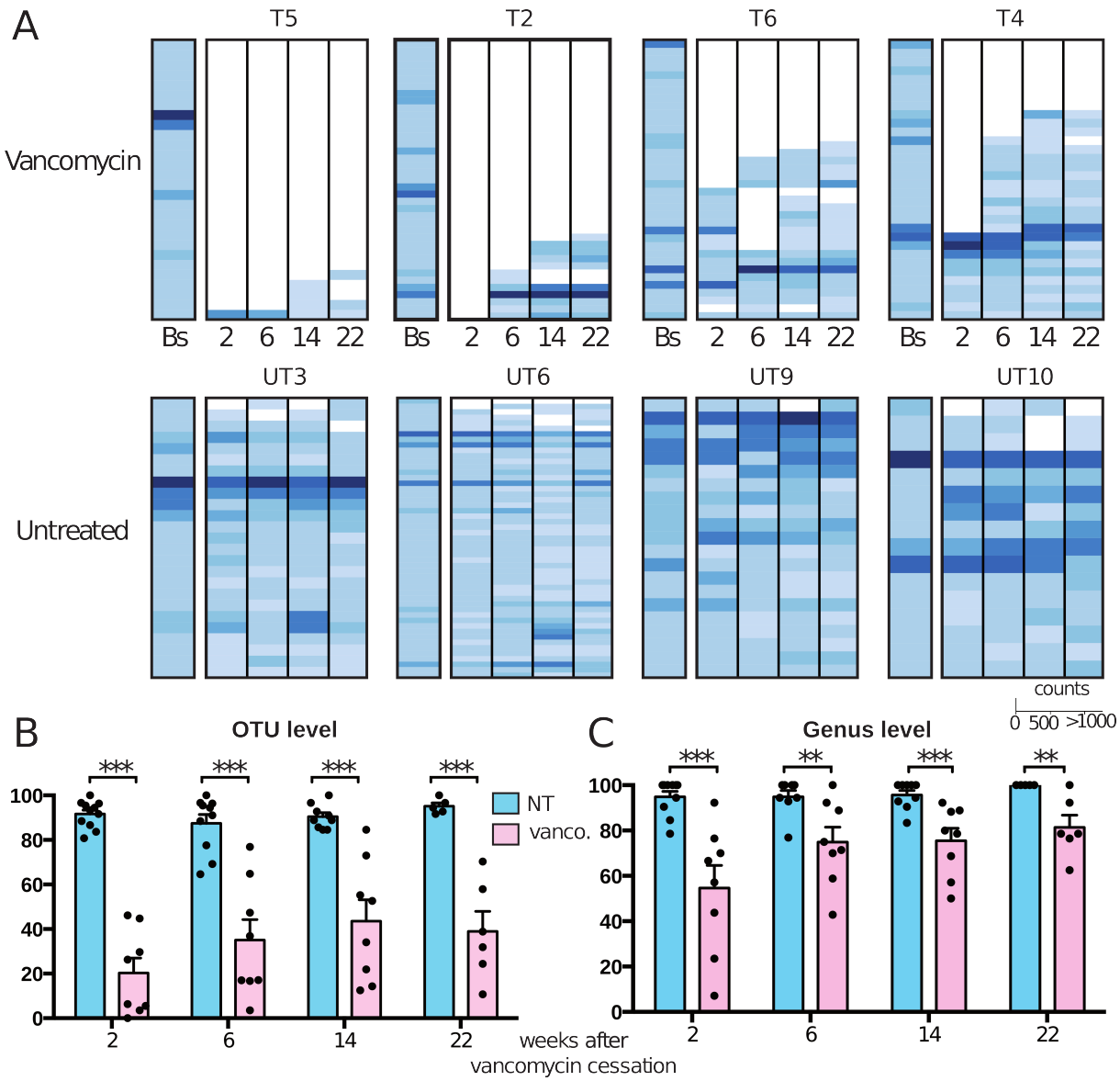


Figure 3.8: Incomplete and individualized recovery of the human microbiota after vancomycin withdrawal. (A) Relative abundance at baseline (Bs) and at different weeks post-antibiotic cessation of OTUs whose abundance was greater than 10 counts in the baseline sample of the analyzed patient. Note that each patient baseline OTUs are distinct. The ID of each patient is indicated. As control, samples at similar time-points were analyzed from patients not receiving vancomycin. 4 representative patients from each group are shown (the 4 untreated patients for which we were able to collect and analyze fecal samples at every time point and 4 out of the 5 vancomycin treated patients for which we were able to collect and analyze fecal samples at every time). (B) Percentage of detected OTUs or (C) genera at different time-points post-antibiotic cessation, whose abundance was greater than 10 counts in the baseline sample. ***p<0.001,**p<0.01; two-tailed t test. N=6-12 per time point.

weeks post-antibiotic withdrawal, while on patient T4, 70% of its most abundant OTUs could be detected. Untreated patients did develop a few changes over time, and some OTUs were absent in a given time point. However, in most cases, the missing OTUs were detected at subsequent sampling. Overall, in untreated patients, $90.6 \pm 8.1\%$ of the most abundant OTUs at baseline could be detected at any time point (Figure 3.8B). By contrast, in vancomycin treated patients, we could only detect $39.0 \pm 21.9\%$ of the baseline most abundant OTUs at the last time point. A similar result was obtained at the genus level, although the recovery rate was greater than the one observed for OTUs (Figure 3.8C).

3.1.5 Microbiota recovery rate after vancomycin cessation influences the level of susceptibility to VRE colonization.

We have demonstrated that upon vancomycin cessation, the human microbiota recovers to a different extent in a subject-specific manner. It is conceivable, therefore, that antibiotic-driven dysbiosis and the microbiota recovery rate observed after vancomycin cessation could impact susceptibility to infection, with "slow recoverers" being at higher risk. Because vancomycin also induces long lasting intestinal microbiota perturbations in mice, allowing for intestinal colonization by VRE [175], we used this model of infection to investigate if a different microbiota recovery pattern upon vancomycin cessation could influence susceptibility to infection. We focused on the microbiota recovery at 2 weeks post-antibiotic cessation since: 1) previous reports indicate that CDI patients are mainly at-risk for the development of intestinal infections during the first 3 weeks after vancomycin cessation [250]; and 2) we have found that at 2 weeks post-vancomycin withdrawal, there are already major differences in the microbiota recovery among different patients (Figure 3.8).

We first examined whether vancomycin induces similar microbiota changes in mice compared to humans. As shown in Figure 3.9, alterations observed in the murine microbiota during vancomycin treatment were strikingly similar to those observed in patients, including: a) decrease in microbiota richness (Figure 3.9B), b) depletion of all Bacteroidetes OTUs and most Firmicutes OTUs, and c) increase of Proteobacteria OTUs (Figure 3.9A).

Moreover, 2 weeks post-antibiotic cessation, mice recovered a subset of their baseline OTUs (Figure 3.9A), leading to an increase in the phylodiversity index and the number of OTUs (Figure 3.9B). Noticeably, and in line with the observation in humans, this recovery was variable. We next evaluated, using this mouse model, the extent to which a different microbiota recovery rate after vancomycin administration could impact VRE intestinal colonization. As shown in Figure 3.9C and previously reported [175], untreated mice were resistant to VRE colonization, while mice that received oral vancomycin were highly susceptible to VRE colonization. 2 weeks after antibiotic cessation, mice were still susceptible to VRE colonization, but with a high degree of interindividual variability. We then analyzed the fecal microbiota composition immediately before infection, on mice that recovered from vancomycin treatment and correlated the degree of microbiota recovery with the VRE levels. Notably, there was a significant negative correlation between the

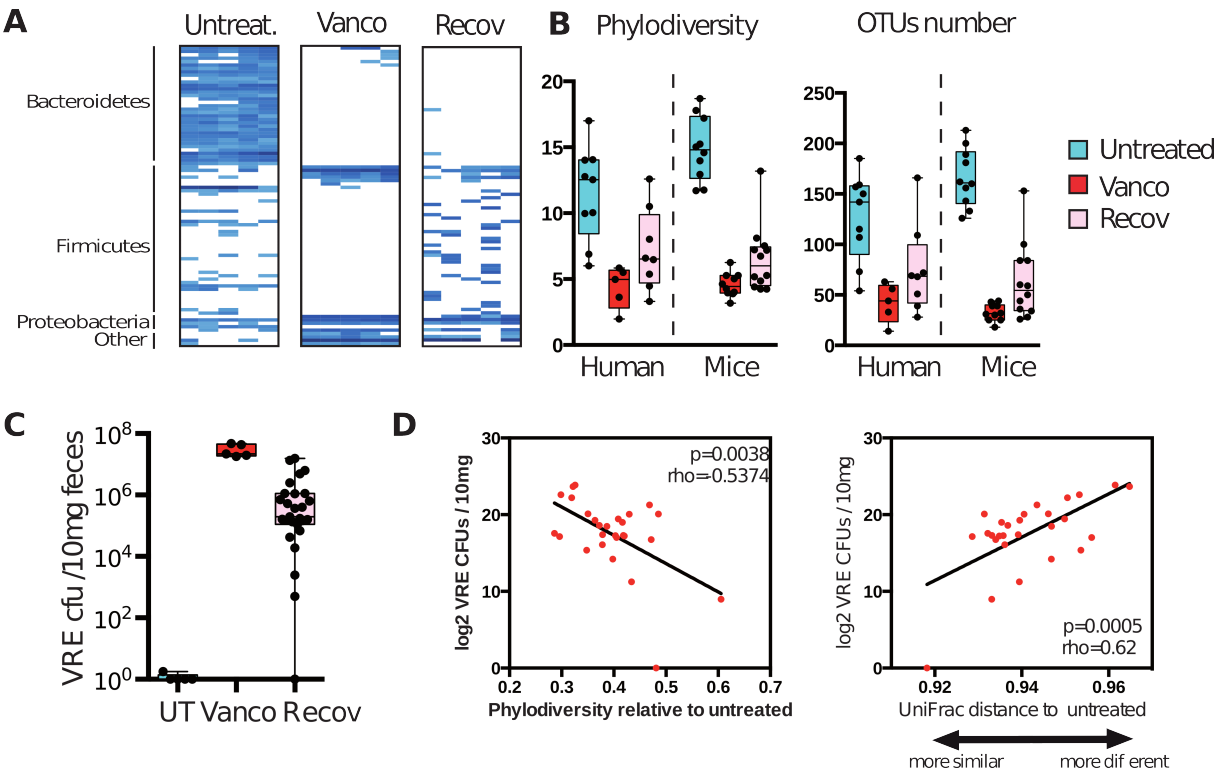


Figure 3.9: Microbiota recovery rate after vancomycin cessation influences the level of susceptibility to VRE intestinal colonization. (A) Heatmap showing the most prevalent OTUs (>10 counts per group of mice) found in the feces of mice treated with vancomycin for seven days (Vanco), two weeks after antibiotic cessation (Recov) or in mice that remained untreated (Untreat.). (N=5 per group). (B) Number of OTUs and phylodiversity index identified in fecal samples from untreated mice or humans, or mice/humans that were treated with oral vancomycin, the day the treatment was stopped (Vanco) or two weeks post-antibiotic cessation (Recov). Boxes extent from the 25th to 75th percentiles. The line at the middle of the box represents the median. Whiskers extent from the minimum to the maximum values. (N=5-12 per group). (C) VRE CFUs/10mg of feces 2 days post-infection in untreated mice (UT), mice that received vancomycin for 1 week and were infected immediately after vancomycin therapy (vanco) or two weeks post-antibiotic cessation (Recov). N=5 for NT and Vanco group, N=27 for Recov group. (D) Correlation analysis between variable in y axis: log2 VRE CFUs/10mg of feces detected the second day after infection in mice that were infected 2 weeks after vancomycin cessation, and variable in x axis: (i) phylodiversity index identified in the infected mice, the day of infection, divided by average phylodiversity index identified in untreated mice or (ii) the average UniFrac distance between infected mice, the day of infection, and untreated mice. N=27. ***p<0.001,**p<0.01; two-tailed t test. N=6-12 per time point.

phylodiversity index and the number of VRE CFUs in feces, indicating that mice that recovered a phylodiversity index closer to that of untreated mice were less susceptible to VRE colonization (Figure 3.9D). In addition, mice that recovered a microbiota more similar to that of untreated mice (lower UniFrac distance) were less susceptible to VRE colonization (Figure 3.9D). In conclusion, the microbiota recovery rate after vancomycin cessation influences the level of susceptibility to intestinal colonization by VRE.

3.1.6 Verification that the sequencing depth does not affect the conclusions of the study.

Additional sequencing analysis were performed using a different rarefaction strategy to demonstrate that the results obtained in this study do not depend on utilizing just a subset of 2883 sequences per sample.

First, we increased the number of sequences analyzed from human fecal samples by rarefying samples collected from a particular patient to the lowest number of sequences found in the samples collected from that particular patient, instead of rarefying to the lowest number of sequences in the entire dataset (i.e. 2883). The individual (intra-patient) rarefaction step allowed for an increase in the number of sequences analyzed (see figure 3.10, where the exact number of sequences included for each patient sample is indicated).

Subsequently, we calculated, for each patient, the number of OTUs, phylodiversity index and Shannon index using the newly obtained set of sequences. We then calculated the fraction of OTUs identified at each time-point (post-treatment) as compared to the number of OTUs obtained from the baseline sample for each particular patient. The same analysis was performed for the number of OTUs obtained using 2883 sequences for all samples (the number of sequences used in the main Figures). As shown in the Figure 3.10 A, the same richness (number of OTUs) changes upon vancomycin treatment were observed using a higher number of sequences per sample. Similar results were obtained for the Phylodiversity index and Shannon index (Figure 3.10 B). Thus, independently of the number of sequences used per sample, we observed a reduction of richness and diversity respect to baseline, both immediately after vancomycin treatment or weeks after antibiotic cessation.

In order to demonstrate that a subset of 800 sequences (utilized in Figure 3.9) is representative of the changes observed in the microbiota of mice after vancomycin administration, we performed a rarefaction curve with all the sequences obtained from the murine samples (Figure 3.11).

As shown in this figure, as the number of analyzed sequences is increased, the number of OTUs detected also increases. Nevertheless, the conclusions on microbiota richness changes after vancomycin treatment does not seem to be affected by analyzing a lower number of sequences. As shown in Figure 3.11, independently of the sequence depth, the number of OTUs is greatly reduced during vancomycin treatment. However, two weeks after vancomycin cessation, some of the mice partially recover their microbiota OTUs richness, although this recovery was variable depending on the mouse.

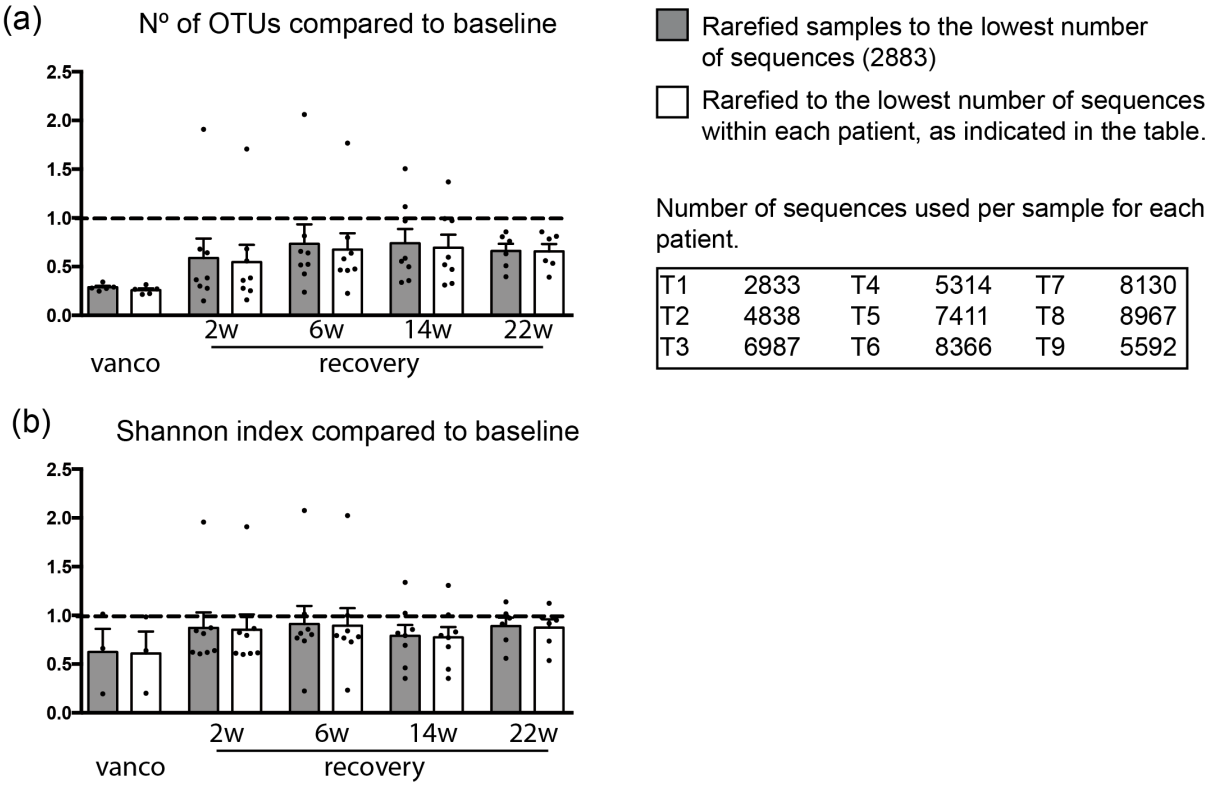


Figure 3.10: Similar microbial richness reduction after vancomycin treatment was observed using different sequencing depth. The number of (a) OTUs or (b) Shannon index was calculated for each sample after rarefying to the lowest number of sequences found in any sample (2883) or rarefying to the lowest number of sequences within each patient, as indicated. The fold change difference between the richness and diversity obtained in a given sample compared to its respective baseline sample was calculated and plotted for each patient. The dash line indicates those samples with no fold change difference as compared to the baseline sample. The same level of richness and diversity reduction, immediately after vancomycin treatment (vanco) or weeks after antibiotic cessation (recovery), was observed using a higher number of sequences per sample.

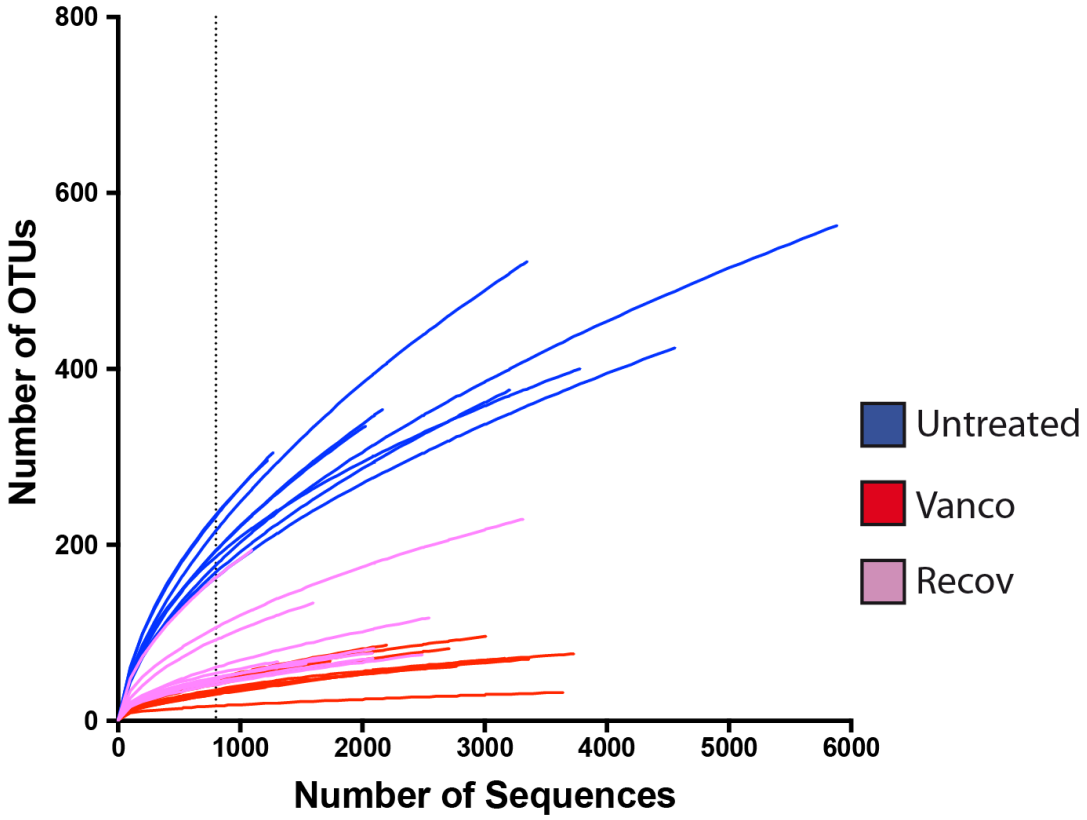


Figure 3.11: The number of sequences included in the analysis does not affect the changes in microbiota richness observed after vancomycin administration in mice. The number of OTUs identified for each sample using different number of sequences was calculated and plotted. Fecal samples analyzed were obtained from mice that were treated with vancomycin for 1 week (vanco), mice that were allowed to recover for 2 weeks after vancomycin treatment (recov) or mice that did not receive antibiotics (untreated). The dash line intercepts the x-axis at the value 800, the number of sequences used in the analysis shown in Figure 3.9B. Rarefaction curves show that the conclusions obtained with the mouse sequencing data are independent on the number of sequences included in the analysis.

3.2 Effect of antibiotics on gut microbiota and influence on VRE colonization

It has been previously described that antibiotics, by disrupting the gut microbiota, annihilate the CR normally proportionated by a healthy gut microbiota [17,112,251]. We just demonstrated that the rate of recovery after vancomycin treatment (alternatively, the extent of perturbation) of the microbiota correlate significantly with the level of colonization by VRE [252]. It has also been shown that recovery of the microbiota through a fecal transplant restores the CR to VRE lost upon antibiotic treatment [112]. Thus, commensal bacteria have a key role in conferring protection against VRE intestinal colonization. On the other hand, not every bacteria is important for suppressing VRE intestinal colonization. For example, administration of *Klebsiella* to antibiotic treated mice do not prevent VRE colonization [214]. Consequently, it is probable that specific commensal bacteria and functions are responsible for the CR against VRE infection. However,these bacteria and functions have not been not fully identified nowadays.

Taking advantage of the mouse model that we developed in the previous study to assess the effect of vancomycin on gut microbiota and VRE colonization, we decided to study how antibiotics of various spectrum affect the gut microbiota and promote VRE intestinal colonization. This data will improve our understanding of the association between antibiotic treatments, alteration of the microbiota and secondary infections.

Moreover, these data, associating different alterations of the gut microbiota and the level of VRE intestinal colonization will enable us to identify bacteria that are associated with the resistance against this opportunistic pathogen. We expect that VRE will colonize in high levels the intestinal tract when the antibiotic treatment suppresses commensal bacteria that are relevant for conferring resistance against VRE. These relevant bacteria will still be present in those mice receiving an antibiotic that do not promote VRE intestinal colonization. Of course, the antibiotic should not have any direct effect on VRE growth. Thus, by analyzing the effect of several treatments with different spectra of action on the microbiota composition and capabilities of VRE to colonize the intestine, we expect to identify the commensal bacteria whose presence is related with a low VRE load while its elimination is consistently related with a higher VRE colonization. The identified bacteria may be key in conferring protection against VRE colonization.

For this purpose, we used antibiotics with a described spectrum of action against (I) gram positive bacteria (vancomycin), (II) gram negative bacteria (neomycin), (III) gram positive and anaerobes (clindamycin), (IV) a mix activity against both gram positive and gram negative bacteria (ceftriaxone, ciprofloxacin, ampicillin) (Table 2.1 and Figure 2.2).

For each treatment, we analyzed the microbiota composition before challenging the mice with VRE. The alterations in the microbiota and the capability of VRE to colonize the intestine were evaluated both during the antibiotic treatment and also after a two-week period of recovery (Figure 2.1). Thus, we can also evaluate how the microbiota recovers in each case and how it does affect the colonization by VRE. in the following sections, we will name these two different conditions: (i) “during treatment” or “without recovery” and (ii) “with recovery” respectively.

The present study was performed in 2013, using the Roche 454 platform to sequence the samples (see 2.5.1.2). Using this approach, we obtained a mean of 2652 sequences per

sample. With the advent of Illumina technology, we decided to sequence again these samples to obtain a higher sequencing coverage. The following results were obtained thanks to this second sequencing approach. We identified similar changes in the microbiota composition as the ones detected previously with 454 but, moreover, we were able to detect also low abundant species.

3.2.1 The amplitude of the gut microbiota dysbiosis depends on the type of antibiotic treatment

To assess the extent of the alterations provoked by each treatment, we first estimated the total bacterial load calculating the biomass (quantity of DNA/g faeces) as previously described [228] (Figure 3.12 A). A trend to a biomass diminution was detected for all the given treatments, being ceftriaxone the treatment causing the highest diminution but also the most variable effect among different mice. The diminution in biomass was significant for all the treatments except for ciprofloxacin.

Subsequently, we assessed the bacterial richness (the number of OTUs) present during the different treatments. All the treatments caused a significant diminution of the bacterial richness by comparison with the level observed in untreated mice. The smallest diminution was detected after neomycin administration, an intermediate diminution was detected after ciprofloxacin, ceftriaxone or ampicillin treatment and the highest diminution occurred after the administration of clindamycin or vancomycin (Figure 3.12 B).

To have a better idea of the microbiota alterations resulting from the antibiotic administration, we calculated the shannon index. This index accounts both for the number of species present and for the relative abundance of each one: for the same number of species present, if they are equally abundant, the score will be higher than if there is a dominant species and the other ones are very low abundant. The shannon index calculated in ciprofloxacin treated mice was similar as the one calculated in untreated mice. Except for this treatment, all the antibiotics administered caused a significant diminution of the diversity calculated with the shannon index. Neomycin caused a small diminution of the diversity respect to the index calculated in untreated mice, ceftriaxone and ampicillin caused a significant and similar diminution whereas the biggest diminution of diversity was registered after clindamycin and vancomycin treatment (Figure 3.12 C).

Thus, as expected, antibiotics of different spectrum have a different effect on the total intestinal biomass, microbiota richness and diversity.

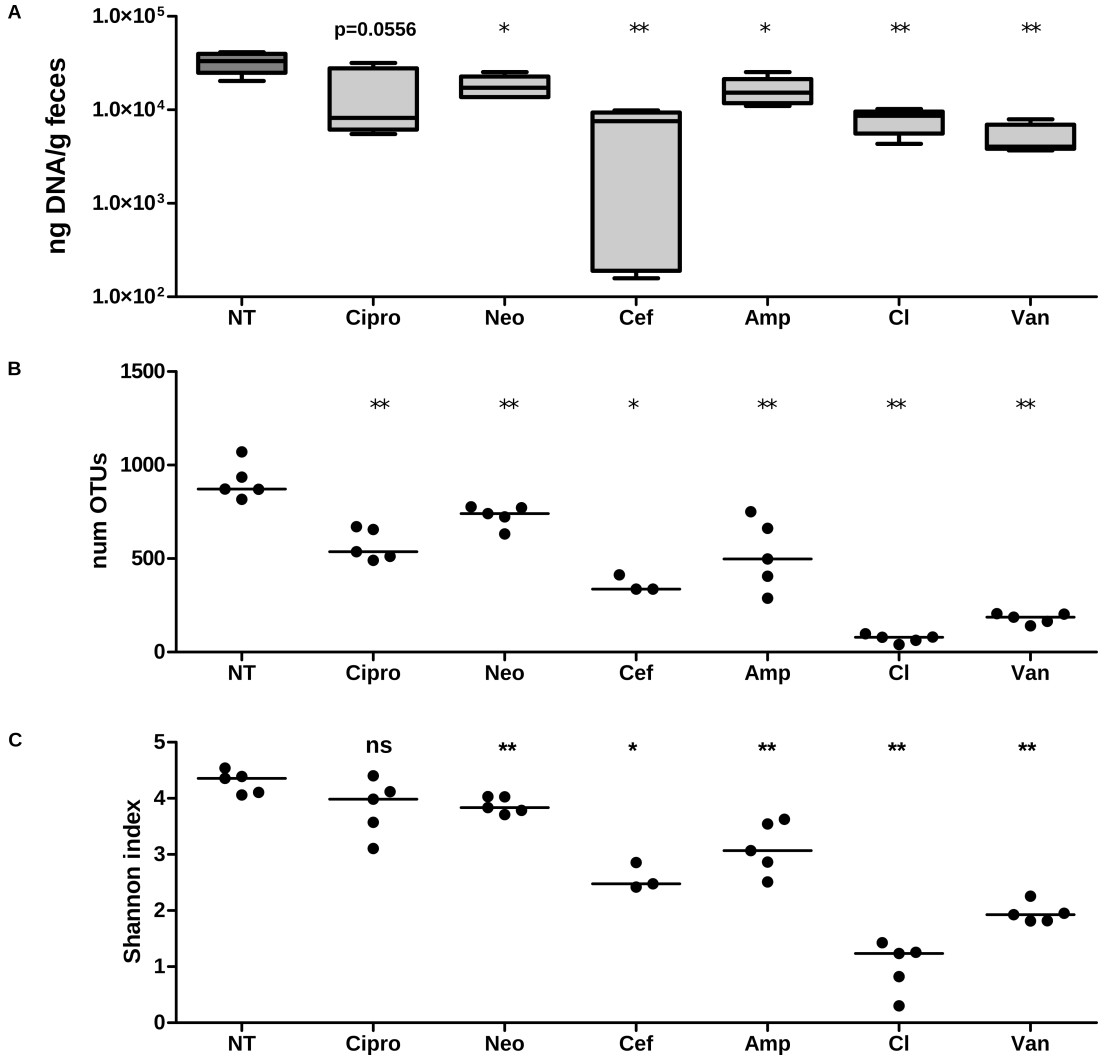


Figure 3.12: Alterations of the biomass, microbiota richness and diversity after administration of different antibiotics Mice were treated during 7 days with different antibiotics. The day of antibiotic withdrawal, a fecal pellet was collected and analyzed as described in 2.5.5. A) Biomass measured as ng of DNA per g of feces. This value represents an approximation of the total bacteria load as indicated in [228]. B) Number of OTUs detected in each sample out of 29010 sequences. This is a measure of richness. C) Shannon index. NT: Untreated, Cipro: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: clindamycin, Van: Vancomycin. n=5 per group except for Cef were N=3. The asterisks depict the significance of the Mann-Whitney test respect to values of untreated mice: ns not significant, * p.value<0.05, ** p.value<0.01. In case the p.value was almost significant, the exact p.value is indicated.

3.2.2 Antibiotic treatments of different class alter the abundance of different intestinal bacterial taxa

As we have just shown, different antibiotics cause different amplitude of alterations in the gut microbiota total bacterial load, richness and diversity. We next investigated whether the alterations are specific of each treatment or if some patterns are shared.

First, we verified that the samples were segregated according to the treatment administered. As shown on the non-metric multidimensional scaling (NMDS) plot, antibiotics affect the microbiota composition differently and specifically (Figure 3.13, see Material and Methods 2.5.5 for the parameters of the analysis). For example, vancomycin and ciprofloxacin cause contrary effects, vancomycin induced changes being characterized by an increased in the abundance of Proteobacteria (i.e. *Escherichia/Shigella* and *Parasutterella*). In the same way, clindamycin treated mice displayed a microbiota diametrically opposite to the one of the untreated mice and of the neomycin-treated mice. The untreated mice present a higher abundance, among others, of *Alistipes* and Unclassified Porphyromonadaceae, while clindamycin treated mice contained a higher abundance of *Enterococcus* and *Bacteroides*. We have just mentioned antibiotics that affect very differently the microbiota. On the opposite, ampicillin and ceftriaxone, both antibiotics belonging to the class β-lactams, seem to produce relatively similar alterations (intermediate between the untreated microbiota and the drastic alterations associated with clindamycin or vancomycin).

Taking into account that this NMDS plot is a two-dimensional representation of the variability in the microbiota composition among samples, we performed a ANOSIM test to assess whether the samples really cluster due to the treatment administration. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. An R value close to 1 indicates dissimilarity between groups while an R value close to 0 indicates that there are no differences between groups [253]. The ANOSIM test R value was 0.78 (significance: 0.001, 999 permutations), therefore we can conclude that the antibiotics affect the microbiota composition differently.

In order to have a general view of the changes in the microbiota produced by each antibiotic, we plotted the relative abundance of the most abundant genera identified in all the mice analyzed (mean abundance minimal of 5% in at least one group) (Figure 3.14). In decreasing order of abundance, untreated mice were dominated by *Barnesiella* (35%) and unclassified Porphyromonadaceae (10%). After treatment with (1) ciprofloxacin, the microbiota was dominated by *Barnesiella* (37%) and unclassified Lachnospiraceae (31%); (2) neomycin: the microbiota was dominated by *Barnesiella* (24%), *Bacteroides* (14%) and *Akkermansia* (13%); (3) ceftriaxone: the microbiota was dominated by *Bacteroides* (31%), *Barnesiella* (24%) and *Akkermansia* (22%); (4) ampicillin: the microbiota was dominated by *Bacteroides* (30%), *Barnesiella* (26%) and *Akkermansia* (15%); (5) clindamycin: the microbiota was dominated by *Bacteroides* (55%) and *Barnesiella* (28%); (6) vancomycin: the microbiota was dominated by *Akkermansia* (55%), *Lactobacillus* (16%) and *Escherichia/Shigella* (12%). Thus, the most abundant bacteria, *Barnesiella*, is maintained upon ciprofloxacin and neomycin treatment. The two $\beta-$lactams, ceftriaxone and ampicillin, present similar dominant bacteria (accounting for more than 70% of the total microbiota). Notably, the dominant one was *Bacteroides* which represents only 3% of

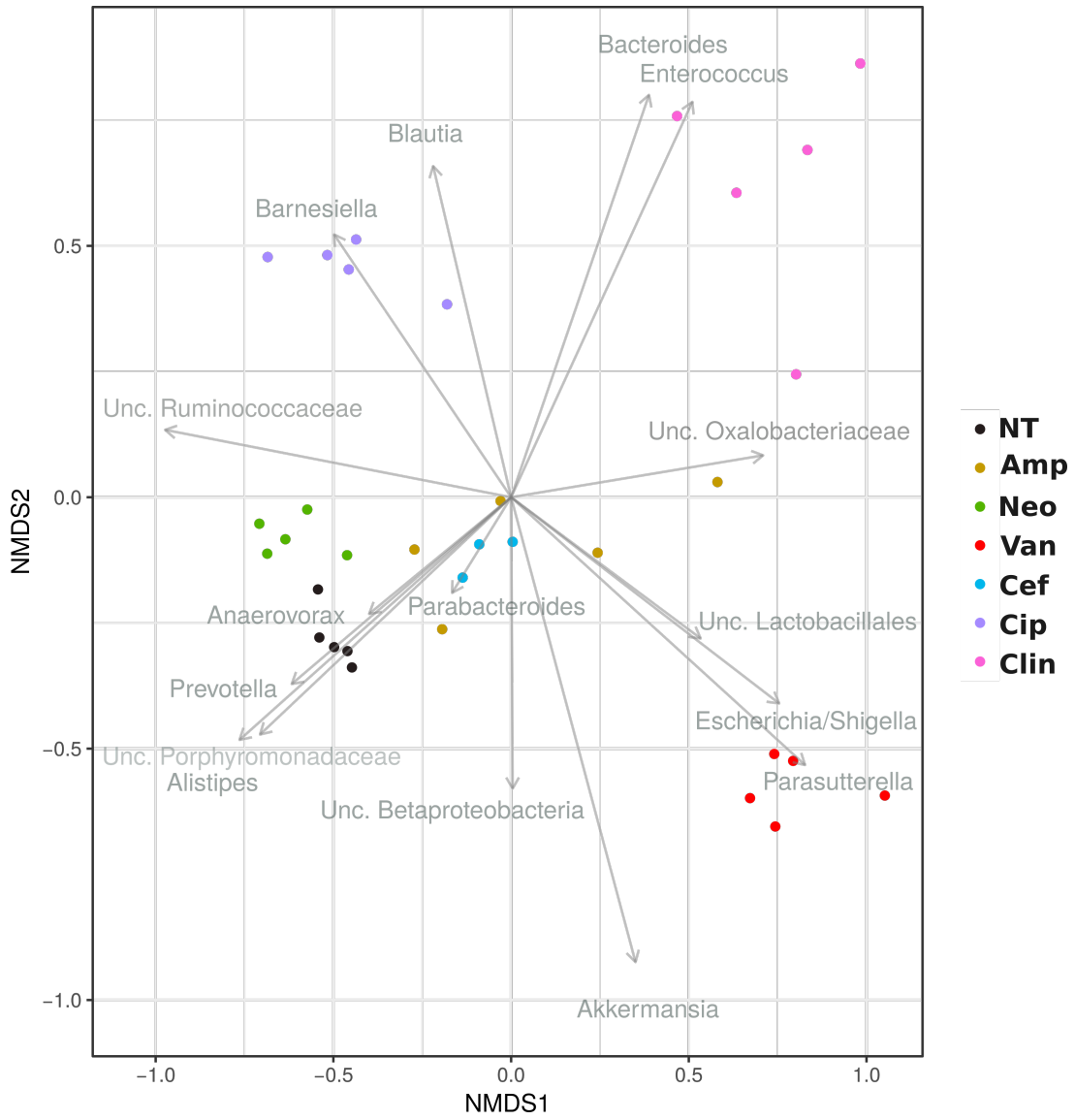


Figure 3.13: Microbiota composition separation according to the antibiotic treatment administered Mice were treated during 7 days with different antibiotics. The day of antibiotic withdrawal, a fecal pellet was collected and analyzed as described in 2.5.5. NMDS representing the differencessimilarities in the microbiota among different samples. Taxa that presented the best correlation with the topology of the samples are displayed. NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Clin: clindamycin, Van: Vancomycin. NMDS was calculated with Braycurtis distance on two dimensions. Stress: 0.1

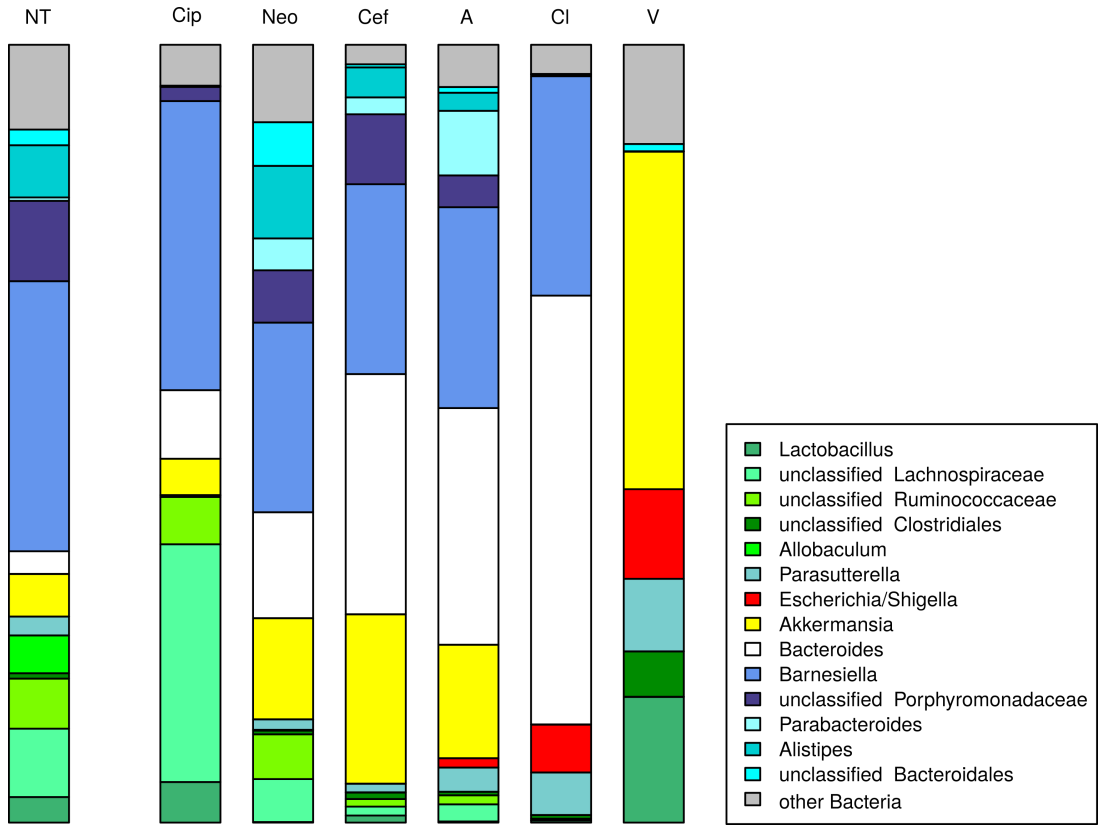


Figure 3.14: Abundance of the most prevalent genus in untreated mice and upon different antibiotic treatments. Barplot of the microbiota composition in untreated mice and mice treated with antibiotics for one week. Genera whose mean abundance reach 5% in untreated mice or upon administration of an antibiotic are represented. The other genera are grouped in "other bacteria". NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxon, A: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ by group, except for ceftriaxone $n = 3$.

the microbiota in untreated mice. In the case of clindamycin treatment, this bacterium represents also more than half of the gut microbiota.

Finally, the major alteration was observed upon vancomycin treatment: the most abundant bacteria, which sum up 83% of the total microbiota, are normally relatively low abundant in untreated mice. Indeed, in untreated mice the mean abundance of *Akkermansia*, *Lactobacillus* and *Escherichia/Shigella* represents 5%, 3% and 0.06%, respectively.

Knowing that the different antibiotics tested alter differently the most abundant bacteria, we next focused on the specific taxa altered by each treatment, included the low abundant ones. As depicted in the figure 3.15 A, all the antibiotics cause significant alterations already visible at the phylum level. Two low abundant phyla, Actinobacteria and the candidate division TM7, were consistently diminished upon the administration of any antibiotic. On the opposite, the phylum Verrumicrobia increased after the administration of most of the treatments tested except clindamycin. Indeed, this treatment causes the diminution of most of the phyla and an increase of Proteobacteria.

In the case of neomycin treatment, only the low-abundant phylum (Actinobacteria, Deferribacteres, Tenericutes and TM7) were statistically diminished and only Verrumicrobia was increased.

The two β-lactams, ceftriaxone and ampicillin caused similar alterations at the phylum level (i.e. a diminution of Firmicutes, Actinobacteria, TM7 and an increase of Verrumicrobia), except that ceftriaxone also diminished the abundance of the phylum Proteobacteria.

Concordantly with the opposite situation of vancomycin and ciprofloxacin in the NMDS plot (Figure 3.13), ciprofloxacin caused a decrease in Proteobacteria abundance whereas the abundance of this phylum increased in after vancomycin treatment. Surprisingly, except for these two antibiotics and neomycin, most of the treatment tested caused a significant diminution of the phylum Firmicutes. It is especially striking that vancomycin, an antibiotic treatment with a spectrum against gram positive (Figure 2.2) did not cause significant alteration of the phylum Firmicutes, whose members are mainly gram positive.

Vancomycin caused a significant increase of the very low abundant phylum (i.e Deferribacteres, Proteobacteria, Tenericutes) and of the phylum Verrumicrobia, whereas the abundant phylum Bacteroidetes was significantly diminished after vancomycin treatment. Specifically, the increase of the phylum Tenericutes is not surprising because these bacteria do not have a cell wall, the target of the vancomycin treatment.

To understand better the discrepancy between the spectrum of action and the alterations detected upon vancomycin administration, we examined the modifications of the microbiota at the genus level (Figure 3.15 B).

The decrease in the phylum Actinobacteria observed upon administration of any treatment is associated with the diminution of the three taxa *Aldercreutzia*, *Olsenella* and *Bifidobacterium*.

The alteration of each of the phyla Verrucomicrobia, Deferribacteres and Tenericutes were related with changes in a unique genera in each phyla (i.e *Akkermansia*, *Muscispirillum* and *Anaeroplasma*, respectively).

The increase of the low abundant phylum Tenericutes observed after the administration of vancomycin, was associated with an increase in an unique genus, *Anaeroplasma*. As we mentioned, it is not surprising as this bacteria do not present any cell wall and thus cannot be directly affected by the antibiotic.

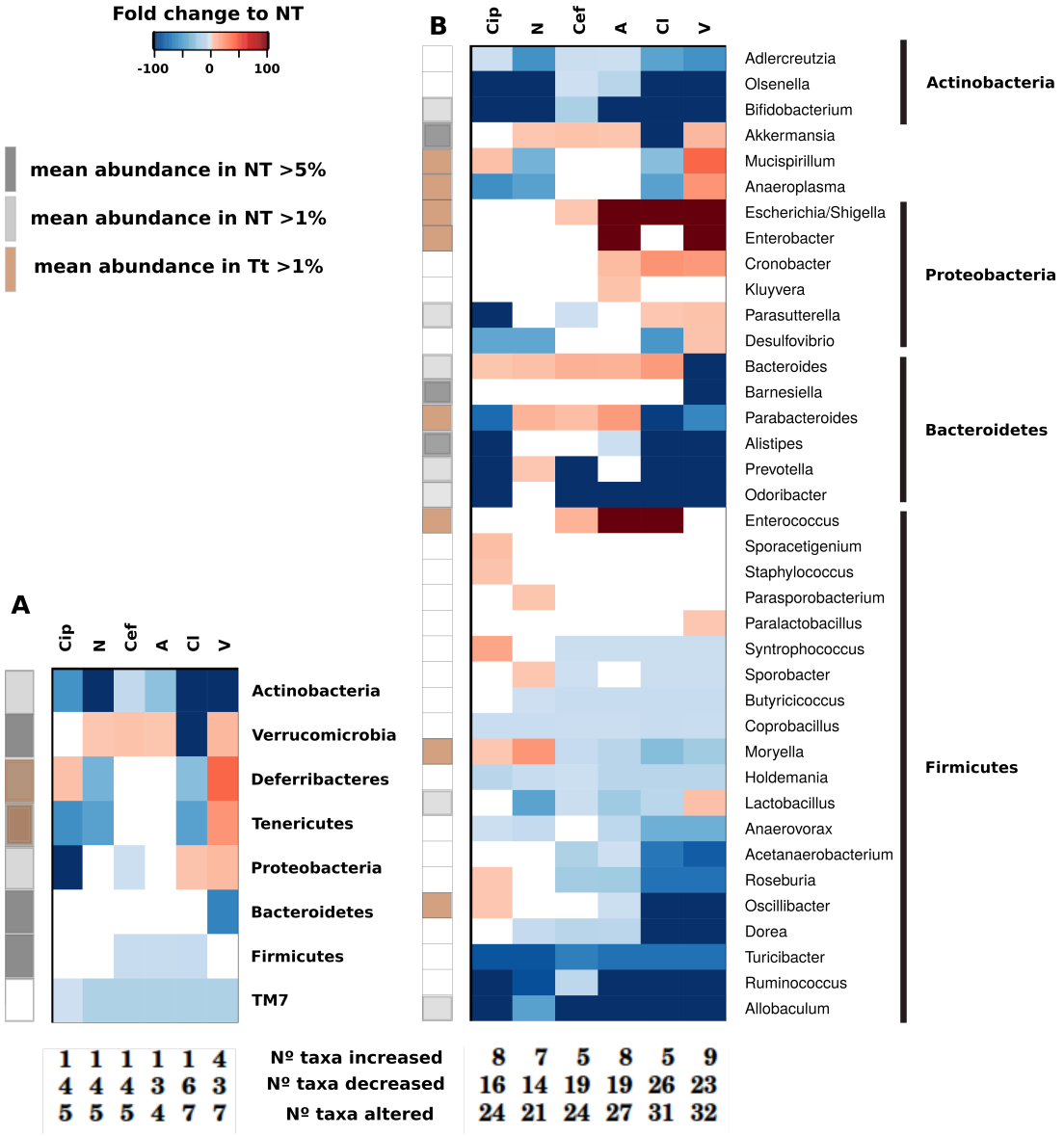


Figure 3.15: Taxa significantly altered according to the antibiotic treatment administered. Mice were treated during 7 days with different antibiotics. The day of antibiotic withdrawal, a fecal pellet was collected and analyzed as described in 2.5.5. A) heatmap representing the statistically significant fold changes respect to untreated mice at the phylum level. B) heatmap representing the statistically significant fold changes respect to untreated mice at the genus level. Genus are ordered by taxonomy and, inside each phylum, by fold change in decreasing order. Fold change respect to untreated mice was calculated for each treatment. Only taxa whose FDR adjusted p value was < 0.05 and with a fold change (increase or decrease) superior to 2 are colored (white : taxa that do not fulfill these criteria). The color bar on the left indicates the relative abundance of the bacteria, different shades of grey indicate that the bacteria is abundant in untreated mice and brown indicate that this bacterium reach a relative abundance superior to one percent after administration of antibiotic treatment (Tt). NT: Untreated, Amp: Ampicillin, Neo: Neomycin, Van: Vancomycin, Cef: Ceftriaxone, Cip: Ciprofloxacin, Cl: Clindamycin. $n = 5$ per group, except for ceftriaxone where $n = 3$.

The phylum Proteobacteria, is normally also very low abundant in the gut. On the other hand, it includes numerous known oportunistic pathogens belonging to the genera *Escherichia, Klebsiella* or *Salmonella*. The Proteobacteria genera *Escherichia* and *Cronobacter* were found to be increased in 3 out of the 6 treatments (ampicillin, clindamycin and vancomycin) while *Parasutterella* and *Desulfovibrio* diminished after neomycin and ciprofloxacin treatment.

After comparing how the antibiotics tested affect the phylum Proteobacteria, we focused our attention on the phylum Bacteroidetes. The effect of antibiotics on this phylum was highly variable both considering the antibiotic administered and the genus affected. For example, *Bacteroides* increased after administration of most of the antibiotics, meanwhile, *Alistipes*, *Prevotella* and *Odoribacter* usually decreased. Regarding the effect of each antibiotic on the genus from the phylum Bacteroidetes, we noticed that most of the antibiotics treatment tested affected differently various genus, increasing one specific genus at the same time they do decrease another one. On the opposite, vancomycin consistently decreased the abundance of all members of the phylum Bacteroidetes (as described also in the previous chapter of my thesis), while neomycin increased their abundance.

Finally, the antibiotic effects on the phylum Firmicutes, normally abundant in the gut microbiota, were also different depending on the treatment. For example, the genus *Enterococcus* was highly increased after the administration of ampicillin, clindamycin and, in a lower extent, by ceftriaxone. On the other hand, after the administration of ciprofloxacin, *Oscillibacter* and other genus increased. On the opposite, numerous genera diminished upon administration of the antibiotics tested (i.e. *Dorea*, *Turicibacter*, *Ruminococcus*, *Allobaculum*, *Butyricicoccus*, *Coprobacillus*, *Holdemania*, *Anaerovorax* and *Acetanaerobacterium*). Within the different antibiotics, ciprofloxacin and neomycin did not affect significantly the relative abundance of the Firmicutes phylum. Indeed, they caused the increase of some genus and the decrease of others, within this phylum. In the case of ceftriaxone, ampicillin and clindamycin, the diminution was significant at the phylum level as most of the genus were diminished and the only taxa that increased, *Enterococcus*, represents a very low abundant genus. In the case of vancomycin treatment, most of the genus from the phylum Firmicutes were also diminished. However, the genus *Lactobacillus*, one of the most abundant genus of the phylum Firmicutes, increased. Thus, the overall abundance of the phylum Firmicutes was not significantly diminished after vancomycin treatment.

As a summary of the overall alterations at the genera level, we identified the total number of genera significantly altered after the administration of each antibiotic to evaluate the extent of their impact. Clindamycin and vancomycin caused the highest number of alterations whereas ciprofloxacin, neomycin, ceftriaxone and ampicillin altered a lower number of taxa.

We also identified the total number of genera significantly diminished after the administration of each antibiotic to evaluate the extent of their impact. The decrease of taxa can be due to the direct action of the antibiotic or to an indirect effect by the loss of a syntrophic member of the microbiota. On the opposite, the increase of one taxa is mainly an indirect effect of the antibiotic. Taking into account only the genus significantly diminished, we could separate the antibiotics in three levels of alteration: ciprofloxacin and neomycin cause fewer alterations, ceftriaxone and ampicillin a medium number of alterations whereas clindamycin and vancomycin decreased a higher number of taxa (Figure

3.15 B). This separation of the antibiotics was also concordant with the shannon index (Figure 3.12 C).

In the present data, we described the significant alterations of the microbiota produced by each antibiotic. Although the number of alterations detected is high, it is important to highlight that most of the altered taxa are normally present in very low abundance, as indicated by the color code on the left of the figure 3.15 B.

3.2.3 Antibiotic treatments diminish the diversity within specific taxa

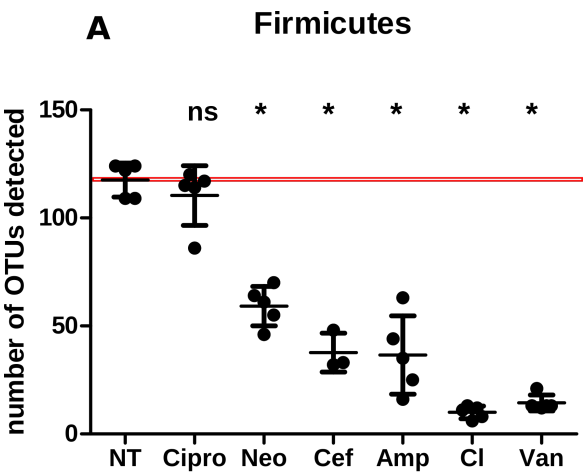
We saw that specific genus, such as *Bacteroides* can account for more than half of the microbiota composition. This genus includes numerous species but, unfortunately, we are not able to define them accordingly to the data obtained with the 16s rRNA sequencing approach. For this reason, we used the OTU approximation to explore the alteration of the gut microbiota to a deeper level than the genera level. This approach allowed us to estimate the extent of the diversity included within the genus of interest and also in the unclassified sequences that could not be attributed to a taxonomic level more precise than the family, as for example the unclassified Lachnospiraceae. We focused this analysis on the most prevalent OTUs (OTU detected in at least 80% of the samples of the group and that present a minimum relative abundance of 0.1% in at least one sample (see section 2.5.5 for the parameters of this analysis)). We first represented the relative abundance of these OTUs in every mice analyzed (Figure 3.16). Seeing that some antibiotics depleted an important number of OTUs, we calculated the richness within the phylum represented (number of OTUs identified within the phylum) as well as within the genus *Bacteroides*, *Barnesiella*, *Parabacteroides* and *Alistipes* (the genus identified that presented at least 5 prevalent OTUs).

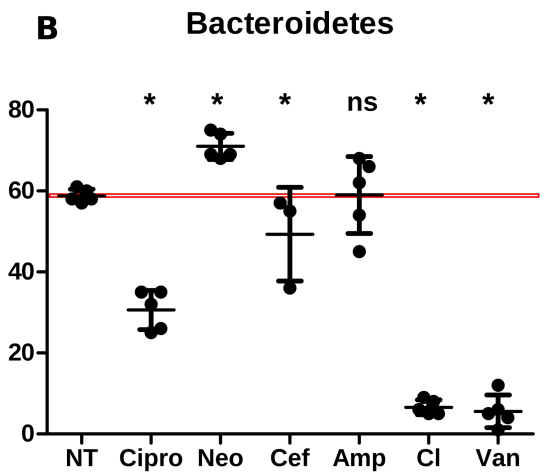
Interestingly, the genera *Bacteroides* and *Parabacteroides* presented a high number of OTUs detected only after administration of specific antibiotics (Figure 3.18 C-D). In the case of ciprofloxacin and clindamycin treatment, few OTUs are detected within these genera and indeed only one OTU of the genus represented the majority of the genus *Bacteroides* (98 % and 99%, respectively) in mice treated with theses antibiotics. In both treatments, the most abundant OTU is different, maybe reflecting a difference in resistance or a difference in metabolism which would influence their fitness in the presence of the antibiotic-altered bacterial community.

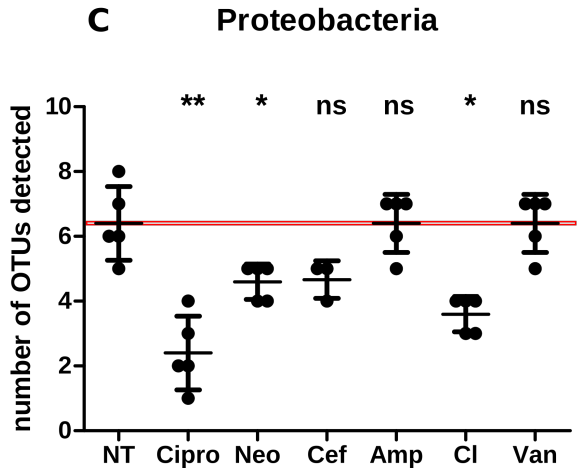
As we have just mentioned, ciprofloxacin is characterized by the presence of few OTUs of the genus *Bacteroides*. One of them is very abundant and responsible for the increase in the relative abundance of the genus 3.16. This antibiotic is associated with a diminution of the diversity of different genus of the phylum Bacteroidetes (*Bacteroides*, *Barnesiella*, *Parabacteroides*, unclassified Porphyromonadaceae and *Alistipes*). Thus, even if there is no significant diminution in the relative abundance of the phylum Bacteroidetes, ciprofloxacin diminish the diversity within this phylum (Figure 3.17 A).

In the case of neomycin treatment, only the low-abundant phylum (Actinobacteria, Deferribacteres, Tenericutes and TM7) were statistically diminished. whereas the abundance of the phylum Bacteroidetes, Firmicutes and proteobacteria were not statistically altered. Although the relative abundance of the phylum Bacteroidetes did not vary, its intra-phylum richness(number of OTUs within the phylum) was increased respect to the

Figure 3.16: Abundance of the most prevalent OTUs in untreated mice and upon different antibiotic treatments. Heatmap of microbiota composition in untreated mice and during antibiotic treatment. Each column represents one mouse, each row represents one OTU (grouped by phylum). The redder, the higher the number of sequences of the represented OTU. Only OTUs present in at least 80% of samples per treatment group and with an abundance superior to 0.1%, in at least one sample, were selected. NT: Untreated, Cip: Ciprofloxacin, N: Neomycin, Cef: Ceftriaxon, A: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ per group, except for ceftriaxone where $n = 3$.







Average number of OTUs detected

	Firmicutes	Bacteroidetes	Proteobacteria
NT	117.6	58.8	6.4
Cipro	110.4	30.6	2.4
Neo	59.2	71.0	4.6
Cef	37.7	49.3	4.7
Amp	36.6	59.0	6.4
Cl	10.0	6.6	3.6
Van	14.4	5.6	6.4

Figure 3.17: Number of prevalent OTUs detected within different phyla after antibiotic treatments. A-C) Number of OTUs detected within each phyla. The same criteria as in figure 3.16 were followed. Basically, OTUs present in at least 80% of samples per treatment group and with an abundance superior to 0.1%, in at least one sample, were selected. NT: Untreated, Cipro: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ per group, except for ceftriaxone where $n = 3$. Mann-Whitney test comparing each group of samples with the group NT, ns not significant, * p-value <0.05, ** p-value <0.01.

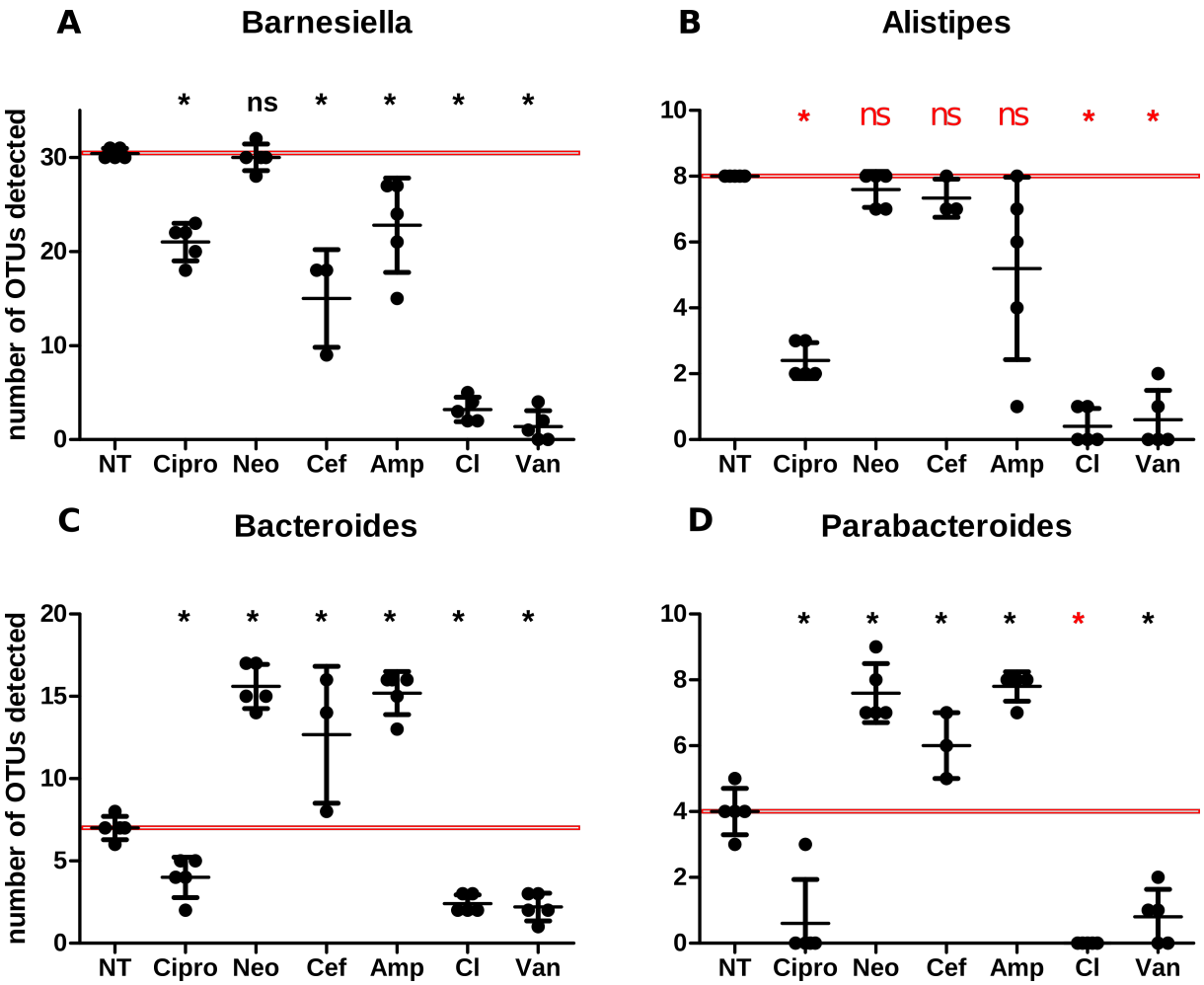


Figure 3.18: Number of prevalent OTUs detected within different genus after antibiotic treatments. A-D) Number of OTUs from specific genera of the phylum Bacteroidetes. The same criteria as in figure 3.16 were followed. Basically, OTUs present in at 80% of samples per treatment group and with an abundance superior to 0.1% in at least one sample were selected. NT: Untreated, Cipro: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ per group, except for ceftriaxone where $n = 3$. Mann-Whitney test comparing each group of samples with the group NT, ns not significant, * p-value <0.05. In red : when one of the compared group presented in all mice the same number of a particular OTU, a t-test was performed.

richness calculated in untreated mice (Figure 3.17 B). Curiously, the few genera whose abundance were increased after neomycin treatment, *Bacteroides* and *Parabacteroides*, also presented an increase in richness (higher number of OTUs detected) (Figures 3.18 C and D). On the opposite, the intra-genus richness of *Barnesiella* and *Alistipes*, two genera of the phylum Bacteroidetes whose relative abundance did not vary upon neomycin treatment, was not altered (Figures 3.18 A and B).

The $\beta-$lactams antibiotics ceftriaxone and ampicillin caused both similar alteration at the genus and phylum level, although the amplitude of the fluctuations can vary (Figure 3.15 A and B). The alterations produced in the richness within phylum were mainly observed within the Firmicutes phylum, where a reduction of the number of OTUs was observed upon treatment with these two antibiotics(Figure 3.17 A).

Clindamycin treatment induced an important loss of richness, as seen on figure 3.16. The microbiota after this treatment contained very few OTUs that were present in high abundance. It is very striking that only one OTU from each of these genera: *Bacteroides* (55%), *Barnesiella* (28%), *Escherichia/Shigella* (6%), *Parasuterella* (5%) and *Enterococcus*(3%), accounted for 97% of the total microbiota.

As seen on figure 3.16, after vancomycin treatment, the phylum Bacteroidetes and Firmicutes were broadly depleted. Some very low abundant OTUs of the phyla Bacteroidetes and Firmicutes were detected (relative abundance <0.001%) and one OTU of the phylum Firmicutes (identified as *Lactobacillus*) presented a higher abundance. (Figures 3.17 A-B, 3.18 A-D and 3.16). Unlike clindamycin treatment, there was no decrease in the number of OTUs from the phylum Proteobacteria, as seen on figure 3.17 C). Nevertheless, the relative abundance of these OTUs clearly increased upon vancomycin treatment (Figures 3.15 A and 3.16)

In summary, we have demonstrated that each antibiotic cause specific alterations on the microbiota. Similar alterations of the abundance at the phylum level can produce very different patterns of alterations at lower taxonomic level, such as the genus level. Moreover, antibiotics can cause drastic changes in the richness within each genera. In the most extreme case, this disminution in the richness can lead to the domination of the complete microbiota by a few OTUs, as observed after administration of clindamycin.

These important alterations in the composition of the bacteria and the drastic diminution of the richness are worrying (1) for the capacity of the microbiota to perform its normal functions (such as the colonization resistance) and (2) for the possibility of not recovering a healthy microbiota after the withdrawal of the antibiotic treatment. We mentioned in the introduction that the microbiota presents a high resilience only if the alterations are not too drastic and that its stability rely on a high richness. For this reason, we next investigated the recovery of the gut microbiota after the administration of the same antibiotics.

3.2.4 The microbiota recovers partially after the withdrawal of the antibiotic treatment

Seeing the huge variability in magnitude and type of microbiota alterations associated with different treatments, as well as the loss of diversity, we decided to assess to which extent these alterations persist after the withdrawal of the antibiotic. Thus, after admin-

istering the antibiotic for one week, we allowed the microbiota to recover for two weeks and analyzed the resulting microbiota (Figure 2.1).

As expected, the alterations (measured by the biomass, the number of OTUs and the shannon index) are smaller if we allow the microbiota to recover for two weeks after the antibiotic treatment (Figure 3.19). Except for ciprofloxacin, no treatment caused a significant loss of biomass two weeks after its withdrawal (Figure 3.19 A). This is especially important in the case of ceftriaxone since a high decrease of biomass was detected during the treatment.

On the other hand, after this period of recovery, we still identified a significant diminution of the richness (number of OTUs detected) in four out of the six treatments tested (Figure 3.19 B, ciprofloxacin, ceftriaxone, clindamycin and vancomycin). Neomycin displayed levels of richness similar to the one of the untreated mice (Figure 3.19 B). In the case of ampicillin, most of the mice had lower levels of microbiota richness but, due to the small number of samples and the high variability, this decrease was not significant.

The shannon index, that mesures the diversity in a sample, was lower in all the antibiotic-treated groups except for neomycin (Figure 3.19 B). Notably, only clindamycin-treated mice experimented an important increase in diversity upon recovery (Mann-Whitney test, p-value$<$0.01). In the case of ceftriaxone treatment, the shannon index was very variable among different mice.

The number of OTUs detected and the shannon index indicate that in most cases the microbiota does not fully recover two weeks after the withdrawal of the antibiotic treatment.

Next, we performed a NMDS analysis to assess the relative similarity between the microbiota observed during the treatment and upon recovery. In agreement with the low number of differences detected between neomycin-treated and untreated mice, samples obtained during the treatment and after the withdrawal were very close to those of untreated mice on the NMDS plot (Figure 3.20). In addition, the NMDS analysis showed that the microbiota after two weeks of recovery is relatively similar to the one obtained during the treatment in the case of neomycin, ciprofloxacin, ampicillin and vancomycin treatment. Surprisingly, as we will describe later, more alterations of the microbiota were detected after two weeks of recovery than during the treatment with ceftriaxone. Indeed, the recovered microbiota was drastically different from the one observed during the treatment. After clindamycin treatment as well, we observed very different states during the treatment and upon recovery. In this case, both states seem to be very different to the untreated one (Figure 3.20).

As there are more groups to segregate than in the first analysis performed only on the sample obtained during the treatment, the taxa indicative of their separation can differ slightly from the previous analysis. The neomycin-treated and untreated samples were characterized by an increase in *Alistipes* and, to a less extent, by the presence of *Adlercreutzia* and *Anaerovorax*. This result is similar as the one obtained comparing only the samples of mice treated with antibiotics without a recovery period (Figure 3.13). Ciprofloxacin treatment promoted a higher abundance of *Oscillibacter* (genera from the Ruminococcaceae family) and unclassified Ruminococcaceae. As we described previously, clindamycin treatment was characterized by very high levels of *Bacteroides*. However, the group after recovery shifted to dominance by unclassified Lachnospiraceae. Similarly, the group of mice treated with ceftriaxone presented high levels of unclassified

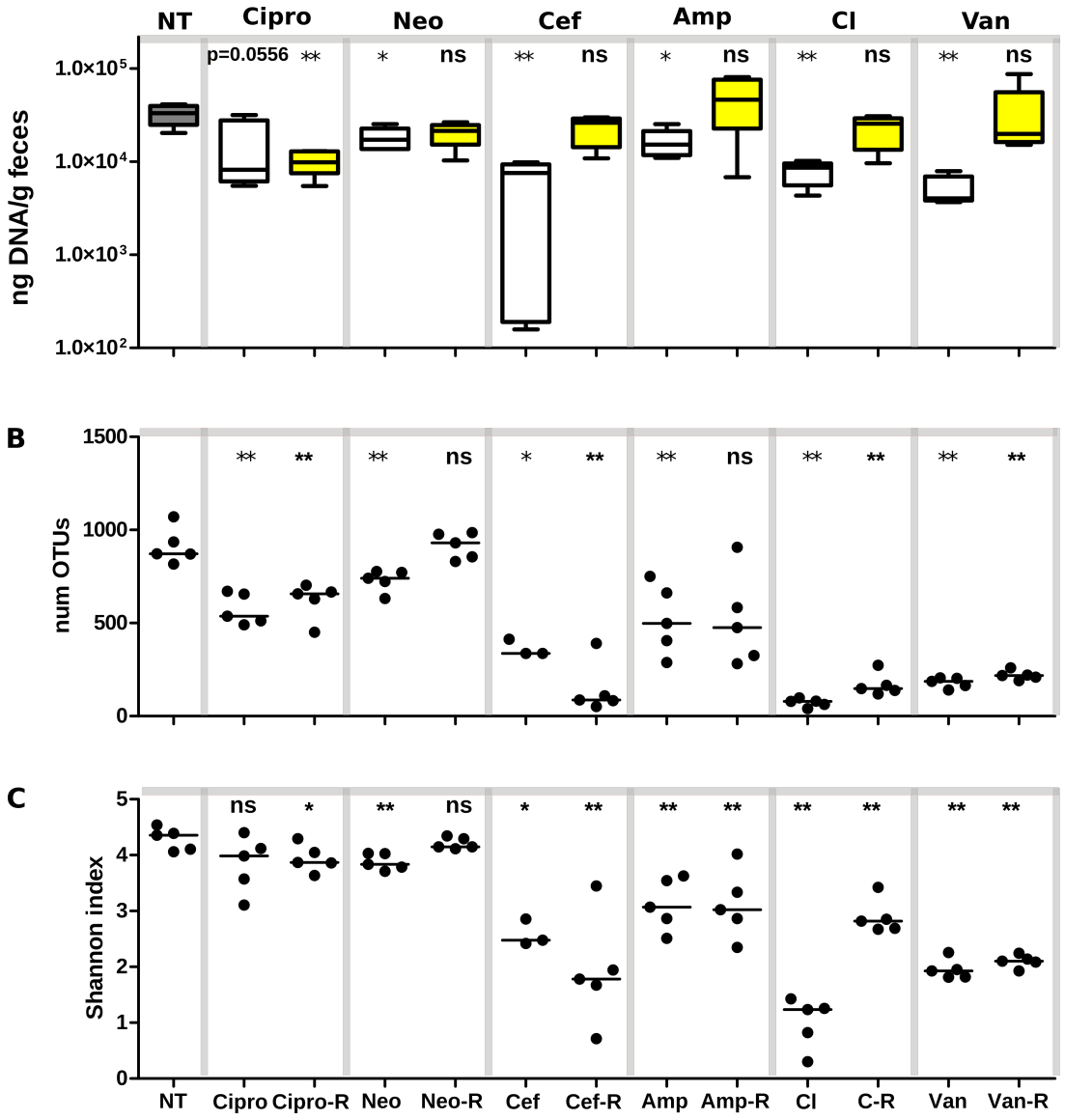


Figure 3.19: Alteration of the microbiota two weeks after the withdrawal of antibiotics A) Biomass measured as ng of DNA per g of feces. This value represents an approximation of the total bacteria load as indicated in [228]. B) Number of OTUs detected in each sample out of 29010 sequences, which is considered as a measure of microbiota richness. C) Shannon index, which is considered as a measure of microbiota diversity. Data during the treatment are proportionated for comparison. NT: Untreated, Cipro: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. Data after two weeks of recovery: Cipro-R, Neo-R, Cef-R, Amp-R, Cl-R, Van-R. n=5 per group except for Cef in which n=3. The asterisks depict the significance of the Mann-Whitney test respect to untreated mice: ns not significant, * p.value<0.05, ** p.value<0.01, the values almost significant are indicated.

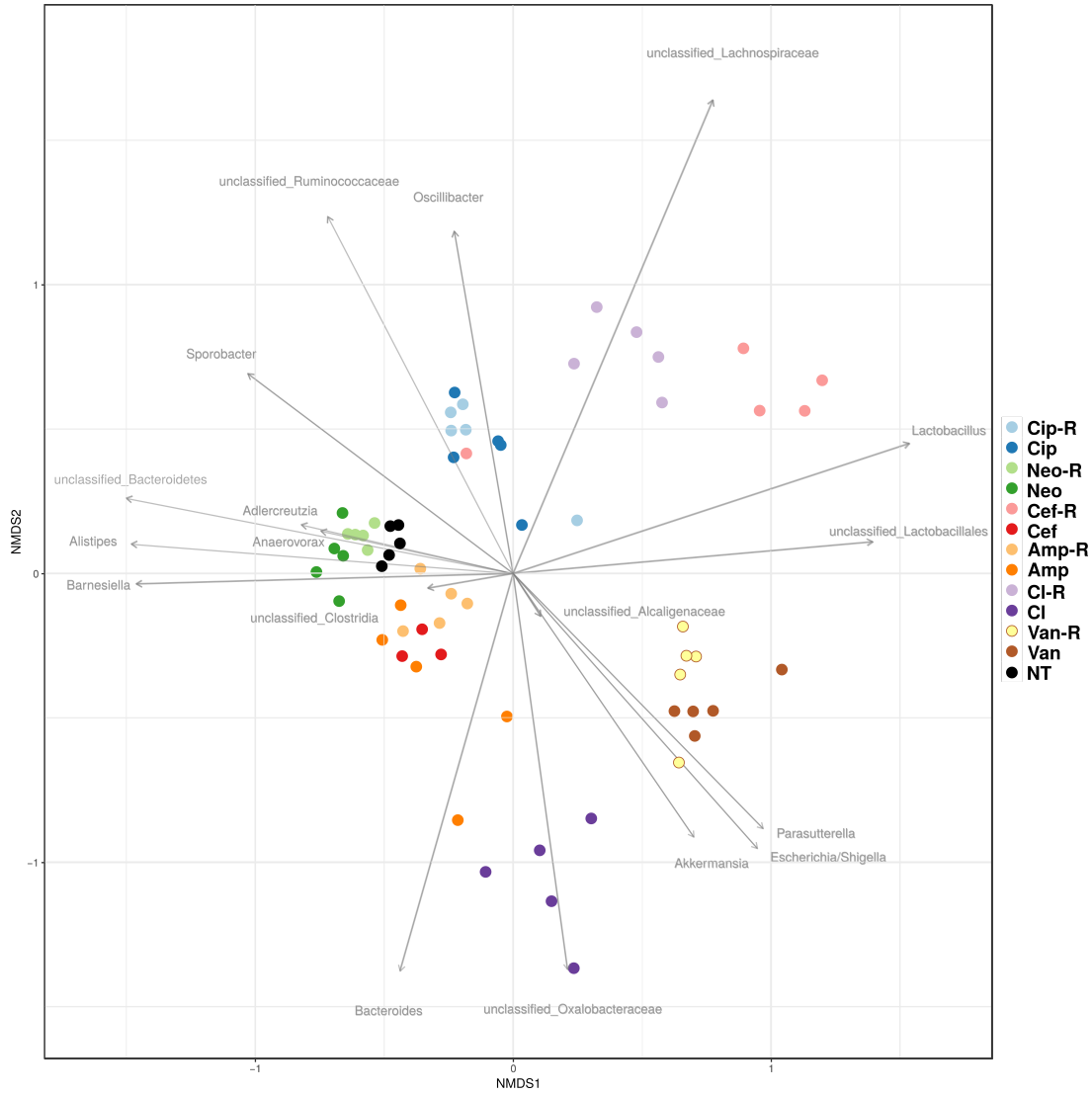


Figure 3.20: Microbiota composition separation according to the antibiotic treatment administered Taxa that presented the best correlation with the topology of the samples are displayed. NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. Data after two weeks of recovery: Cip-R, Neo-R, Cef-R, Amp-R, Cl-R, Van-R. NMDS was calculated with Braycurtis distance on two dimensions. Stress: 0.16

Lachnospiraceae and *Lactobacillus* upon recovery. In the case of vancomycin treatment, both groups during the treatment and upon recovery are characterized by high levels of *Akkermansia* and Proteobacteria (i.e *Escherichia/Shigella* and *Parasuterella*).

We have seen that the microbiota after two weeks of recovery is relatively similar to the one obtained during the treatment in the case of neomycin, ciprofloxacin, ampicillin and vancomycin treatment. To assess whether there was a significant recovery of the microbiota, we calculated the Braycurtis distance from each sample to the untreated ones. If the distance between samples after two weeks of recovery to untreated mice is smaller than the distance during the treatment to untreated mice, we can deduce that there is a recovery (Figure 3.21).

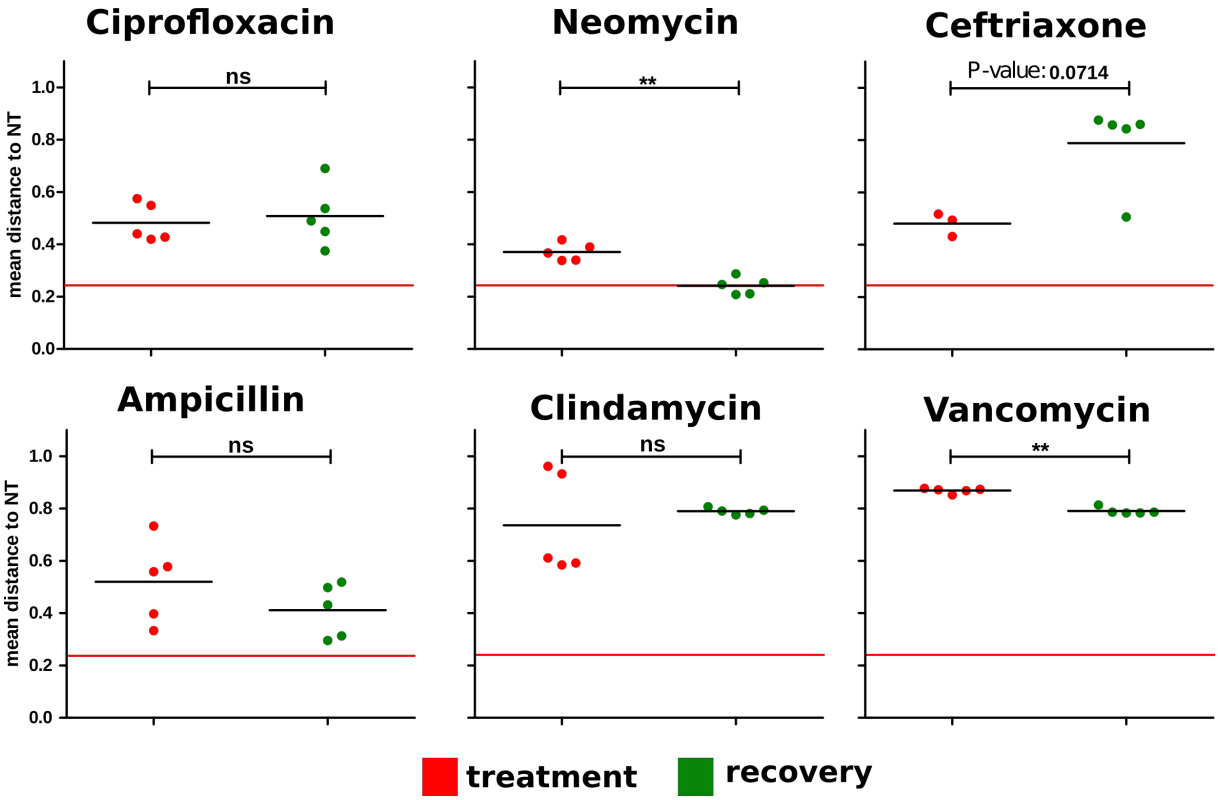


Figure 3.21: Recovery of microbiota after administration of different antibiotic treatments. Mice received an antibiotic treatment for one week than a fecal sample was collected to determinate the microbiota composition (treatment). Alternatively, mice were allowed to recover for two weeks between the withdrawal of the antibiotic treatment and the colletion of the sample used to analyse the microbiota composition (recovery). Mean Braycurtis distance to untreated samples. The red line indicates the distance comparing the untreated samples between them. It is the baseline distance due to the intra-group variation. NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: clindamycin, Van: Vancomycin.

That way, we determined that two weeks after ciprofloxacin or ampicillin treatments, there was no significant recovery of the initial microbiota state (Figure 3.21). On the opposite, the recovery was clear after the withdrawal of neomycin, the microbiota being similar to the initial microbiota state (Mann-Whitney test to untreated mice, not sign-

nificant) (Figure 3.21). Two weeks after the withdrawal of clindamycin, the microbiota composition was as dissimilar to the untreated samples as the microbiota observed during the treatment (Figure 3.21). In the case of ceftriaxone treatment, a higher number of alterations were detected after the recovery than during the treatment (Figure 3.15), resulting in a microbiota more different from the one of the untreated mice (Figure 3.21). As described when we performed the NMDS plot, upon two weeks of recovery the microbiota adopted an alternative state, different from the untreated one after the administration of ceftriaxone and clindamycin (Figure 3.20).

Consistent with the results from the first section of the thesis, the treatment with vancomycin caused important alterations of the microbiota that persist after the antibiotic withdrawal, as indicated by the high Braycurtis distance to untreated mice observed both in treated mice and after two weeks of recovery (Figure 3.21). Nevertheless, a trend to recovery, visible in the NMDS plot ((Figures 3.20), was confirmed by the significant lower distance to untreated mice after the recovery (Figure 3.21).

Then, we assessed, as before, the overall composition of the microbiota two weeks after the recovery from each antibiotic treatment (Figure 3.22). In decreasing order of abundance, untreated mice were dominated by *Barnesiella* (35%) and unclassified Porphyromonadaceae (10%). After treatment with (1) ciprofloxacin, the microbiota was dominated by unclassified Lachnospiraceae (36%) and *Barnesiella* (29%); (2) neomycin: the microbiota was dominated by *Barnesiella* (31%), unclassified Lachnospiraceae (14%) and unclassified Porphyromonadaceae (12%); (3) ceftriaxone: the microbiota was dominated by unclassified Lachnospiraceae (60%) and *Lactobacillus* (15%); (4) ampicillin: the microbiota was dominated by *Barnesiella* (31%) and *Akkermansia* (21%) and *Bacteroides* (18%); (5) clindamycin: the microbiota was dominated by unclassified Lachnospiraceae (56%); (6) vancomycin: the microbiota was dominated by *Akkermansia* (50%) and unclassified Lachnospiraceae (14%);

As described, we still observed alterations of the microbiota two weeks after the withdrawal of the antibiotic treatments. Thus, we explored the taxa altered at the phylum on genus level. Taking into account that most samples obtained after the recovery period grouped with the ones obtained during the treatment and far apart from the untreated mice, it was not surprising that we still detected numerous significant alterations after 2 weeks of recovery (Figure 3.23).

At the phylum level, three of the six treatments (ciprofloxacin, ampicillin and neomycin), display alterations that affect the very low abundant phyla (Figure 3.23 A). Ciprofloxin caused a small decrease of Proteobacteria and alteration of the low abundant phyla Actinobacteria, Deferribacteres and Tenericutes. Ampicillin administration also caused a small decrease of Proteobacteria, of the low abundant phyla TM7 and an increase of Verrucomicrobia. Neomycin only caused a small diminution of very low abundant phyla (i.e Deferribacteres, Tenericutes and TM7) and of Verrucomicrobia.

The three other treatments, ceftriaxone, clindamycin and vancomycin affect significantly almost all of the phyla.

Both ceftriaxone and clindamycin decreased the majority of the phyla and especially the Verrucomicrobia. In the case of clindamycin treatment, the phyla Bacteroidetes also suffered a drastic diminution. These two antibiotics increased the phyla Firmicutes and moreover ceftriaxone increased the phylum Tenericutes whereas ceftriaxone increased the Proteobacteria. In the case of clindamycin, the alterations were similar to the ones ob-

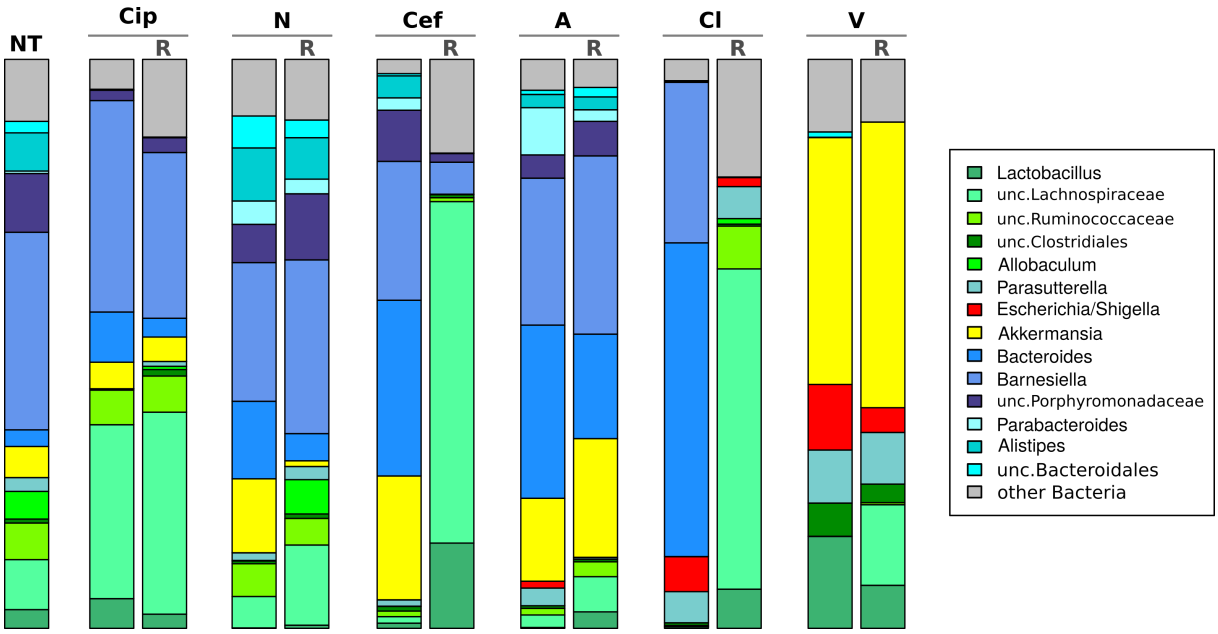


Figure 3.22: Abundance of the most prevalent genus. Barplot of the microbiota composition in untreated mice (NT), in treated mice (data proportionated for comparison) and two weeks after the withdrawal of antibiotic treatment (R: recovery). Genus whose mean abundance reach 5% in untreated mice or upon administration of an antibiotic are represented. The other genus are grouped in "other bacteria". NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ by group

served during the treatment except for the significant diminution of the Bacteroidetes. Interestingly, ceftriaxone caused a higher number of alterations after the recovery than during the treatment.

The alterations observed upon recovery from vancomycin treatment were similar to the ones observed during the treatment. Actinobacteria and TM7 decreased whereas the other low abundant phyla (namely Verrucomicrobia, Deferribacteres and Tenericutes) increased. The abundant phyla also experiment the same alterations seen during the treatment: the Proteobacteria increased whereas the Bacteroidetes were depleted and no significant alteration was detected for the phylum Firmicutes.

At the genus level, some taxa experimented a diminution upon recovery from most antibiotic treatments (Figure 3.23 B). That is the case for (i) the Bacteroidetes *Barnesiella*, *Alistipes*, *Prevotella* and *Odoribacter*, (ii) the Firmicutes *Anaerovorax*, *Allobaculum*, *Turicibacter*, *Ruminococcus* and for (iii) *Adlercreutzia* and *Bifidobacterium*.

On the opposite, some bacteria showed an increase upon recovery from several antibiotics. This is the case for some Proteobacteria genera (i.e *Escherichia*, *Enterobacter*, *Cronobacter*) and a few Firmicutes (i.e. *Enterococcus*, *Blautia*, *Clostridium*, *Anaerostipes*).

In the case of ciprofloxacin, the post-recovery alterations were similar to the ones observed during the treatment although on a smaller scale. For example, the abundance of the Proteobacteria *Parasuterella* was less diminished. In the case of the Bacteroidetes, all the taxa altered went in the same direction (reduction) whereas the alterations among the Firmicutes were in different direction (increase or decrease) depending on the genera.

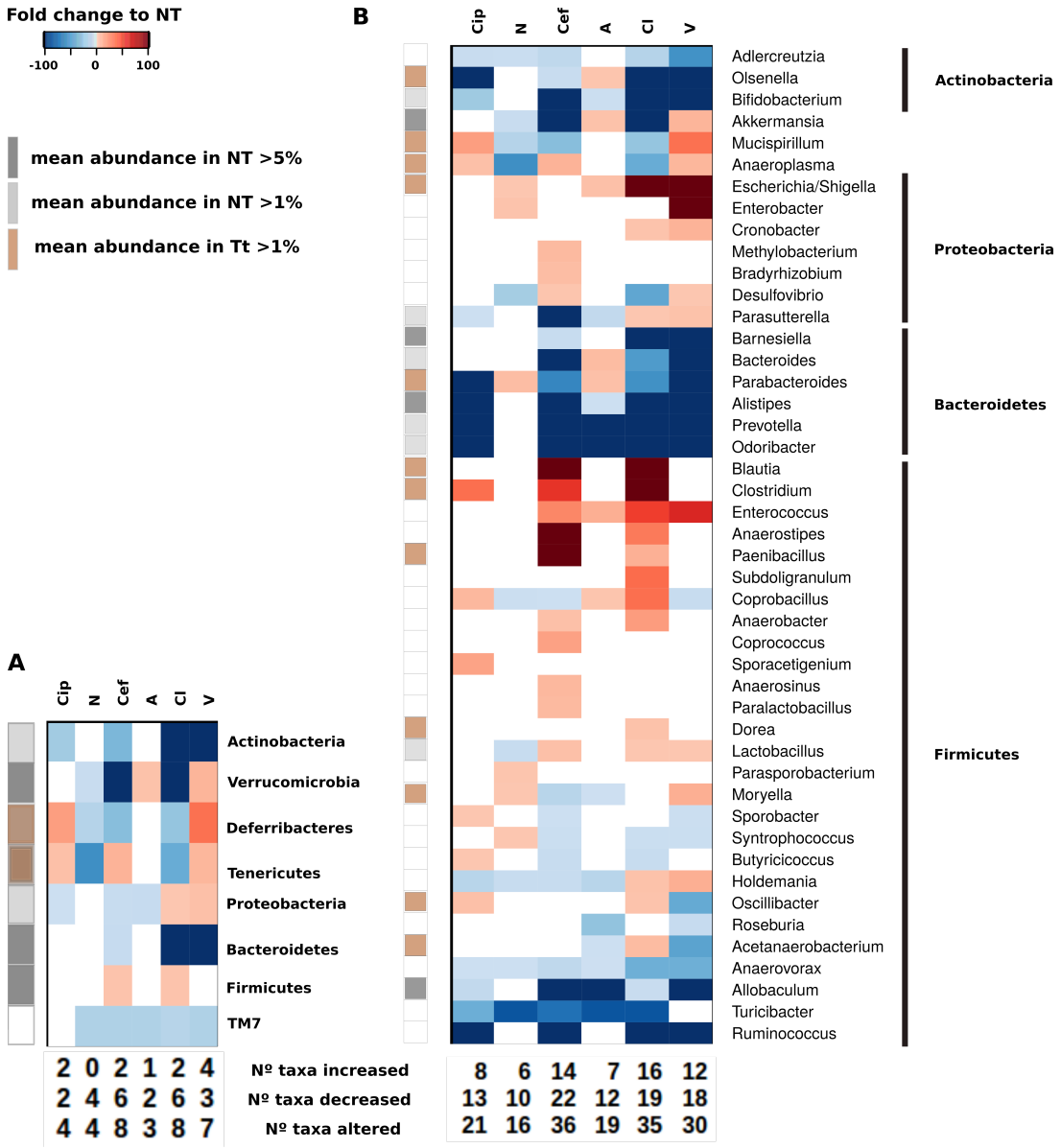


Figure 3.23: Taxa significantly altered two weeks after the withdrawal of the antibiotic administered. Mice were allowed to recover for 2 weeks after withdrawal of the antibiotic treatment and the microbiota was analyzed. A) heatmap of statistically significant fold changes respect to untreated mice at the phylum level. B) heatmap of statistically significant fold changes respect to untreated mice at the genus level. Genus are ordered by taxonomy and, inside each phylum, in decreasing order of fold change. Fold change respect to untreated mice was calculated for each treatment. Only taxa whose FDR adjusted p value was < 0.05 and with an absolute fold change (increase or decrease) superior to 2 are colored. The color bar on the left indicates the relative abundance of the bacteria, different shades of grey indicate that the bacterium is highly abundant($> 1\%$, $> 5\%$) in untreated mice and brown indicate that this bacterium reach a relative abundance superior to one percent after administration of an antibiotic treatment. NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ per group

In the case of neomycin, there were very few alterations of moderate magnitude. This result is in agreement with the small distance detected in the NMDS plot between samples collected from mice treated with neomycin and the samples collected from untreated mice (Figure 3.20).

The microbiota of mice treated with ampicillin also presented relatively few alterations upon two weeks of recovery. Alterations of genera from the phylum Proteobacteria were observed. In addition, within the Bacteroidetes phylum, it was the only treatment that still presented an increase in the genus *Bacteroides* and *Parabacteroides* after two weeks of recovery. It is interesting to highlight that the genus *Bacteroides* was increased during most of the treatments but not upon their recovery. This could indicate its capacity to survive during the dysbiosis although it may not be a good competitor with the rest of the microbiota as it does not maintain its supremacy upon the withdrawal of the treatment.

The three antibiotics that caused the highest number of alterations on the microbiota two weeks after their withdrawal; ceftriaxone, clindamycin and vancomycin, induced some common alterations. First, they caused a significant diminution of all the Actinobacteria and Bacteroidetes. The three of them also increased the relative abundance of the lactic acid bacteria *Enterococcus* and *Lactobacillus*.

In the case of ceftriaxone and clindamycin, an important decrease in the abundance of *Akkermansia* and an increase in the Firmicutes *Blautia*, *Clostridium*, *Anaerostipes* and *Paenibacillus* were observed. The main difference between these two antibiotics concerns the Tenericutes *Anaeroplasma* that was increased upon ceftriaxone treatment and diminished after administration of clindamycin.

Clindamycin and vancomycin shared a high increase in the relative abundance of the taxa *Escherichia/ Shigella*. Unlike ceftriaxone and clindamycin, vancomycin caused a high increase in the abundance of the genera *Akkermansia* and *Muscispirillum*.

In summary, after two weeks of recovery, the effect of the different antibiotics was still detectable although the relative abundance of several taxa was restored. None of the antibiotic-treated microbiota had come back to the initial state, except for neomycin (the microbiota resulting from a 2-weeks recovery after neomycin treatment was not different from the untreated microbiota according to the Braycurtis distance, however, some taxa were still significantly altered). Usually, the alterations detected two weeks after antibiotic cessation were similar to the ones detected during the treatment except for specific taxa such as *Bacteroides*. However, in the case of ceftriaxone and clindamycin treatment, the alterations identified upon recovery were drastically different to the ones detected during the treatment (Figure 3.22 C).

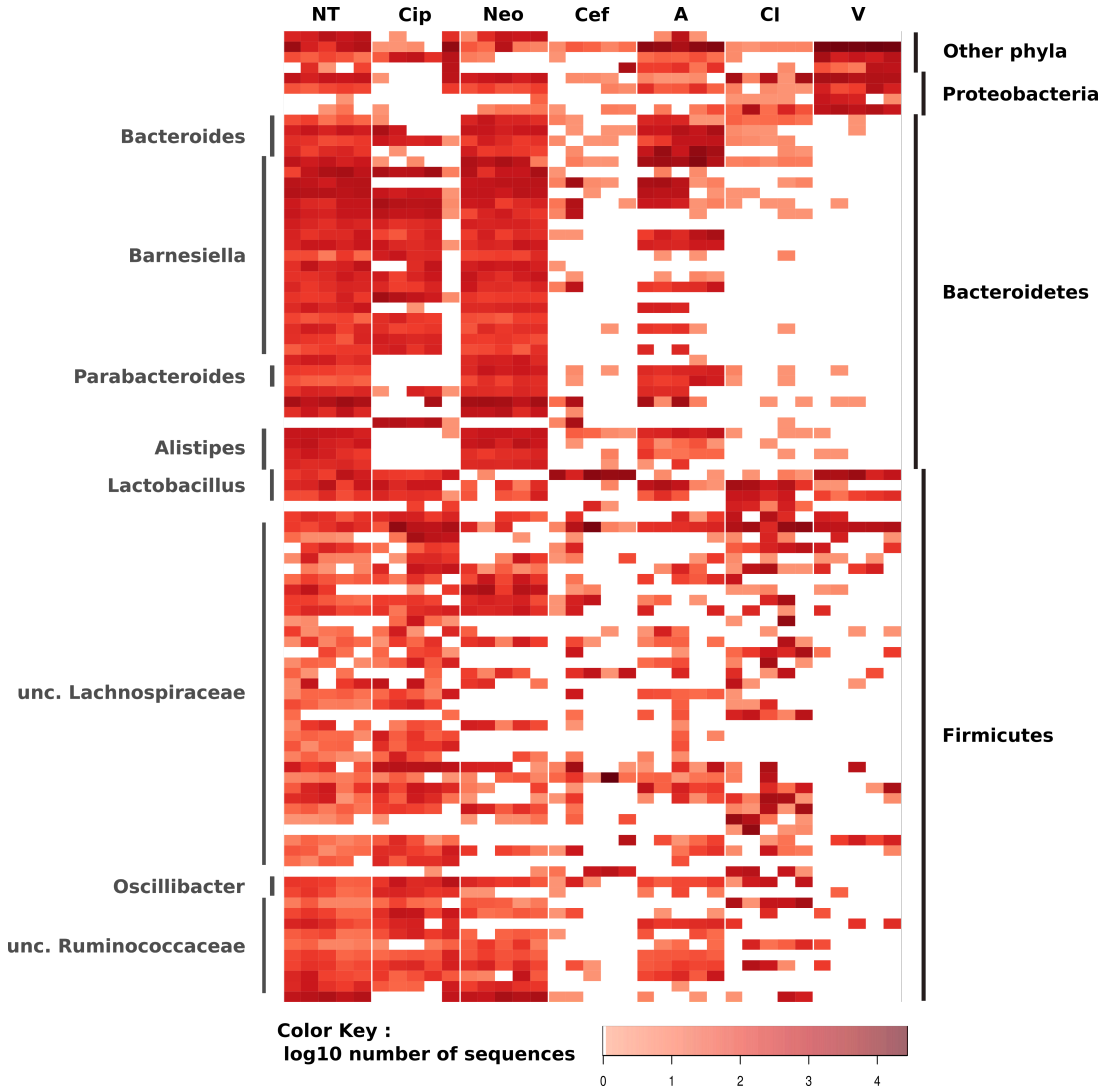


Figure 3.24: Abundance of the most prevalent OTUs in untreated mice and two weeks after the withdrawal of different antibiotic treatments Heatmap of microbiota composition in untreated mice and two weeks after the withdrawal of antibiotic treatments. Each column represents one mouse, each row represents one OTU (grouped by phylum). The redder, the higher the relative abundance. Only OTUs present in at least four out of five mice per treatment group (three in the case ceftriaxone) and with an abundance superior to 1% in a sample were selected. NT: Untreated, Cip: Ciprofloxacin, Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: Clindamycin, Van: Vancomycin. $n = 5$ per group

3.2.5 Determination of putative protective bacteria against VRE colonization

Until now, we have described the effect of the antibiotics on the microbiota during the treatment and upon recovery. In both cases, the mice were latter orally infected with VRE and its levels were assessed two days post-infection as described in the section 2.2.1 (Figure 2.1). Doing this, we were able to link specific alterations of the microbiota with different levels of colonization by VRE.

These data, associating different alterations of the gut microbiota and level of VRE colonization, enabled us to identify bacteria that promote the resistance against the colonization by this opportunistic pathogen. The premise was that the antibiotic treatment would have depleted important bacteria for protection when VRE was able to colonize the gut in high level whereas these relevant bacteria for protection would be present in case of a low VRE colonization. It is important to highlight that each antibiotic also eliminate bacteria that do not affect VRE colonization. Thus, by analyzing several treatments with different spectrum, we wanted to select the bacteria whose presence was related with a low VRE load while its elimination was consistently related with a higher VRE colonization.

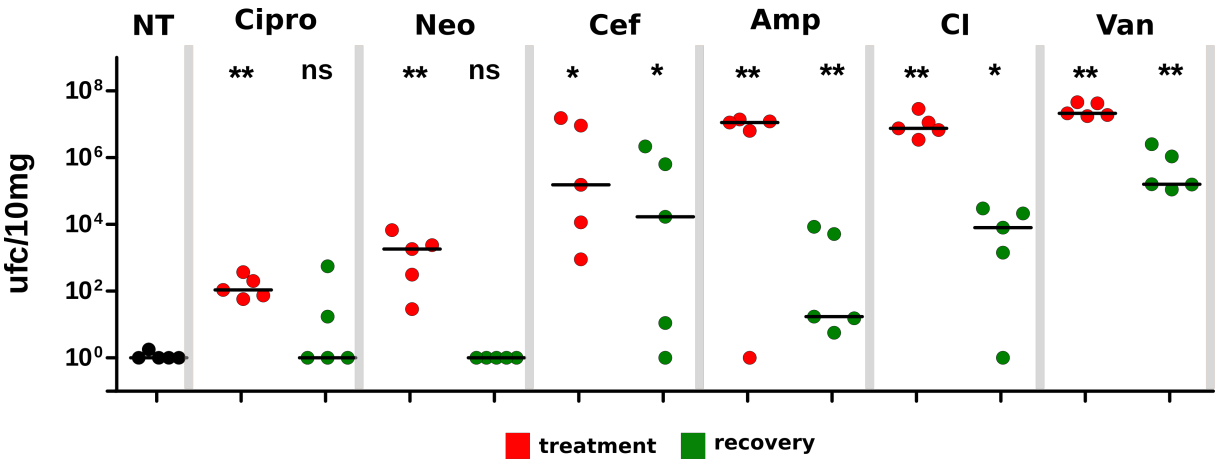


Figure 3.25: Colonization by VRE after administration of antibiotics Mice received an antibiotic treatment for one week than a fecal sample was collected to determinate the microbiota composition (treatment). Alternatively, mice were allowed to recover for two weeks between the withdrawal of the antibiotic treatment and the colletion of the sample used to analyse the microbiota composition (recovery). VRE counts two days post infection. NT: Untreated, Cip: Ciprofloxacin , Neo: Neomycin, Cef: Ceftriaxone, Amp: Ampicillin, Cl: clindamycin, Van: Vancomycin. n=5 by group. The asterisks depict the significance of the Mann-Whitney test respect to values of untreated mice : ns not significant, * p.value<0.05, ** p.value<0.01.

First, we used the model without recovery, in which the mice are challenged with VRE while still under antibiotic treatment (Figure 2.1). We determined the colonization capacity by plating fecal samples at day two post infection on selective media. According to the different alterations of the microbiota associated which each treatment, we also observed different VRE colonization capacities (Figure 3.25). In increasing order of magnitude, VRE present the lowest colonization capacity during the ciprofloxacin treatment,

followed by neomycin, ceftriaxone, clindamycin, ampicillin and finally reached the highest level during the administration of vancomycin. (median level of 1.09E+02, 1.84E+03, 1.54E+05, 7.62E+06, 1.14E+07, 2.12E+07 CFUs/10mg feces, respectively)

In the section 3.2.2, we suggested the separation of the antibiotics in three levels of alteration: ciprofloxacin and neomycin caused fewer alterations, ceftriaxone and ampicillin a medium number of alterations while clindamycin and vancomycin depleted the highest number of genus. Thus, in agreement with the result observed in the section 3.1.5, the treatments that cause the highest number of alterations of the microbiota were associated with a higher capacity of colonization by VRE.

We described in the previous section that when we allowed the microbiota to recover for two weeks after the withdrawal of the antibiotic, we observed a partial recovery or a remodelation of the microbiota (see 3.2.4). Consequently, when we challenged with VRE the mice two weeks after the withdrawal of the treatment (model with recovery), the level of colonization obtained was always lower than the level observed when challenging the mice without letting them recover from the antibiotic treatment (Figure 3.25). For example, VRE levels reach 2±2E+03 CFUs/10mg feces when VRE was administered while the mice were being treated with neomycin. However, VRE was not able to colonize the mice when we allowed them to recover from neomycin for two weeks before VRE challenge.

Again, the level of VRE colonization when mice were allowed to recover from antibiotics treatment before VRE challenge, depends on the level of the microbiota alterations respect to untreated mice. We described in the section 3.2.4 that neomycin, ciprofloxacin and ampicillin recovered a microbiota more similar to that of untreated mice as compared to ceftriaxone, clindamycin or vancomycin (Figure 3.22 A) In agreement, mice that recovered from neomycin, ciprofloxacin and ampicillin treatment presented the lowest median level of colonization by VRE (0, 0 and 1.7E01 CFUs/10mg feces respectively) In the case of ceftriaxone and clindamycin treatment, the VRE levels were intermediate (3.2E+05 and 8.0E+03 CFUs/10mg feces), although lower than when mice were challenged during the treatment administration. Notably, the overall composition of the microbiota was still very different from that of untreated mice (Figure 3.21) but mice treated with these antibiotics recovered a microbiota that was also different from the microbiota identified during treatment (Figure 3.20) Thus, it seems that the new configuration of the microbiota adopted upon removal of ceftriaxone or clindamycin treatment (see 3.2.4) restores partially the CR against VRE. Regarding vancomycin treatment, the microbiota suffered drastic alterations during the treatment and did only slightly recover upon antibiotic withdrawal (Figure 3.21). Consequently, the median VRE level detected upon recovery from vancomycin treatment was still high (1.6E+05 CFUs/10mg feces), although lower than during the antibiotic administration (1.1E+07 CFUs/10mg feces).

The aim of associating different microbiota alterations with the VRE colonization capacity was to identify bacteria that could be responsible for the CR in untreated mice. These bacteria of interest will be depleted after the administration of antibiotics that promote high levels of VRE. Thus, to determine the bacteria that correlate negatively with VRE colonization (the bacteria that are absent when VRE is present in high levels), we performed a spearman correlation test on the data with and without recovery (Table 3.2)(see 2.5.5 for the parameters used).

Notably, we found that Unc.Ruminococcaceae, *Oscillibacter*, unc.Lachnospiraceae, *Barnesiella*, *Allobaculum*, unc.Bacteroidetes, *Alistipes* and unc. Porphyromonadaceae

Table 3.2: Prevalent taxa that present a significant negative correlation with VRE.

Genus	spearman.cor	adj.spearman.p.value.BH	abundance in NT mice (%)
unclassified_Ruminococcaceae	-0.73	1.07E-09	4.45
Oscillibacter	-0.60	3.81E-06	0.80
unclassified_Lachnospiraceae	-0.52	2.14E-04	4.88
Barnesiella	-0.46	1.85E-03	27.10
Allobaculum	-0.46	1.85E-03	30.97
unclassified_Bacteroidetes	-0.45	1.97E-03	0.69
Alistipes	-0.36	1.40E-02	5.00
unclassified_Porphyromonadaceae	-0.30	4.25E-02	7.53

NT: not treated. Some sequences could not be attributed to the genus level. In this case, they were attributed to the last taxonomic level possible preceded by "unc." (standing for unclassified).

correlated negatively with the levels of VRE, suggesting that these bacteria may be associated with the protection against the pathogen (Table 3.2).

As indicated in the figure 3.26, mice that were colonized with low levels of VRE (<10E4 ufc/g) contained high fecal levels of the identified commensal bacteria. On the contrary, mice with high levels of VRE (>10E6 ufc/g) presented low fecal levels of these bacteria (see section 2.5.5 for the definition of the high and low-level VRE groups.) These results indicate that the taxa identified are associated with resistance against VRE colonization.

It is also possible that some bacteria that expanded upon antibiotic treatment promoted the colonization by VRE. Indeed, several bacteria presented a significant positive correlation with VRE, the most significant one being *Escherichia.Shigella* (Table 3.3). These bacteria could also simply be a biomarker of sensibility to VRE colonization, without influencing it. For example, the positive correlation with *Enterococcus* may reflect that changes that promote the growth of the endogenous *Enterococcus* could also promote the growth of the exogeneous VRE administered (Table 3.3).

Table 3.3: Taxa that present a significant positive correlation with VRE.

Genus	spearman.cor	adj.spearman.p.value.BH	abundance in NT mice (%)
Escherichia/Shigella	0.67	6.04E-08	0.0
Enterococcus	0.42	3.40E-03	0.0
Akkermansia	0.42	3.40E-03	1.0
unclassified_Enterobacteriaceae	0.40	5.69E-03	0.0
Parasutterella	0.36	1.30E-02	1.6
unclassified_Burkholderiales	0.32	3.55E-02	0.0
Lactobacillus	0.31	3.66E-02	1.6
unclassified_Desulfovibrionaceae	0.30	4.25E-02	0.0

NT: not treated

Taking advantage of the separation of the samples in a high and low-VRE level group, we applied a Linear Discriminiant Analysis (LDA) using LEfSe software [233] to determine which taxa characterized each group of mice.

By doing so, we wanted to confirm that the bacteria negatively correlated with VRE levels were indeed characteristic of the low-VRE level group. This algorithm selected the continuous variables that best discriminate between two groups.

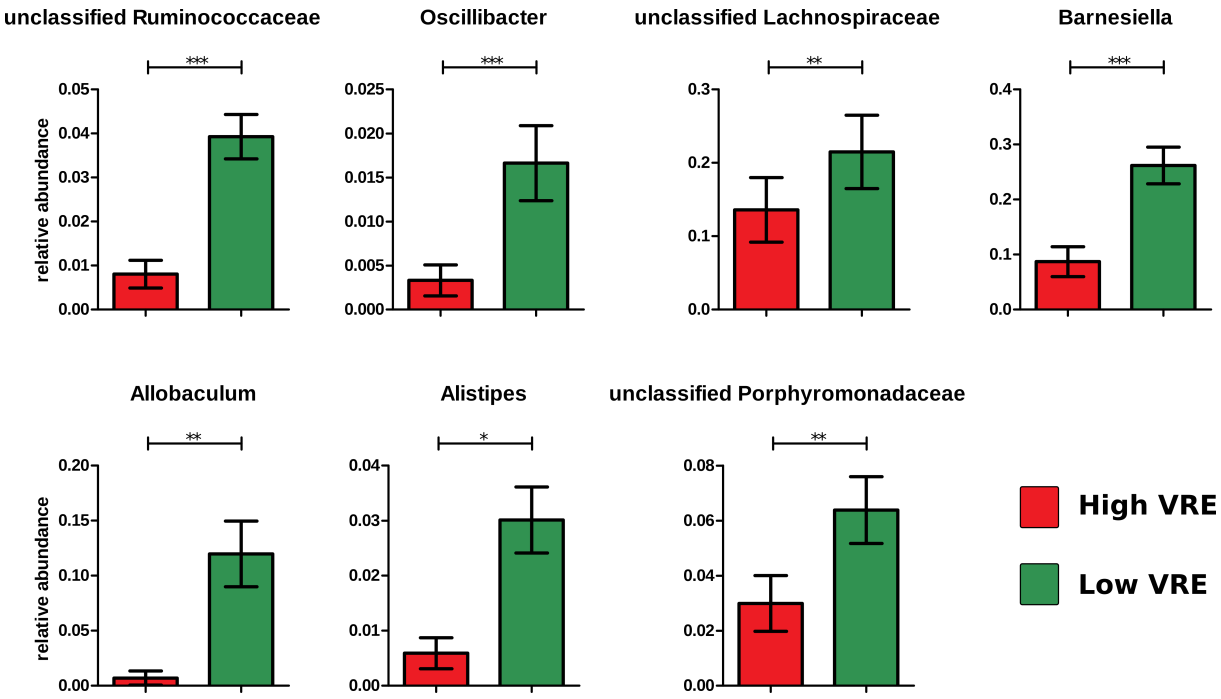


Figure 3.26: Commensal bacteria level in fecal samples of antibiotic-treated mice. The relative abundance of the bacterial genus and family that were negatively associated with VRE colonization are shown. Bacteria are organized from left to right and up to down starting from the highest negative spearman correlation. Sample were separated into those mice with high or low level of VRE in feces two days post-infection. green : low VRE level (<10E4 ufc/g faeces, $N = 23$) red : high VRE level (>10E6 ufc/g faeces, $N = 25$) Mann-Whitney test, * p.value<0.05, ** p.value<0.05, *** p.value<0.001. Mean with SEM.

The results of the LDA analysis, represented as a cladogram, confirmed that *Barnesiella*, Porphyromonadaceae, *Alistipes*, Ruminococaceae and *Oscillibacter* are related with low VRE loads. *Allobaculum* was not detected as being of importance (Figure 3.27). Similarly, as obtained with the spearman correlation, we can highlight an association between high VRE load and high levels of proteobacteria (*Escherichia-Shigella*, *Parasutterella*).

We also performed a spearman correlation test comparing the OTUs identified in the data with and without recovery against VRE levels. The lowest correlation factor was of -0.59, and corresponded to a *Barnesiella* OTU. Indeed, 10 out of 12 of the OTUs with a correlation factor lower than -0.50 corresponded to *Barnesiella* or the Porphyromonadaceae family (unc. Porphyromonadaceae). Thus, different OTUs of the genus *Barnesiella* may exert a similar protective effect. A similar result was obtained for other genera associated with resistance against VRE.

The correlation analysis was also performed on the data obtained by illumina in order to confirm the results obtained with 454. As expected, all the taxa identified to be associated with the VRE resistance when performing the analysis on the 454 data were also identified to negatively correlate with VRE levels when performing the analysis on the data obtained by illumina (not shown).

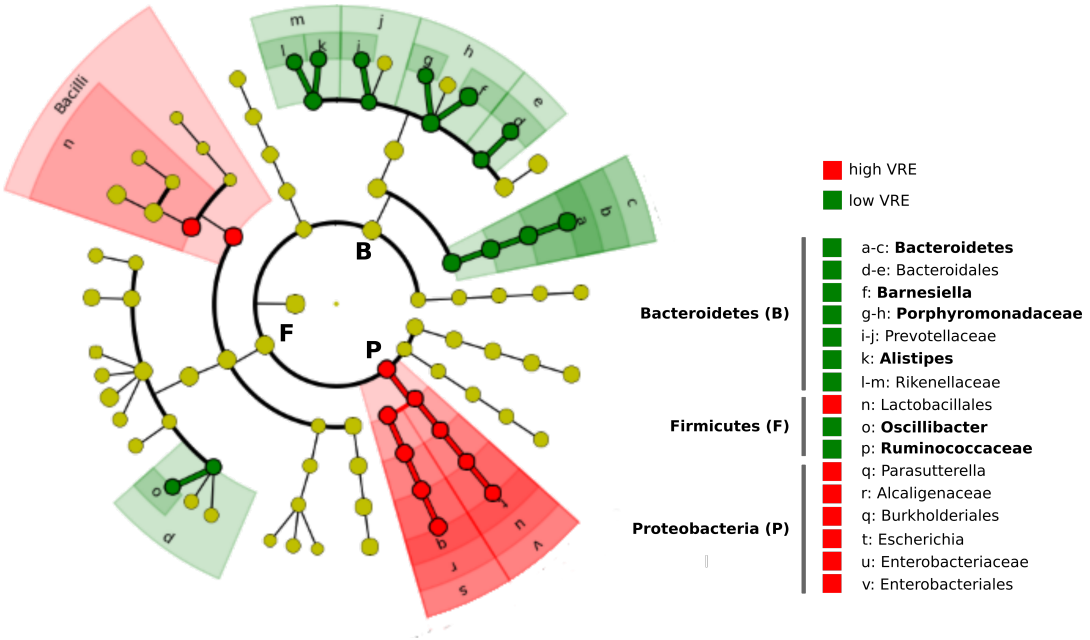


Figure 3.27: Result of the LDA analysis. Cladogramm of the bacteria significantly related with an high or low VRE load post-infection. The bacteria are organized by taxonomy. The taxa characteristic of one of the two group are colored. green : low VRE level ($< 10E4$ ufc/g faeces). red : high VRE level ($> 10E6$ ufc/g faeces). The taxa that present a negative correlation with VRE are indicated by a grey spot.

3.3 Intestinal bacteria isolation

Having determined the bacteria associated with the resistance against VRE colonization, we decided to test them in a mouse model to check their protective activity against VRE. Most of the bacteria available in the biological resource centers were isolates from in human samples. In a first attempt, we determined by comparison of 16s rRNA which bacteria of the DSMZ database were the most similar to the one we were interested in. We test the DSMZ isolates in our mouse model, however, most of the bacteria purchased from DSMZ were not able to colonize the mice (result not shown). Thus, we isolated the bacteria of interest directly from the murine gut.

Although the main purpose was to isolate the bacteria necessary to protect against the VRE intestinal colonization, we also wanted to obtain a library of murine bacteria for other projects of the laboratory. For this reason, we did not use a specific method to isolate the bacteria of interest except if it was indispensable.

Until recently, the specific conditions required for the growth of most of the intestinal bacteria where unknown and thus, an important fraction was uncultivable [254, 255]. To determine which medium could allow us to isolate the targeted bacteria, we sowed the culture medium Columbia Blood Agar, Schaedler, Reinforced Clostridium and Brucella agar with an aliquot of untreated-mice cecum content. We preferentially plated a cecum sample over a fecal sample based on the assumption that some bacteria from feces may not be active and therefore more difficult to grow *in vitro*.

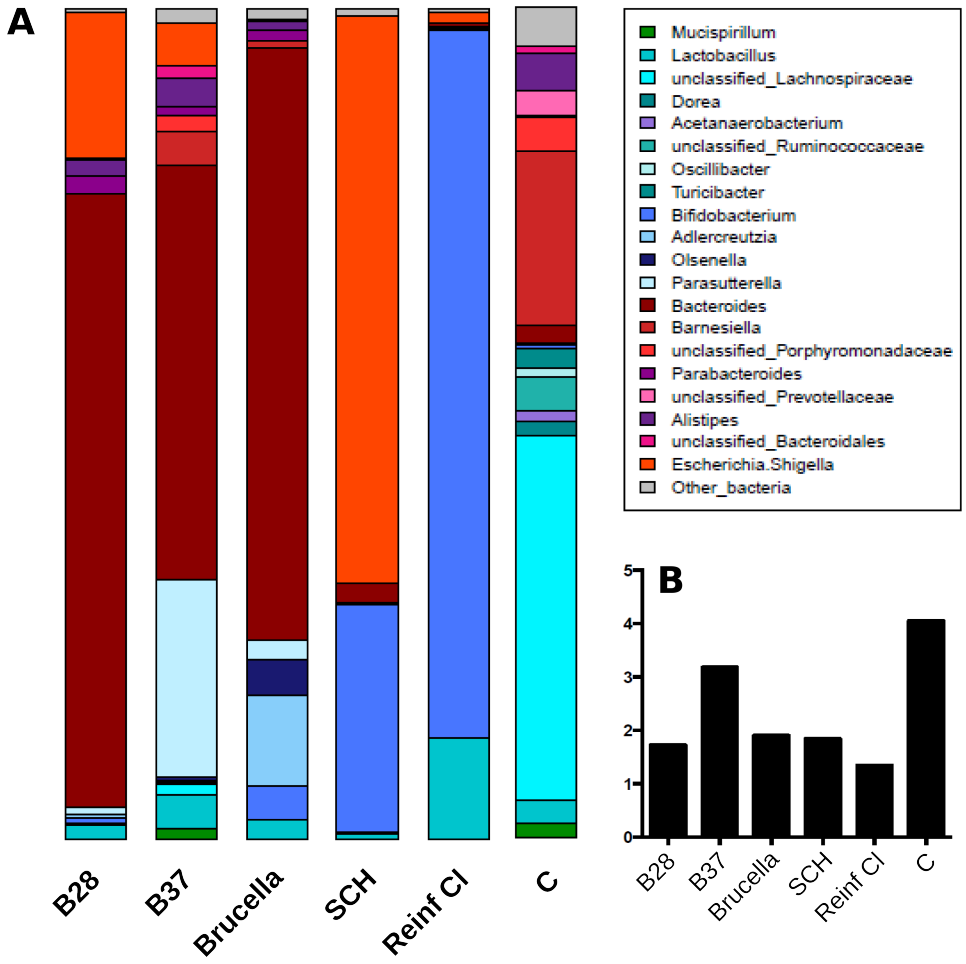


Figure 3.28: Different culture media allow the growth of different bacterial diversity. (A)Phylogenetic classification of 16S rDNA frequencies in the medium tested and from the cecal sample used to sow them. Each bar represents the microbiota composition from one medium. The most predominant bacterial taxa (relative abundance above 1%) are shown and labeled with different colors as indicated. Those bacterial taxa with a relative abundance lower than 1% are combine in the group other bacteria. Bacterial taxa were obtained by classification of 16s rDNA sequences to the genus level using mothur. In case a sequence could not be classified to the genus level, the closest level of classification to the genus level was given, preceded by unclassified. (B)Shannon index. In both figures, the tested medium are : Columbia Blood Agar at 28°C(B28) or at 37°C(B37), Brucella agar (Brucella), Schaedler agar (SCH), Reinforced Clostridium agar (Reinf.Cl) and the cecal sample (C).

Plates were incubated in anaerobic condition for 6 days at 37°C(except for Columbia Blood Agar that was also incubated at 28°C). The medium Columbia Blood Agar is a rich medium, used to grow fastidious taxa, thus we expected a high diversity of bacteria to grow in it. For this reason, we also tried a lower temperature, 28°C, to verify whether it would be possible to grow bacteria that were outcompeted at 37°C. Subsequently, we collected the bacterial colonies from each medium and identified by 16s high-throughput sequencing which bacterial taxa could be grown in each particular medium. As a control,

we also sequenced an aliquot of the cecal sample that was grown in the different media (see section 2.6).

Among the different media tested, we were interested in the one with the highest diversity, as it would allow us to isolate the highest number of bacteria. We evaluated the diversity present in the different media thanks to the Shannon index. As explained in the previous sections, this index account both for the number of OTUs present and for the relative abundance. As indicated by the shannon index, Columbia Blood Agar grown at 37ºC presented the highest diversity, although lower than the cecal sample (Figure 3.28 B). In the heatmap representing the relative abundance of the different genus it is also visible that a higher number of genera were identified in this medium (Figure 3.29).

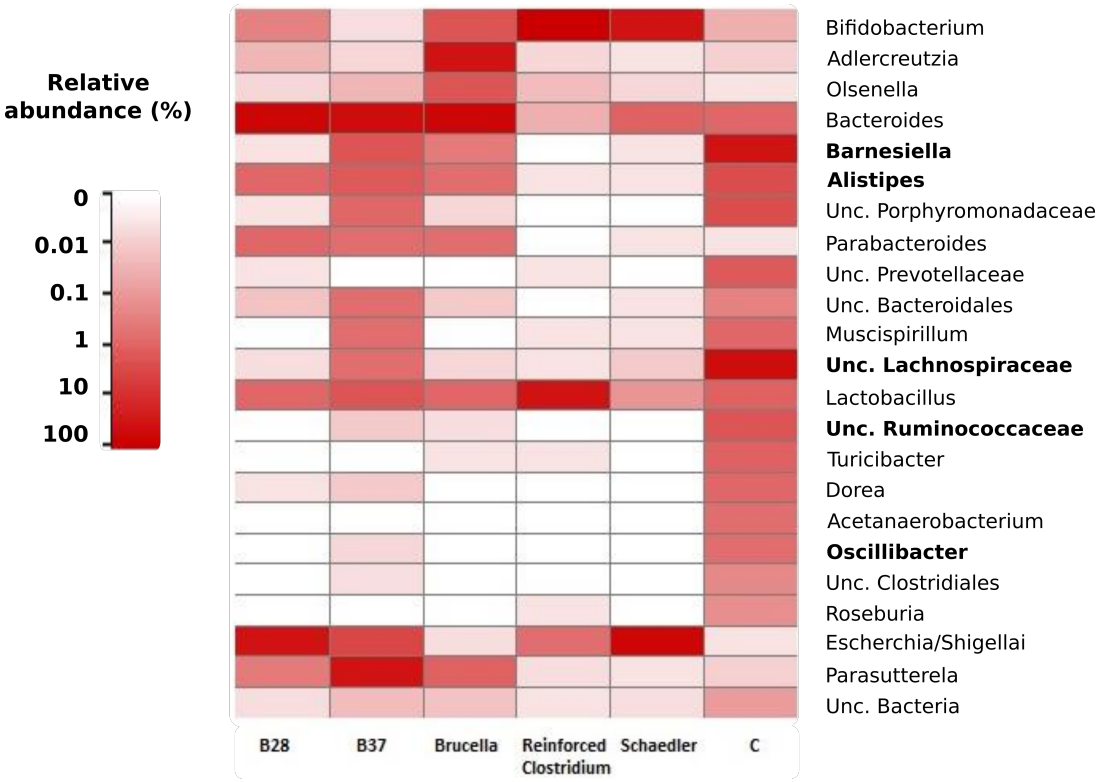


Figure 3.29: Relative abundance of the most abundant taxa in different media. Heatmap representing the relative abundance of taxa identified in the different culture medium tested and in the cecal content of an untreated mouse. Each row correspond to a taxa and each column to a medium. The color represent the relative abundance, darker meaning an higher abundance, as depicted in the key. The relative abundance of the most predominant genus is shown (relative abundance above 0.1% in one of the samples tested). The tested media are : Columbia Blood Agar at 28°C(B28) and at 37°C(B37), Brucella agar (Brucella), Schaedler agar (SCH) and Reinforced Clostridium agar (Reinf.Cl). Bacterial taxa were obtained by classification of 16s rDNA sequences to the genus level using mothur. In case a sequence could not be classified to the genus level, the closest level of classification to the genus level was given, preceded by unc. The genus of interest are in bold.

As mentioned, we wanted to isolate the highest number of taxa possible, but our main objective was to isolate the bacteria that presented a negative correlation with VRE, as

determined in the previous section (Table 3.2). These bacteria grown on the Columbia Blood Agar and no bacteria of interest was present in high levels in any of the other media tested (Figure 3.30). Of note, we already had one strain of *Allobaculum* that was isolated from Schaedler agar (SCH), thus we did not need to design a specific strategy to isolate this genus. Consequently, we decided to use only Columbia Blood Agar grown at 37°C to isolate commensal bacteria.

In this medium, *Barnesiella, Alistipes*, unclassified Porphyromonadaceae and unclassified lachnospiraceae presented a relative abundance superior to 0.1% (figure 3.28 A). Thus, we could isolate them by simply testing an high number of colonies (see section 2.6). With this approach, we were able to isolate most of the taxa of interest (*Barnesiella*, *Alistipes*, *Allobaculum* and unc. Ruminococacceae) as well as other high abundant bacteria from the gut (Figure 3.30). Besides of the bacteria represented in the figure 3.30 (high abundant bacteria), we were able to isolate some low abundant bacteria, that were enriched in the medium used (*Olsenella*, *Aldercreutzia*, unc. coriobacteriaceae, *Desulfovibrio*, *Enterobacter*, *Enterococcus*, *Enterorhabdus*, *EscherichiaShigella* and *Mucispirillum*).

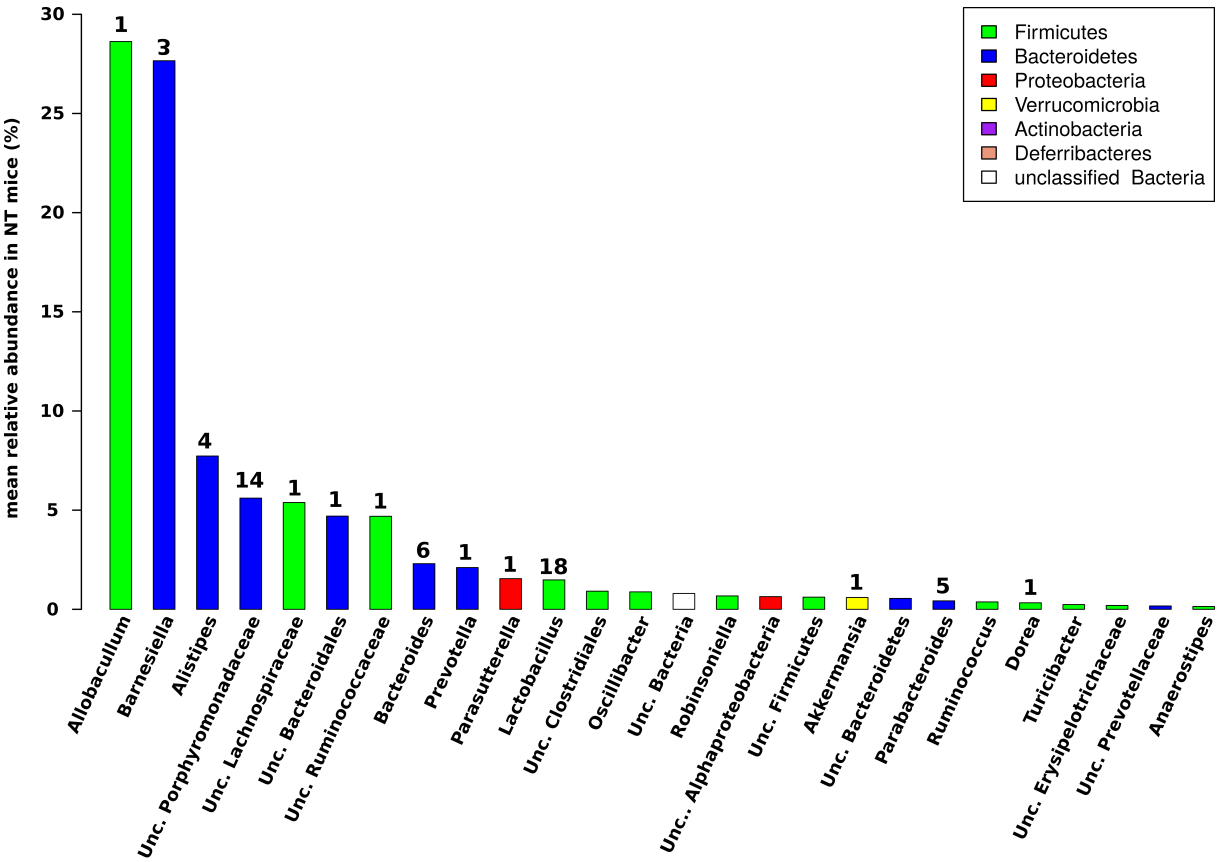


Figure 3.30: Summary of the number of taxa isolated in comparison with their relative abundance in untreated mice. Each bar represent the mean abundance of the taxa in untreated mice (mean calculated on five fecal samples). The color correspond to the phylum specified in the legend. The number above each bar indicates the number of isolates obtained from that particular taxa. Only taxa with a relative abundance superior to 0.1% in untreated mice are shown.

Unfortunately, we were not able to isolate the very low abundant genus *Oscillibacter*, one of the genera that we found to be associated with resistance against VRE. In order to isolate *Oscillibacter*, it was necessary to use a more specific approach. This taxa represents less than 0.01% of the bacteria grown in Columbia Blood Agar at 37℃, and is not present at a higher level in the other media tested (Figure 3.29). Thus, we would have to test more than 10 000 colonies following the previous approach to isolate one strain.

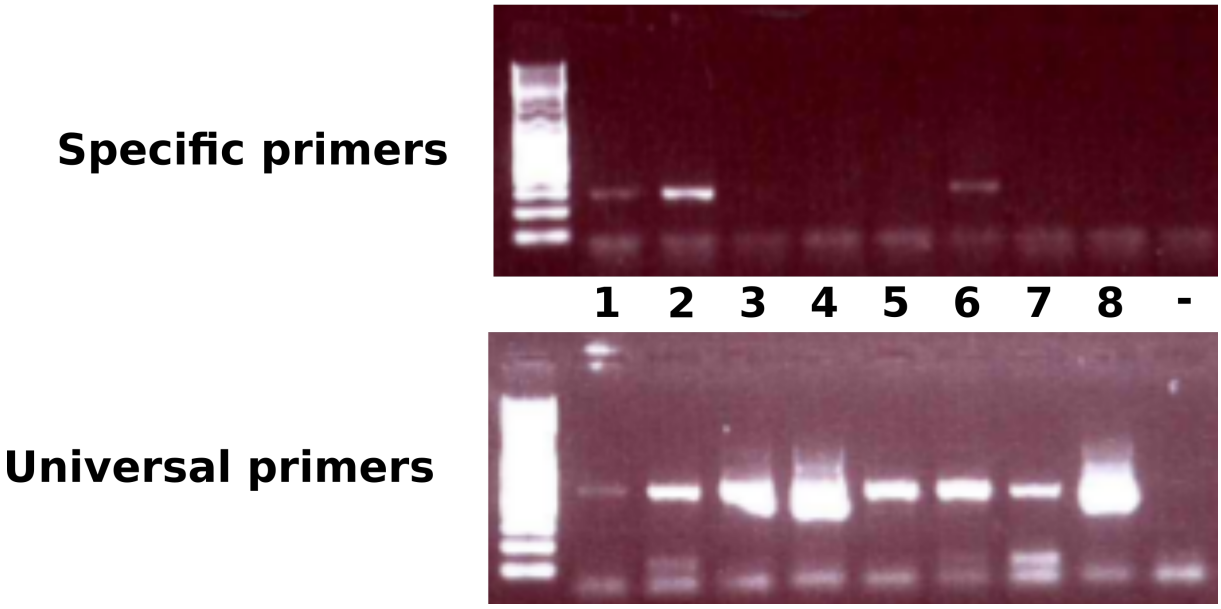


Figure 3.31: Test of the specificity of the primers pair designed. Upper part: specific primers tested. Lower part: universal primers for 16s rRNA. (1) unc.ruminococaceae, (2) unc.lachnospiraceae, (3) *Lactobacillus*, (4) *Bacteroides*, (5) *Parasuterella*, (6) *Barnesiella*, (7) *Allobacullum*, (8) *Escherichia coli*, (-) negative control. 1,4% Agarose gel, electrophoresis of 120V for 30 minutes. Molecular weight ladder: gene ruler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

To isolate *Oscillibacter*, we designed specific primers that could allow us to check the taxonomy of the bacteria grown in the Columbia blood agar medium by performing a PCR from a bacterial colony. Subsequently, we could confirm the taxonomy of positive clones through 16s rRNA sequencing. This strategy could facilitate our task since it would avoid us the need of sequencing thousands of colonies.

The first intention was to design a pair of primers specific for *Oscillibacter*. However, there were very few differences in the 16s rRNA sequence of taxonomically closely related bacteria (*Oscillibacter* vs other bacteria from the family Ruminococaceae). For this reason, we decided to found a primer that would allow us to identify bacteria within the family Ruminococacceae (both *Oscillibacter* and other unc. Ruminococacceae). For this purpose, we used the program PRIMROSE as described in the section 2.8. This program allows the selection of primers that anneal with selected bacteria and not with other bacteria from a given database. The final primers pair selected is indicated in the table 3.4.

To test that the primer pair worked, we used, as a positive control, the unc.ruminococaceae that we had already isolated. In order to assess the specificity of the primers, we used

Table 3.4: Sequence of primers designed to isolate low-abundant bacteria.

Primer	**Sequence (5'-3')**
Ruminococcaceae F16	AACGCCGCGTGARGGAAGAM
Ruminococcaceae R310	CCGCTACTCHVGGAATTCCG

one isolate classified as unc.lachnospiraceae and one isolate of the taxa *Lactobacillus*, *Bacteroides*, *Parasuterella*, *Barnesiella*, *Allobacullum* and *Escherichia coli*.

We performed a PCR with the specific primers and as a control we performed also a PCR with the universal primer pair 27F-553R. As expected, a PCR product of the expected size was detected in all the bacterial isolates tested when the universal primers were utilized. The product of the PCR with the specific primers should be around 250bp (third band from the bottom of the molecular weight ladder). As shown on the figure 3.31, we obtained a band of the expected size (250bp) when we utilized as template the unc. Ruminococacceae isolate. Since the region of 16s rRNA where the primers hybridize is identical in *Oscillibacter*, this indicated that the specific primers designed will allow us to detect it. As it can be seen, a PCR product of the expected size was also detected when isolates from unc. Lachnospiraceae and *Barnesiella* were utilized as template. To avoid amplification of *Barnesiella* and unc. Lachnospiraceae, we increased the annealing temperature to 61^{o}C. Unfortunately, by increasing the temperature, we did not detect a PCR product even when we utilized the unc. Ruminococacceae isolate as template. Nevertheless, the 16s of the most abundant bacteria in the medium Columbia Blood Agar at 37^{o}C (i.e. *Lactobacillus*, *Bacteroides*, *Parasuterella* or *Escherichia coli*) were not amplified by these primers (Figure 3.31). Therefore, this primer pair, although not perfect, was adequate to improve our capacity to isolate *Oscillibacter*. Indeed, thanks to these primers, we were able to isolate *Oscillibacter* and five news strains of the unclassified Ruminococcaceae taxa 3.32.

In summary, we were able to isolate the different genus of interest (those with negative correlation with VRE) plus other commensal bacteria.

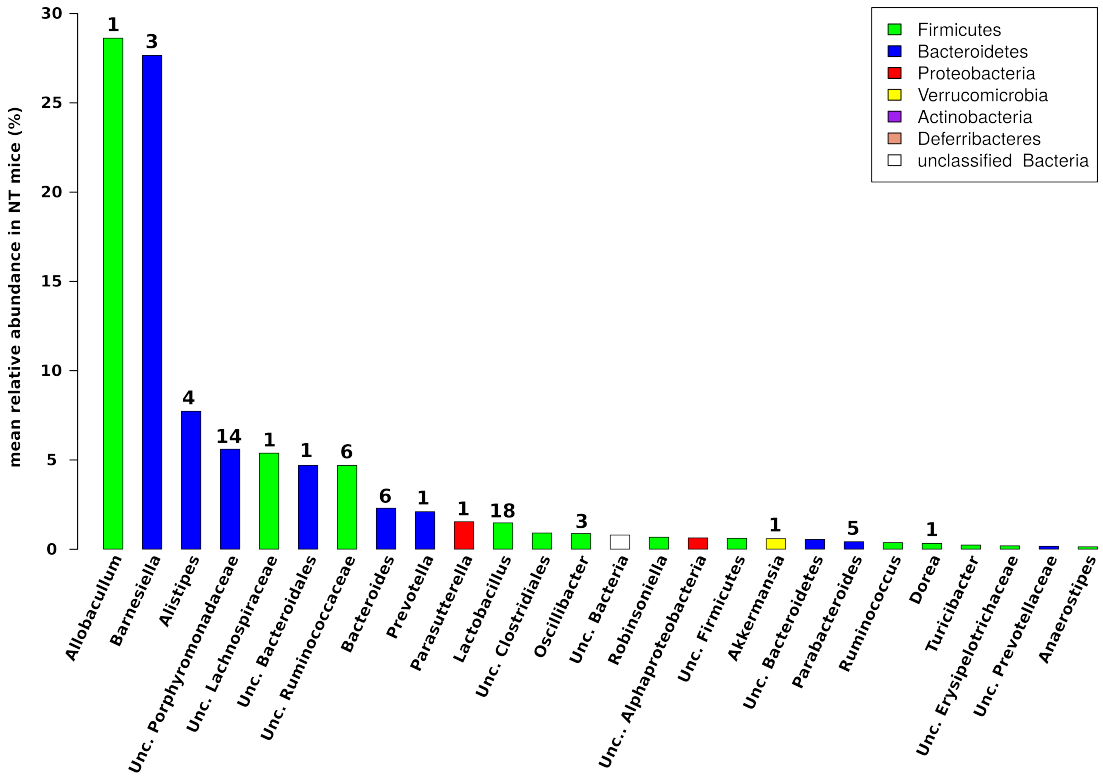


Figure 3.32: Final summary of the number of taxa isolated in comparison with their relative abundance in untreated mice. Each bar represent the mean abundance of the taxa in untreated mice (mean calculated on five fecal samples). The color correspond to the phylum specified in the legend. The number above each bar indicates the number of isolates obtained from that particular taxa. Only taxa with a relative abundance superior to 0.1% in untreated mice are shown.

3.4 Study of the protective effect of the isolated intestinal bacteria against the intestinal colonization by VRE

3.4.1 Selection of the mouse model to test the commensals bacteria.

Ubeda *et al.* [211] demonstrated that the administration of a fecal transplant post-antibiotic treatment restores the microbiota and prevents VRE colonization. That particular study showed that the administration of a complex bacterial population can restore the CR, lost after an antibiotic treatment.

Based on this, we could administrate the specific bacteria in mice that received antibiotics to verify that they restore the CR against VRE. We determined in the previous section (see 3.2.5) that several antibiotics promoted a high level of colonization by VRE. Consequently, we could use this mouse model to test if the bacteria selected are able to prevent VRE colonization. However, the bacteria isolated are sensitive to the antibiotic treatments. For this reason it is necessary to administrate them after the removal of

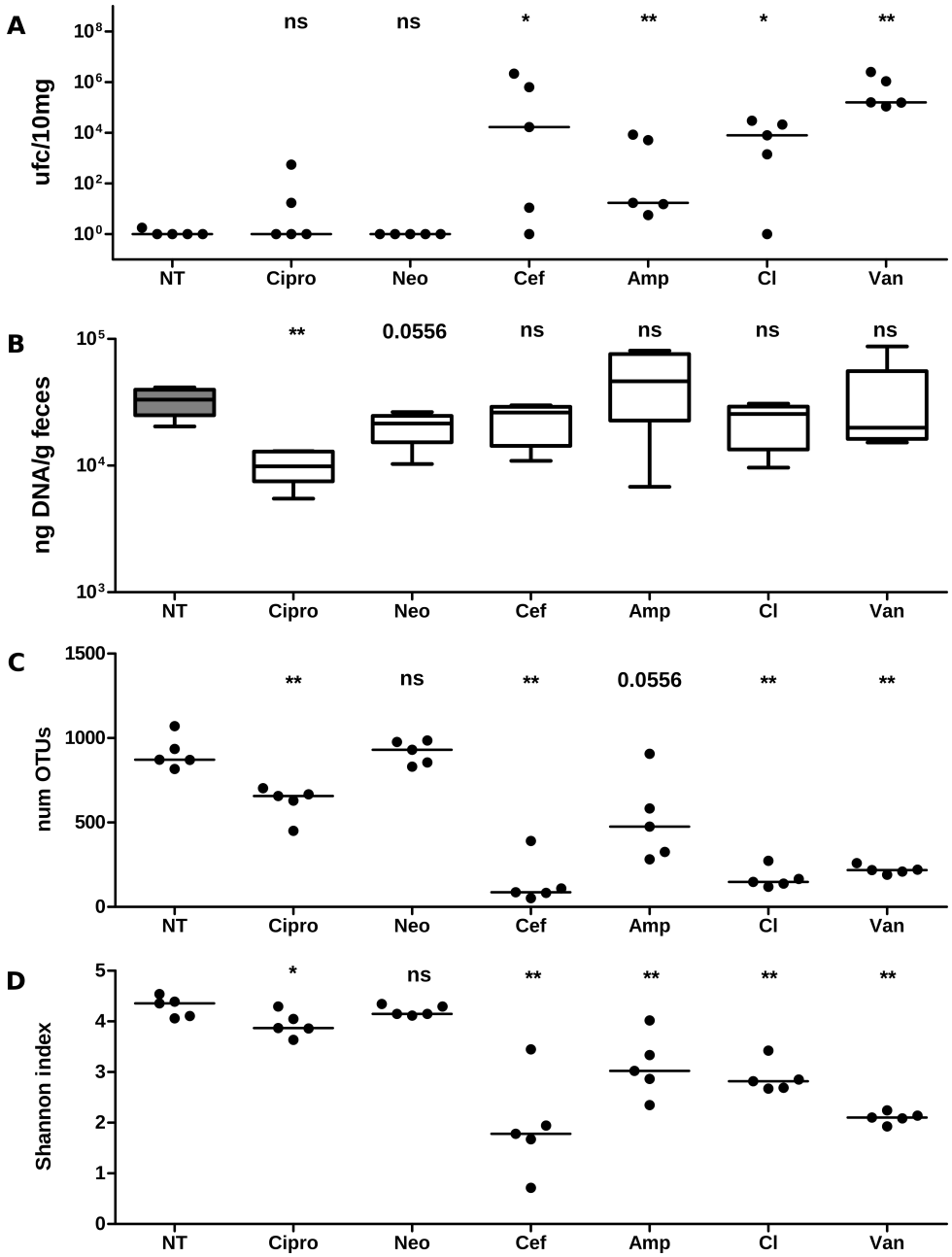


Figure 3.33: Effect of different antibiotics on the gut microbiota richness, diversity and the capacity of colonization by VRE. Mice were allowed to recover for two weeks between the withdrawal of the antibiotic and the determination of microbiota composition as well as subsequent oral infection with VRE. (A) VRE levels observed at day 2 post-infection, (B) biomass, (C) number of OTUs, (D)Shannon index. (B-D) samples were recovered before the infection with VRE. NT:untreated mice, Cipro:ciprofloxacin, Neo:neomycin, Cef:ceftriaxone, Amp:ampicilin, Cl:clindamycin, Van:vancomycin. Mann-Whitney test, ns: not significant, * p.value<0.05, ** p.value<0.01. The exact p.value is indicated when it is close to the threshold of significance.

the antibiotic. Thus, we could use the mouse model in which the antibiotic treatment is followed by a two-weeks recovery. Indeed, when testing the effect of antibiotics on the gut microbiota, we constated that after a two-weeks recovery, important alterations persisted (see subsection 3.2.4) and that these alterations enabled the colonization by VRE (Figure 3.33 A). Similarly as Ubeda *et al.* [211], we administrated the bacteria for three consecutive days from the following day after the withdrawal of the antibiotic treatment. Two weeks after the antibiotic withdrawal, we inoculated mice with VRE and tested the protective effect of the administered bacteria against VRE.

From all the antibiotics that we tested previously, 3.2, we choose to treat mice with vancomycin in order to test the protective effect of isolated bacteria for several reasons: (i) it allows VRE colonization in high levels upon recovery, (ii) the majority of the commensal bacterial species are depleted and do not recover after 2 weeks of antibiotic cessation and (iii) the bacterial density is similar as in untreated mice, thus we can assume that the enhanced colonization by VRE is related to the absence of specific bacteria and not to an unspecific diminution of the total bacterial biomass. As can be seen on the figure 3.33, also clindamycin fulfill this criteria, however, since VRE colonization is higher upon vancomycin cessation than upon clindamycin withdrawal, we preferred to use vancomycin in order to test the protective effect of the commensal bacteria associated with protection against VRE.

Ubeda *et al.* [211] verified that the administration of a fecal transplant after an ampicillin treatment restored the CR to VRE. As we decided to use another antibiotic in our mouse model (vancomycin), we checked that the administration of a fecal transplant would also restore the CR against VRE in our model. Therefore, we could verify that the administration of a complex mixture of commensal bacteria is sufficient to restore the CR against VRE.

For that purpose, we treated mice with vancomycin for one week, then, starting the day following the withdrawal of the treatment, we orally inoculated treated mice with feces from untreated mice during three consecutive days. We allowed the microbiota to recover for two weeks, then we challenged the mice with an oral administration of 10E6 cfu VRE/200µl. As a control, we also inoculated VRE to untreated mice and to a group of mice that was treated with antibiotics but that did not received a fecal transplant. In our model, the fecal transplant diminished VRE colonization.

At day two post-infection, VRE was completely cleared from untreated mice and very low levels were found in the group that received the fecal transplant (Figure 3.34 B). In contrast, the vancomycin treated group that did not received the fecal transplant presented very high levels of VRE the second day post-infection (Figure 3.34 A,B). Thus, in our model, the administration, post-treatment, of a bacterial community proceeding from untreated mice prevents the intestinal colonization by VRE. The protection conferred by the fecal transplant was probably due to the fact that the fecal transplant restored partially the microbial community. Indeed, a smaller weigthed unifrac distance to untreated mice was detected in the group of mice that received the fecal transplant as compared to the distance between untreated mice and mice treated with vancomycin that did not received the fecal transplant (Figure 3.34 C).

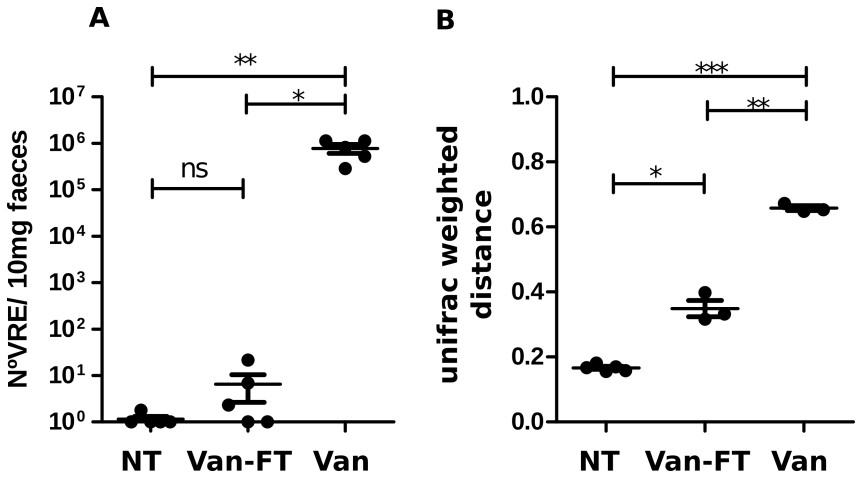


Figure 3.34: A fecal transplant restores the colonization resistance against VRE. Mice were treated with vancomycin for one week, then one group received a fecal suspension from untreated mice for three days. The microbiota composition was determined two weeks after the withdrawal of the treatment, before infecting the mice orally with 10E6 CFUs VRE and determining the colonization levels. (A) VRE levels observed at day 2 post-infection, (B) Weigted unifrac distance to untreated mice (454 sequencing). In (B), each point represents the mean distance to the five untreated mice. In the case of the untreated mice, we represented the distance respect to the four others untreated mice. This indicate the basal distance associated with the intra-group variability. NT:untreated mice, Van-FT:vancomycin- fecal transplant, Van:vancomycin. (A) Mann-Whitney test, ns not significant, * p.value<0.05, ** p.value<0.01, *** p.value<0.001. (B) Unpaired t-test with Welch correction, ns not significant, * p.value<0.05, ** p.value<0.01, *** p.value<0.001.

3.4.2 Selection of the isolated commensal bacteria to be tested against VRE colonization

After selecting the antibiotic to be used in our model, we needed to select the commensal bacterial strains to be tested in our mouse model from the ones that we isolated (section 3.3). According to the results of the spearman correlation (Table 3.2), the taxa unc. Ruminococcaceae, *Oscillibacter*, unc. Lachnospiraceae, *Barnesiella*, *Allobaculum*, *Alistipes* and unc. Porphyromonadaceae were associated with the protection against intestinal colonization by VRE.

During the treatment with vancomycin, the group unclassified lachnospiraceae is significantly increased respect to the levels observed in the untreated mice (Table 3.5). Thereupon, we decided not to administer this taxa.

In the case of the unclassified Porphyromonadaceae group, this taxa is phylogenetically very close to *Barnesiella*, genus within the Porphyromonadaceae family. Moreover, as we mentioned in the section 3.2.5, *Barnesiella* presented a higher negative correlation with VRE and was more abundant in untreated mice than the unclassified Porphyromonadaceae group. Consequently, we decided to administer only *Barnesiella*. The other taxa that could not be classified to the genus level, the unc. Ruminococcaceae, displayed the highest negative correlation (-0.73) and was more abundant than *Oscillibacter* in un-

Table 3.5: Changes of the selected commensal bacteria after administration of vancomycin.

taxon	Fold change from van to NT	Adj fdr p-value
unclassified_Lachnospiraceae	8.340	0.008
Oscillibacter	1.467	0.117
unclassified_Ruminococcaceae	0.459	0.118
Alistipes	0.025	0.008
Barnesiella	0.008	0.008
Allobaculum	0.006	0.008

Fold change were calculated as the mean abundance of the taxa in vancomycin-treated mice after a two-weeks recovery respect to the mean abundance in untreated mice. Wilcoxon test corrected with FDR. n=5 by group.

treated mice (Table 3.2), thus, we administered it. For the different bacteria selected, in case of having several isolates, we selected the isolate whose sequence was more similar to the most abundant OTU of the genus present in untreated mice.

Thus, we finally decided to administrate 5 isolates: *Alistipes* (Cu159), unclassified Ruminococacceae (Cu153), *Barnesiella* (Cu46), *Oscillibacter* (Cu176) and *Allobacullum* (Cu185).

3.4.3 Incidence of the administration of a bacterial mix on VRE colonization

We used the mouse model described in 2.2.3, treating the mice with vancomycin for 1 week, then we administered for three consecutive days the bacteria whose protective activity against VRE had to be tested. Subsequently, mice were allowed to recover for two weeks. After this period, mice were inoculated orally with 10E6 CFUs VRE/200µl. As a control, another group of mice that did not received the commensal bacteria and received instead PBS was also inoculated with VRE.

First, we checked that the bacteria administered did actually colonize the mice. The abundance of *Barnesiella, Alistipes* and *Allobaculum* were significantly diminished upon vancomycin treatment and the administration of the bacteria of interest allowed a recovery to levels similar to the ones observed in untreated mice (the levels reached were slightly higher in the case of *Alistipes* and slightly lower in the case of *Barnesiella* (Figure 3.35 A). The specific OTUs administered within this genus, were responsible for the recovery of the whole genus in untreated mice (Figure 3.35 A). As expected, since several OTUs of the genus *Barnesiella* and *Alistipes* are normally present in the untreated mice, the OTU administered do not account for the abundance of the whole genus (Figure 3.35 A).

The ruminococcaceae family, which includes both *Oscillibacter* and the bacteria unc. Ruminococcaceae, presented similar levels in the treated group that received the bacterial mix or PBS instead. These levels were lower than the ones observed in untreated mice

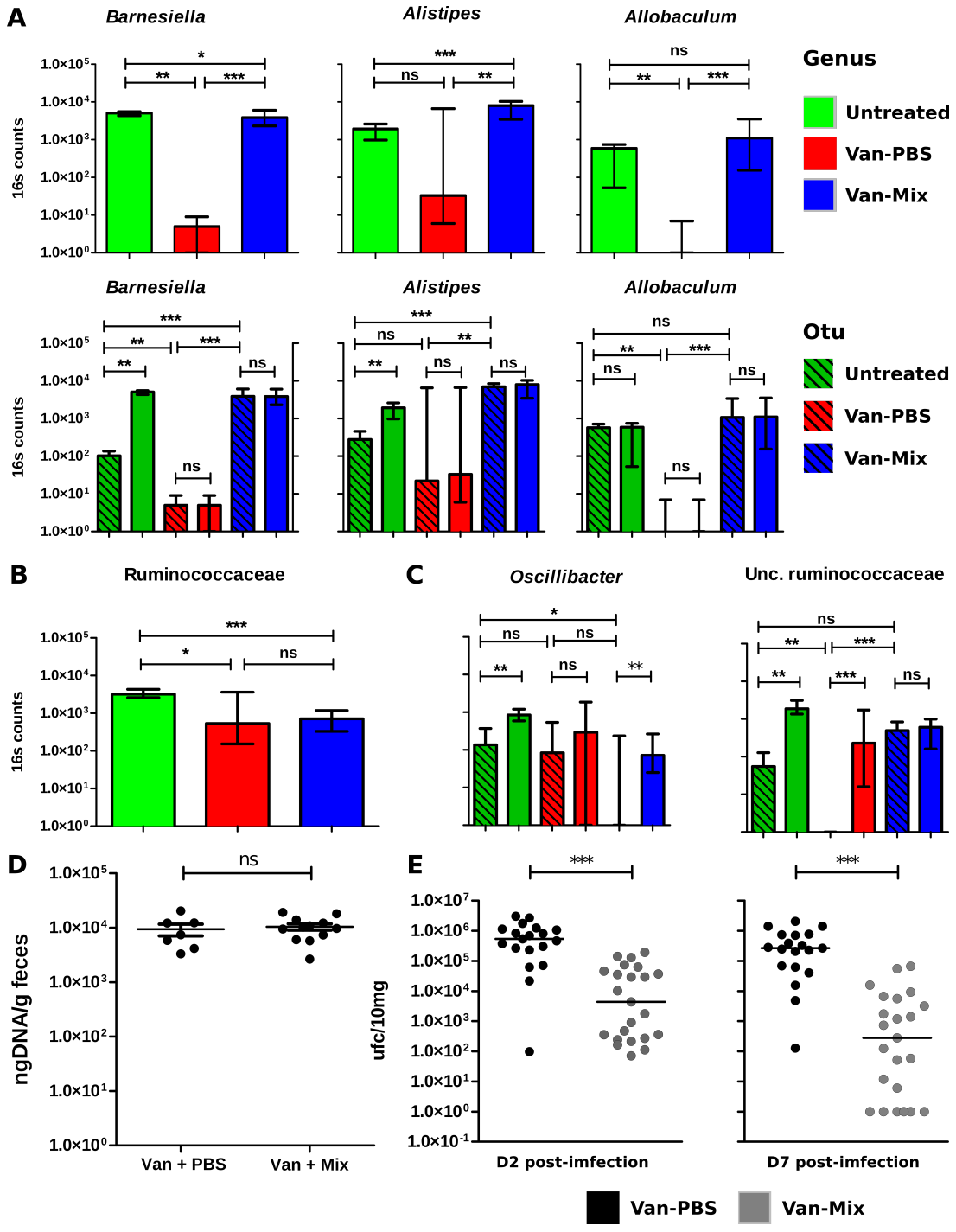


Figure 3.35: Colonization of the bacteria administered and effect on VRE colonization. Mice were treated with vancomycin for one week. Subsequently, mice received 3 consequence days PBS (Van-PBS) or a bacterial mix (Van-Mix) containing *Barnesiella*, *Alistipes*, *Allobaculum*, *Oscillibacter* and unc. Ruminococacceae. Two weeks post antibiotic cessation, the fecal microbiota was analyzed and mice were challenged with 10E6 CFUs VRE. (A) Levels of colonization of the commensal bacteria administered (*Barnesiella*, *Alistipes* and *Allobaculum*). The analysis was performed at the genus and OTU level. For comparison, a group of untreated mice was also analyzed. by probiotics administered, 16s rRNA sequencing determinated at the genus and the OTU level. (B) Levels of colonization of the family Ruminococacceae.

Figure 3.35: (C) Levels of colonization of the commensal bacteria administered (*Oscillibacter* and unc. Ruminococacceae). The analysis was performed at the genus and OTU level. box represent median with range. For (A-C) Untreated n=6, Van-Mix n=10, Van-PBS n=7. (D)Biomass. Van-Mix n=10, Van-PBS n=7. (E)VRE levels detected at day 2 and day 7 post-infection. Black : Van-PBS, n=19. Grey : Van-Mix, n=23. Results of 3 experiments. For (A-E) Mann-Whitney test, * p.value<0.05, ** p.value<0.01, *** p.value<0.001, ns no significant.

(Figure 3.35 B). A similar result was observed when we studied alone the *Oscillibacter* levels and the unc. Ruminococacceae levels.

At the OTU level, the *Oscillibacter* OTU matching the isolate administered presented variable levels in the treated group that received the bacterial mix and the treated group that received PBS instead. The levels of this OTU were similar in both groups of mice (Figure 3.35 C). Moreover, the number of reads assigned to this OTU is low in the group of mice that received the bacterial mix. Thus, the *Oscillibacter* administered did not colonize efficiently the mice to which it was administered.

On the other hand, the specific Ruminococacceae OTU we administered was depleted upon the antibiotic treatment and restored to the level observed in untreated mice through the administration of the bacterial mix (Figure 3.35 C).

In summary, from this analysis, we can indicate that the bacterial isolates (OTUs) present in the mix colonized the mice to which they were administered except for the *Oscillibacter* isolate. Administration of these isolates were sufficient to restore the levels of *Barnesiella*, *Alistipes*, *Allobaculum*, but not the levels of Ruminococacceae.

After checking that most of the probiotics did colonize the mice, we compared the biomass (ng of DNA/ gr feces), an approximation of the total bacteria load [228], between the treated group that received the bacterial mix or PBS instead. The biomass was not significantly different between both groups, indicating that the administration of the bacterial mix does not influence the total bacterial load (Figure 3.35 D). Subsequently, we assessed the effect of the bacterial mix administration on the colonization resistance against VRE by comparing the VRE levels post-infection in these two groups. Mice that did received the bacterial mix contained lower levels of VRE (median, 4.39*10E3 and 2.79*10E2 CFUs/10mg at day 2 and 7 post-infection) than mice that did not received the bacterial mix (median, 5,40*10E5 and 2.67*10E5 CFUs/10mg at day 2 and 7 post-infection) (Figure 3.35 E). Results obtained using the Mann-Whitney test indicated that the difference observed in VRE abundance between both groups was statistically significant (p.value<0.0001 at day 2 and 7 post-infection). Thus, the administration of a mix of 5 bacteria after the antibiotic treatment allowed the partial recovery of CR against VRE.

3.4.4 Simplification of the bacterial mix

Having checked that the administration of the bacterial mix post vancomycin-treatment proportionated a protection against intestinal VRE colonization, we tried to understand the individual action of the bacteria included in the mix. The aim was to verify whether the protective action was due to an individual bacteria or if the complete bacterial mix was required. For this purpose, we administered to vancomycin treated mice each individ-

ual bacteria instead of the complete mix. As a control, we used a group of mice that did received the complete mix and a group of mice that received PBS instead of the bacterial suspension.

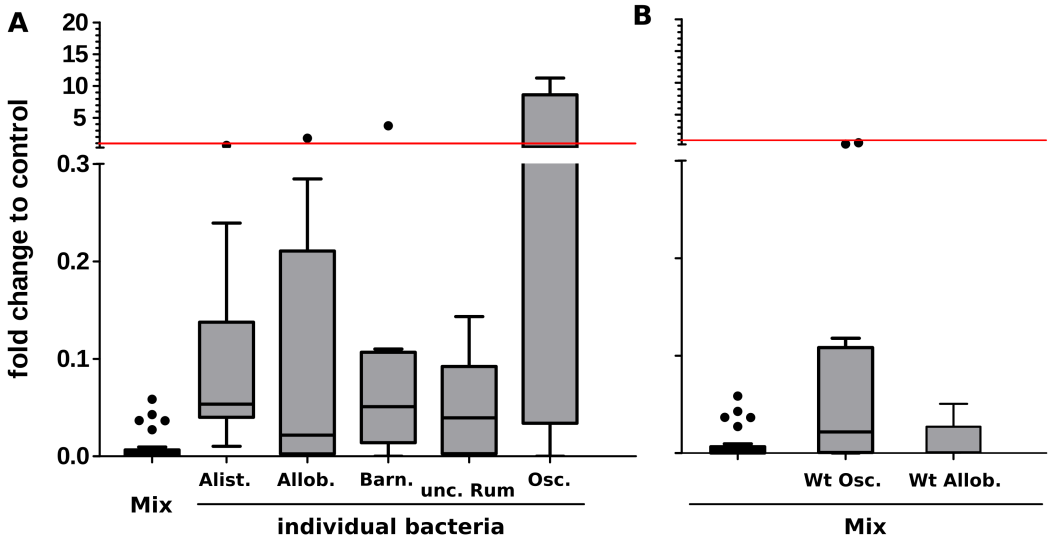


Figure 3.36: Impact of commensal bacteria administration on the colonization capabilities of VRE after antibiotic treatment. Mice were treated with vancomycin for one week and than were allowed to recover. One day after stopping the antibiotic treatment, mice received PBS, specific comensal bacteria or a bacterial mix. Two weeks after antibiotic cessation, mice were orally inoculated with 10E6 CFUs and the levels of colonization of VRE were quantified in feces 2 days post-VRE inoculation. The figure shows the fold change of VRE level observed at day 2 post-infection in the group that received a specific comensal bacteria or a bacterial mix, respect to the group that received PBS of the same experiment. A) from left to right, the complete mix (*Alistipes*, *Allobacullum*, *Barnesiella*, unclassified Ruminococacceae and *Oscillibacter*), and the individual bacteria :*Alistipes*, emphAllobacullum, *Barnesiella*, unclassified Ruminococacceae, and *Oscillibacter*. B) from left to right,the complete mix (*Alistipes*, *Allobacullum*, *Barnesiella*, unclassified Ruminococacceae and *Oscillibacter*), the mix without *Oscillibacter* and the mix without *Allobacullum*. Boxes extent from the 25th to 75th percentiles. The line at the middle of the box represents the median. Whiskers were done with Tukey. The red line is placed at 1 on the y axis (no VRE level change as compare to control). Every group displays a significant diminution of the colonization by VRE except the group that received the individual bacteria *Oscillibacter* (wilcoxon test respect to 1).

Due to the high number of mice required, it was necessary to perform several experiments separately. We observed a similar difference of the VRE colonization loads between the treated groups that did received the complete bacterial mix as compared to those that received PBS, across all the experiments (fold change between 0.002 and 0.008). Nevertheless, the absolute level of VRE detected can change across experiments. Thus, to be able to join the different experiments performed, we normalized each experiment respect to its own control by calculating the fold change between the VRE load observed in the different groups receiving the different bacteria respect to the treated-group that did not

received any bacteria. Thus, values inferior to one indicate a diminution of the colonization by VRE after administration of a particular bacterium, whereas a value superior to one indicates an increase.

The administration of the individual bacteria *Alistipes*, *Allobacullum*, *Barnesiella* or unclassified Ruminococacceae caused a significant diminution of the colonization by VRE, as it can be seen by their fold change inferior to one (Figure 3.36 A). The VRE load range from 20 to 50 times lower than control mice receiving PBS, depending on the bacteria administered. In the case of *Oscillibacter*, the levels detected were very variable and there was no significant diminution of the VRE load (Figure 3.36 A, the fold change is not significantly different from one, Wilcoxon signed rank test). Although the individual bacteria diminished the VRE colonization, the diminution was higher in the group that received the complete bacterial mix (Figure 3.36 B, diminution by 1000 times according to the median fold change).

Since *Oscillibacter* did not seem to exert a protective action against VRE, we decided to test if removing *Oscillibacter* from the mix would have any impact on the capacity of the mix to confer protetion against VRE colonization. In addition, we shown in the figure 3.35 B that this bacterium could be naturally recovered after the administration of vancomycin. Thus, it was reasonable to assume that *Oscillibacter* may not exert a protective effect against VRE in this particular mouse model. Surprisingly, the mix without *Oscillibacter* was less efficient than the complete mix, the median fold change was similar to the one observed when administrating an individual bacteria (median fold change of 0.02, thus a diminution by 50 times in VRE levels as compared to the control)(Figure 3.36 B). Consequently, we decided to maintain this bacterium in the mix.

Second, we tried to remove *Allobacullum* from the mix because, when we determined the bacteria that were associated with the protection against VRE, *Allobacullum* was negatively correlated with VRE but this association was not confirmed in the LDA analysis (see section 3.2.5 and figure 3.27). As shown on figure 3.36, the mix without *Allobacullum* produced a diminution of VRE colonization similar to the complete mix (Mann-Whitney test, not significant).

In summary, we could achieve a high level of CR by administering a mix containing four bacteria (i.e. *Alistipes*, *Barnesiella*, unclassified Ruminococacceae and *Oscillibacter*). We did not have an objective reason to remove any particular bacteria from the bacterial mix as they confer similar protection against VRE individually (except for *Oscillibacter*, whose removal effect was already tested). Thus, we decided to maintain this mix of four bacteria to explore mechanisms by which these bacteria confer protection.

3.4.5 Administration of commensal bacteria not associated with VRE protection does not inhibit VRE colonization

Our previous results indicate that administration of commensal bacteria that associate with protection against VRE do not inhibit the capacity of VRE to colonize the intestinal tract. To confirm that specific bacteria confers protection and that not any bacteria can exert a protective effect against VRE, we colonized antibiotic treated mice with *K. pneumoniae*.

On one hand, it has already been demonstrated that *K. pneumoniae* an VRE co-localized in the intestine without competing with each other [214]. On the other hand,

we can easily assess the levels of *K. pneumoniae* by plating the fecal samples on specific media. We, thus, decided to use *K. pneumoniae* (Cu39) as a control. As can be seen on the figure 3.37, *K. pneumoniae* was able to colonize the intestine of mice, however it did not confer protection against VRE.

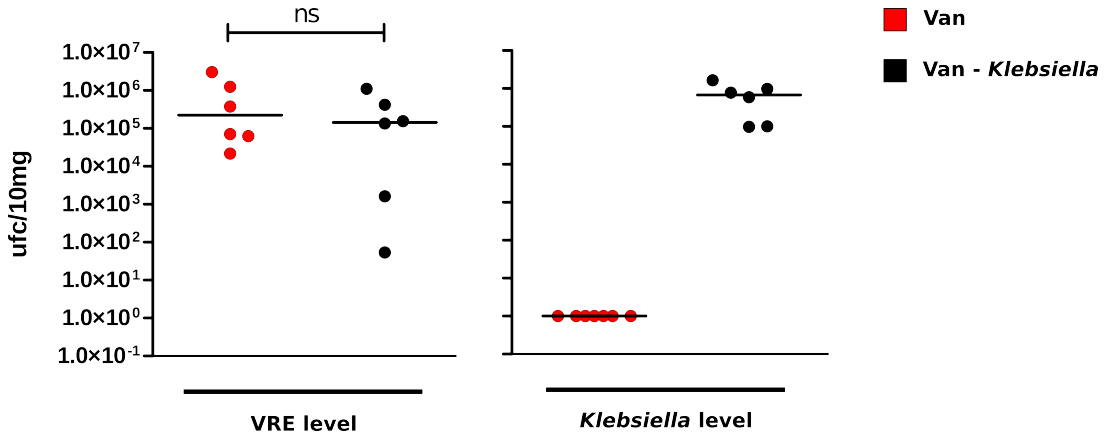


Figure 3.37: VRE or *K. pneumoniae* levels observed at day 2 post-infection. Mice were treated with vancomycin for one week. A group of mice received orally *K. pneumoniae* strain after antibiotic withdrawal (Van-*Klebsiella*), while the other group received PBS. Two weeks after antibiotic cessation, mice were orally inoculated with 10E6 VRE CFUs. VRE levels and *K. pneumoniae* levels were identified in both groups of mice two days after VRE infection. n=6 mice per group. Mann-Whitney test, ns: not significant. VRE levels observed on both group were similar and *Klebsiella* was detected only in the group of mice that received it.

3.5 Study of the bacterial mediated mechanism of protection against VRE colonization : *in vivo* meta-transcriptome and metabolome analysis

Before performing the meta-transcriptome and metabolome analysis described in this section, we performed several in-vitro experiments to try to identify a possible mechanism by which the commensal bacteria could confer protection against VRE colonization. First, we grew VRE in (1) the filtrates of cecum obtained from treated mice that received the bacterial mix or PBS and (2) the same filtrates complemented with BHI (a culture medium that support VRE growth). The growth observed after 2, 4, 6 and 22 hours was similar in all cases (result not shown). Moreover, to test if one of the bacteria administered produced a bacteriocin, we co-cultivated in agar media each of the bacteria administered and VRE. We did not observed any inhibition (result not shown). Thus, in the *in vitro* conditions used for both tests, we were not able to detect any inhibition by the commensal bacteria. Thus, we decided to study in more detail *in vivo* which bacteria functions express the protective commensal bacteria and how these functions change the gut metabolica landscape to identify possible mechanisms of protection (e.g. inhibitory products, competition for nutrients).

3.5.1 Verification of the recovery of a similar microbiota in co-housed mice

In order to decipher possible mechanisms of protection of the bacterial mix containing the four bacterial strains, we performed an *in vivo* transcriptomic and metabolomic study on the cecal content from mice that were treated with vancomyin and subsequently received the bacterial mix or PBS. Samples were taken two weeks after antibiotic cessation in mice that were not infected with VRE so that we could identify changes in the metabolome and transcriptome induced exclusively by the protective bacterial isolates and not derived from the metabolism of VRE. Since we observed some variability in VRE colonization among different mice experiments and also, in a particular experiment, between different mice of the same group, we decided to check that the mice that were going to be analyzed behave as expected in terms of CR against VRE. This test must be done indirecly (we cannot test the level of CR in the mouse that will be analyzed because we do not want VRE to interfere with the metabolome and transcriptome). For this reason, we decided to co-house two mice per cage, one of this mouse will serve as a reporter of CR (it will be colonized with VRE) while the other one will supply the cecal samples for metatranscriptomic and metabolomic analysis. Mice are coprophagous and previous results from Ubeda *et al.* [112], have shown that mouse from the same cage recover a similar microbiota after antibiotic cessation.

To demonstrate that mice from the same cage will behave similarly in terms of CR capabilities against VRE we calculated the intraclass correlation coefficient (ICC) on the fecal VRE levels detected at day two post-infection in co-housed mice. Taking into account 25 cages of co-housed mice (vancomycin + PBS: n=11, vancomycin + bacterial mix: n=14), we demonstrated the agreement in the VRE colonization levels of co-housed mice (ICC = 0.91 [0.81,0.96], within group variance = 0.23, among group variance = 2.26) as depicted in figure 3.38 A.

On the other hand, we sequenced the 16s rRNA of a subset of these co-housed mice to check that they shared a similar microbiota. As expected, sample were grouped (i) according to the treatment received (vancomycin + PBS or vancomycin + bacterial mix) and (ii) by cage, indicating that those mice that were housed in the same cage recovered a similar microbiota (Figure 3.38 B).

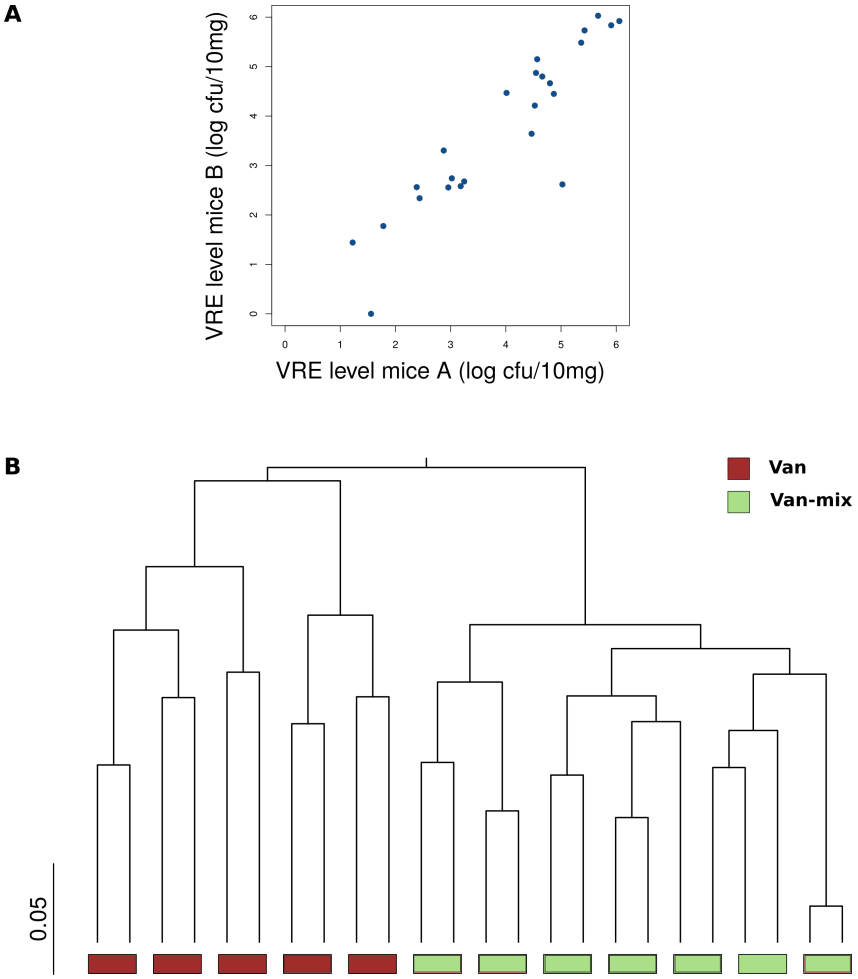


Figure 3.38: Co-housed mice share similar microbiota and sensibility to VRE colonization. (A) Comparison of VRE levels detected in co-housed mice. Representation of logarithms in base 10 of VRE count in feces at day 2 post-infection (cfu/10mg) for co-housed mice A and B. N=25. (B) Microbial community distance. Tree representation of the unweighted unifrac distance between samples of co-housed mice that received vancomycin (N=10) or vancomycin and the mix of bacteria (N=14). Each rectangle represent co-housed mice. Data was obtained from 2 experiments.

3.5.2 Selection of samples for the metatranscriptomic study

After verifying that (i) the administration of the selected bacteria in our vancomycin-treated mouse model confers protection against VRE colonization and (ii) co-housed mice present similar microbiota and levels of VRE colonization upon oral challenge with the pathogen, we performed an experiment to obtain the samples necessary to study the mechanism of protection. Briefly, mice were treated with vancomycin for one week, then after the antibiotic treatment cessation, pairs of mice were housed in the same cage. A group of pairs of mice received PBS while the other group received the bacterial mix. Two weeks after the antibiotic cessation, one mouse was sacrificed to obtain the cecal sample free of VRE while the other mouse from the cage was infected with VRE to check the level of CR.

The administration of the bacterial mix caused a significant diminution of VRE colonization, by 237 times at day two post infection (median mice recaiving the bacterial mix=497.5 and mice receiving PBS=117900 CFUs/10mg feces)(Figure 3.39 A). Seven days after the VRE challenge, the difference of colonization between both groups was even bigger, the median VRE level being 7351 times lower in the group that received the bacterial mix (median Vancomycin + mix=20.5 and Vancomycin + PBS =147400 CFUs/10mg feces). It is noteworthy that VRE was no longer detected in 6 out of the 12 mice that received the bacterial mix (Figure 3.39 B). We checked the VRE colonization by plating fecal samples to be able to follow the evolution of VRE levels until day 7. Then, we sacrificed the mice and plated also the cecal content on day 7 to verify we obtained similar results, since we will be using the cecal content instead of feces for the metabolomic and transcriptomic study (Figure 3.39 C). Similar VRE levels were detected both in feces and fecal contents in each group of mice. Then, we sequenced the 16sr-RNA to assess the microbiota composition of the samples and verify that the bacteria administrated could colonise the intestine. First, we assessed the shannon index in order to investigate if the administration of the bacterial mix increased the overall diversity (Figure 3.39 D). Notably, the bacterial diversity was similar in the two groups of treated mice, independently on the administration of the commensal bacteria, and lower in these two groups than the diversity of the untreated mice samples. Thus the administration of the commensal bacterial mix did not increase the overall diversity of the microbiota.

To vizualize the effect of the treatment and the administration of the bacterial mix on the bacterial composition of the mice included in the experiment, we performed a PCA on the unweighted unifrac distances (which takes into account the phylogenetic relations). The first axis separated the untreated mice from the treated ones. The second axis, however, separated the treated mice that received the bacterial mix from the ones that did not (Figure 3.39 E). Thus, as expected, the microbiota of mice that received the bacterial mix differs from those that received just PBS, and the bacterial mix did not restore the complete microbiota.

We then focused on the analysis of the commensal bacteria that we administered (*Barnesiella*, *Alistipes*, unc. ruminococcaceae and *Oscillibacter*). As shown in figure 3.40, and consistent with the results obtained in the previous experiment (3.35 A and B), *Barnesiella* and *Alistipes* levels were significantly increased by the administration of the bacterial mix. As expected, the increase in these two genera was due to the bacteria that we administered since a single OTU of each genus, with the same sequence as the isolate

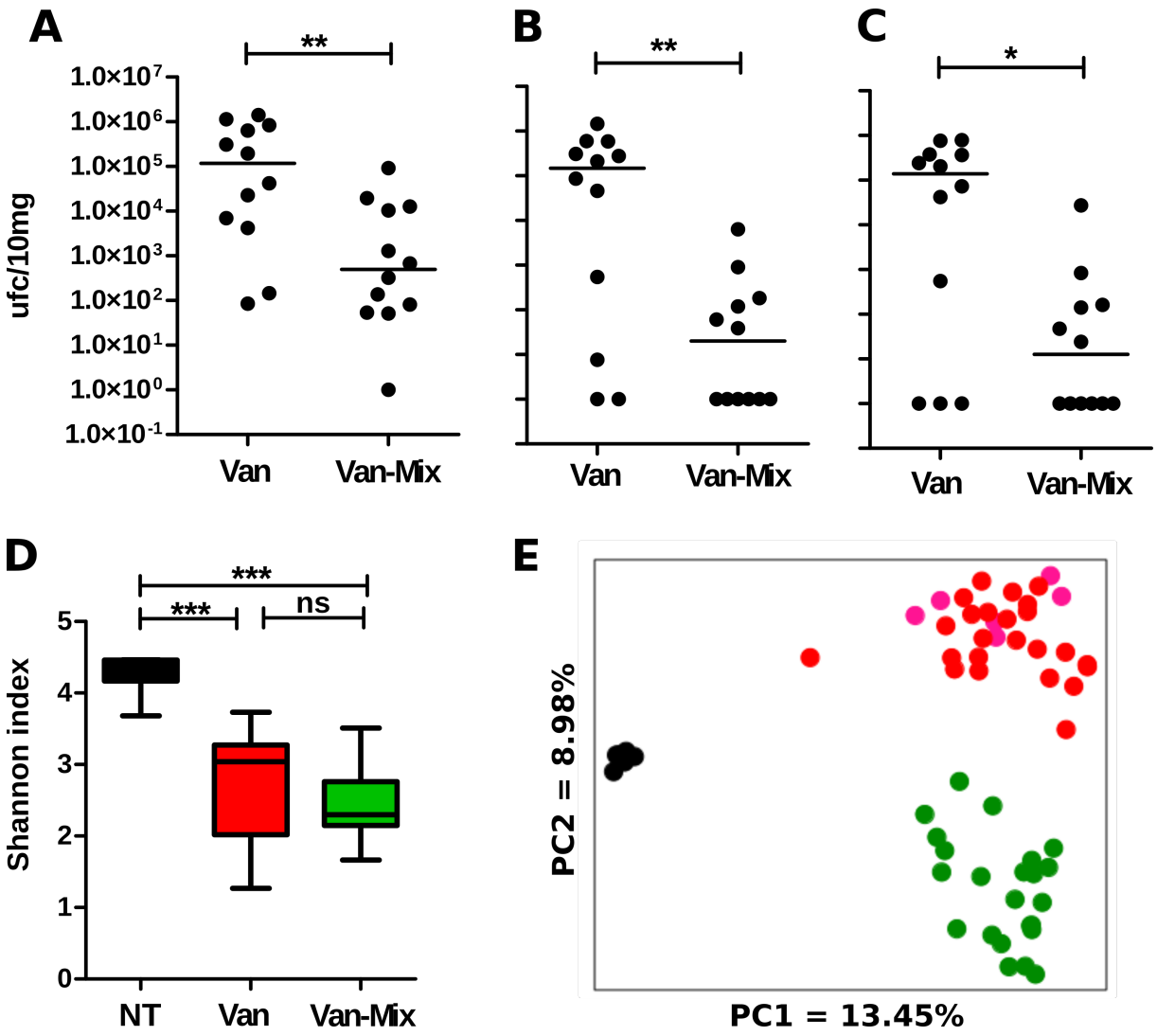


Figure 3.39: Characteristics of the samples collected to analyze by metabolomics and transcriptomics the effect of the protective bacteria. Mice were treated with vancomycin for one week, then one group received the protective bacterial mix for three days. The microbiota composition was determined two weeks after the withdrawal of the treatment, before infecting the mice orally with 10E6 CFUs VRE and determining the colonization levels. We used 12 cages with two co-housed mice for each group included in the experiment and 3 mice-pair for a control group to which we administered *Klebsiella* (sequenced : vancomycin treated (n=24), vancomycin-mix (n=24), untreated mice (n=6), control (n=6)). A: VRE levels in fecal samples at day 2 post-infection. B: VRE levels in fecal samples at day 7 post-infection. C: VRE levels in cecum samples at day 7 post-infection. D: Shannon index. E: PCoA of unweighted unifrac distances. black = untreated mice (NT), red = vancomycin, green = vancomycin-mix, pink = control, vancomycin-Klebsiella ns not significant, * p-value<0.05, ** p-value<0.01, *** p-value<0.001. Mann-Whitney U test. line represent the median.

we administered, was responsible for the increase observed in these two genera (Figure 3.40 B and C).

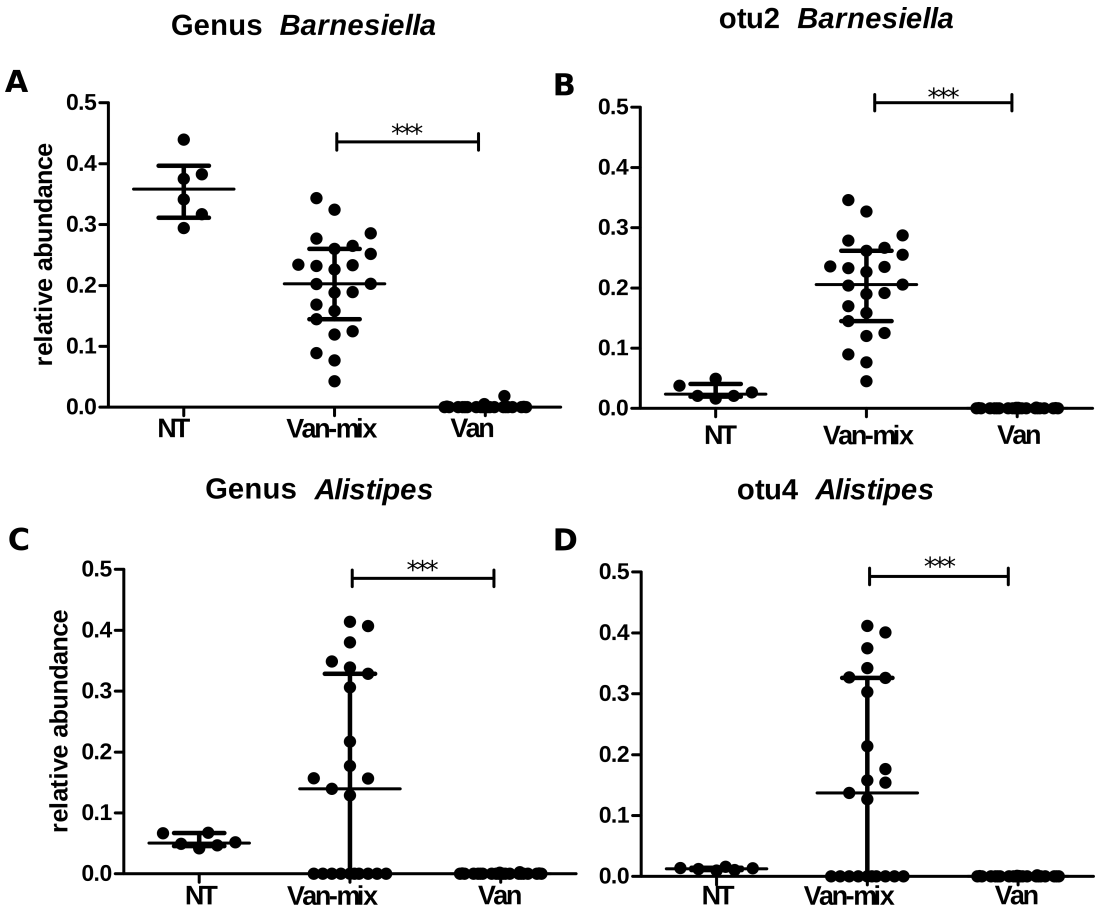


Figure 3.40: Levels of *Barnesiella* and *Alistipes* in fecal samples of mice that will be used for the metatranscriptomic and metabolomic studies. Mice were treated with vancomycin for one week, then one group received the protective bacterial mix for three days. The microbiota composition was determined two weeks after the withdrawal of the treatment, before infecting the mice orally with 10E6 CFUs VRE and determining the colonization levels. NT: untreated mice Van-mix: vancomycin treated mice that received the bacterial mix. Van: vancomycin treated mice that received PBS. ***: p-value<0.001, Mann-Whitney test

On the other hand, unclassified ruminococcaceae levels were similar in mice that were treated with vancomycin, independently if they received the bacterial mix or PBS instead (Figure 3.41 A). At the OTU level, however, as previously shown, the relative abundance of the specific unc. Ruminococacceae OTU that we administered, was found to be higher in mice that received the bacterial mix as compared to mice that received PBS, and did not differ from the levels identified in untreated mice (Figure 3.41 B). In the case of *Oscillibacter*, as previously shown (Figure 3.41 C), the levels were similar between the three groups (untreated mice, mice treated with vancomycin and those that received the bacterial mix after antibiotic treatment) both at the genus and the OTU levels (Figure 3.41 D). This last result indicates that in these particular mice, *Oscillibacter* may not

be relevant to explain the differences in CR observed among the different groups of mice analyzed.

In summary, as previously shown in the experiment shown in the figure 3.35, here also (i) the administration of the bacterial mix significantly diminished the capacity of VRE to colonize the gut and (ii) reconstituted the levels of the genera *Barnesiella* and *Alistipes* and the unc. ruminococcaceae OTU42.

After checking whether the bacteria administered were able to colonize the mice, we investigated further alterations of the microbiota that could be induced by the administration of the commensal bacteria. For this, we determined the bacteria whose levels were significantly different in the treated groups that received the bacterial mix or the PBS instead (Table 3.6). As expected, the bacteria most significantly increased in the group that received the bacterial mix were *Alistipes* and *Barnesiella*, the taxa that best colonized. Moreover, The genus *Olsenella* and some unclassified family were also increased. Interestingly, some genus were more abundant in the treated group that did not received the bacterial mix (Table 3.6).

Table 3.6: Taxa whose abundance is significantly different between the group of mice treated with vancomycin that received either PBS (van) or the protective bacterial mix (van-mix).

taxon	**fold change - increase in van-Mix group**	**adj.fdr.p.value**
Alistipes	442.93	6.73E-04
Barnesiella	166.51	7.69E-08
unclassified_Porphyromonadaceae	18.78	4.30E-08
Olsenella	3.35	3.52E-04
unclassified_Coriobacteriaceae	2.21	8.73E-03
unclassified_Sphingobacteriaceae	1.48	1.81E-02
taxon	**fold change - increase in van group**	**adj.fdr.p.value**
Enterococcus	5.66	6.90E-03
Anaeroplasma	5.35	1.81E-02
Escherichia/Shigella	4.30	1.92E-02
Lactobacillus	2.56	2.79E-03
Parasutterella	2.35	5.54E-03
unclassified_Lactobacillaceae	2.19	1.78E-02
unclassified_Lachnospiraceae	1.71	4.59E-02

Fold changes were calculated as the mean abundance of the taxa in vancomycin-treated mice after a two-week recovery, both in the group that received the bacterial mix or PBS. Bacteria administered are in bold. Wilcoxon test corrected with FDR. n=6 by group.

After verifying that the bacterial mix administered effectively diminished the colonization by VRE, we sought to understand the mechanism of the protective action associated to the administration of this bacterial mix. For that, we decided to analyze, using high throughput sequencing, the gut meta-transcriptome of treated mice that received the bacterial mix and compare it to the metatranscriptome from untreated mice or mice that were treated with vancomycin and received PBS instead of the bacterial mix. However, due to the high cost of this technique and in order to achieve a good coverage per sample, we decided to limit the number of samples analyzed to 6 per group. Representative samples from each group were selected as described in the section 2.13. The VRE levels and a

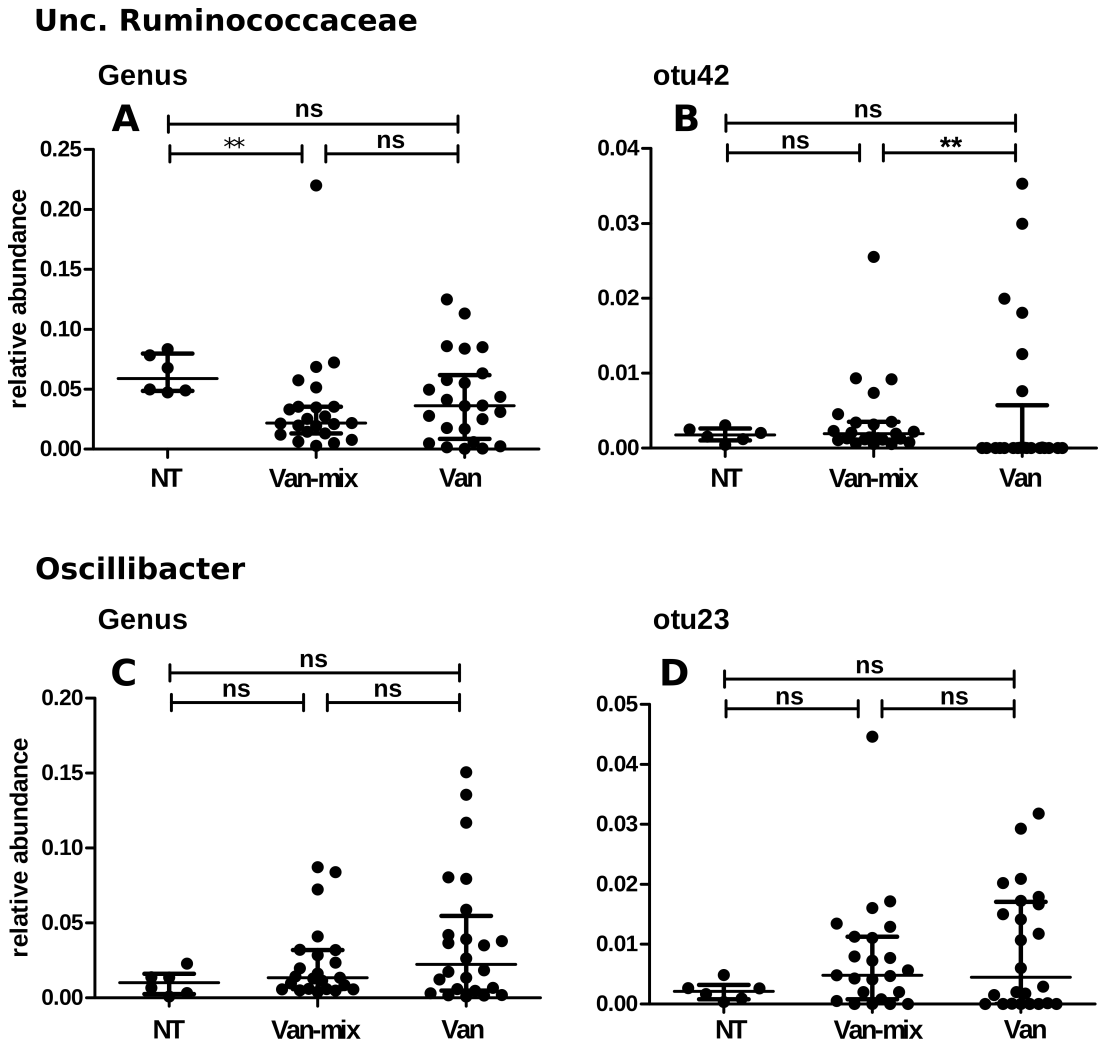


Figure 3.41: Levels of Unc. Ruminococacceae and *Oscillibacter* in fecal samples of mice that will be used for the metatranscriptomic and metabolomic studies. Mice were treated with vancomycin for one week, then one group received the protective bacterial mix for three days. The microbiota composition was determined two weeks after the withdrawal of the treatment, before infecting the mice orally with 10E6 CFUs VRE and determining the colonization levels. Unclassified ruminococcaceae(A-B). Relative abundance at genus (A) or OTU (B) level. Oscillibacter(C-D). Relative abundance at genus (C) or OTU (D) level. Relative abundance- ns: not significant, ** p-value<0.01, Mann-Whitney test.

PCA performed on the unweighted unifrac distance of the selected samples is represented on the figure 3.42.

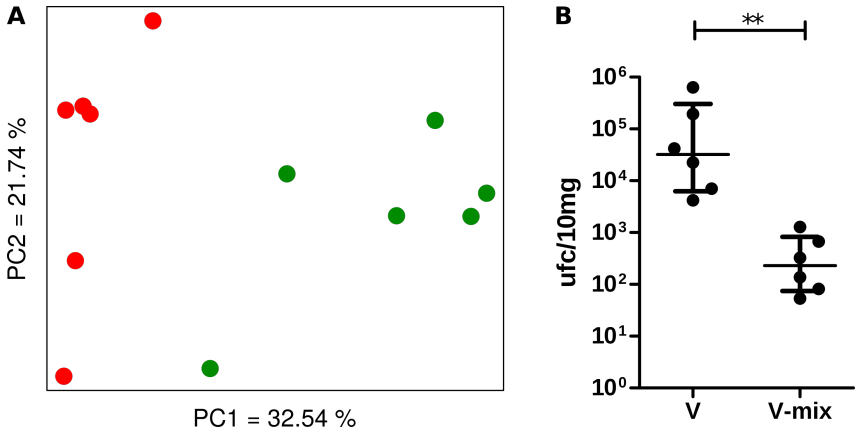


Figure 3.42: Samples selected for the metatranscriptomic study. Samples for the metatranscriptomic study were selected as mentioned in methods section 2.13. A) PCA on the weighted unifrac distance among samples that received vancomycin +PBS(red) or samples that received the vancomycin + bacterial mix (green) B) VRE level in fecal samples at day 2 post-infection of mice that received vancomycin +PBS(V) or the bacterial mix (V-mix) ** p-value<0.01, Mann-Whitney U test.

On the other hand, we also sequenced an untreated group of mice to assess the basal level of expression of the different microbiota genes.

3.5.3 Metatranscriptomic study

3.5.3.1 Segregation of samples according to their metatranscriptomic profile.

The samples obtained for the meta-transcriptomic study were processed as described in the section of material and methods 2.13. After processing the raw data, a database of the unique genes present in the samples was created and annotated. We obtained a total of 1.030.675 non redundant (nr) genes. In average, the untreated group presented 120.453 nr genes per samples, the group that received vancomycin treatment, 80.609, and the group that received the bacterial mix after antibiotic treatment had on average 93.696 nr genes per sample. 132.291 of the nr genes could be annotated using the KEGG database (HMMER e-value lower than 0.05). These genes can inform us on possible mechanisms by which the bacteria administered exert their protective effect.

To identify differences in the detected genes among different samples, we focused our study on the most prevalent ones (genes present in at least five out of six samples of one group of mice). Following this criteria, 74.658 nr genes were taken into account across the three groups, of which 13.798 were annotated in the KEGG database (HMMER e-value lower than 0.05).

As displayed on the PCoA (Figure 3.43 A), this sub-sampling allowed a clear separation of the untreated group from the other two ones (the three groups are different, adonis test with 1000 permutations, p.value< 0.001). A similar result was obtained taking into account only the genes annotated with the KEGG database (Figure 3.43 B and

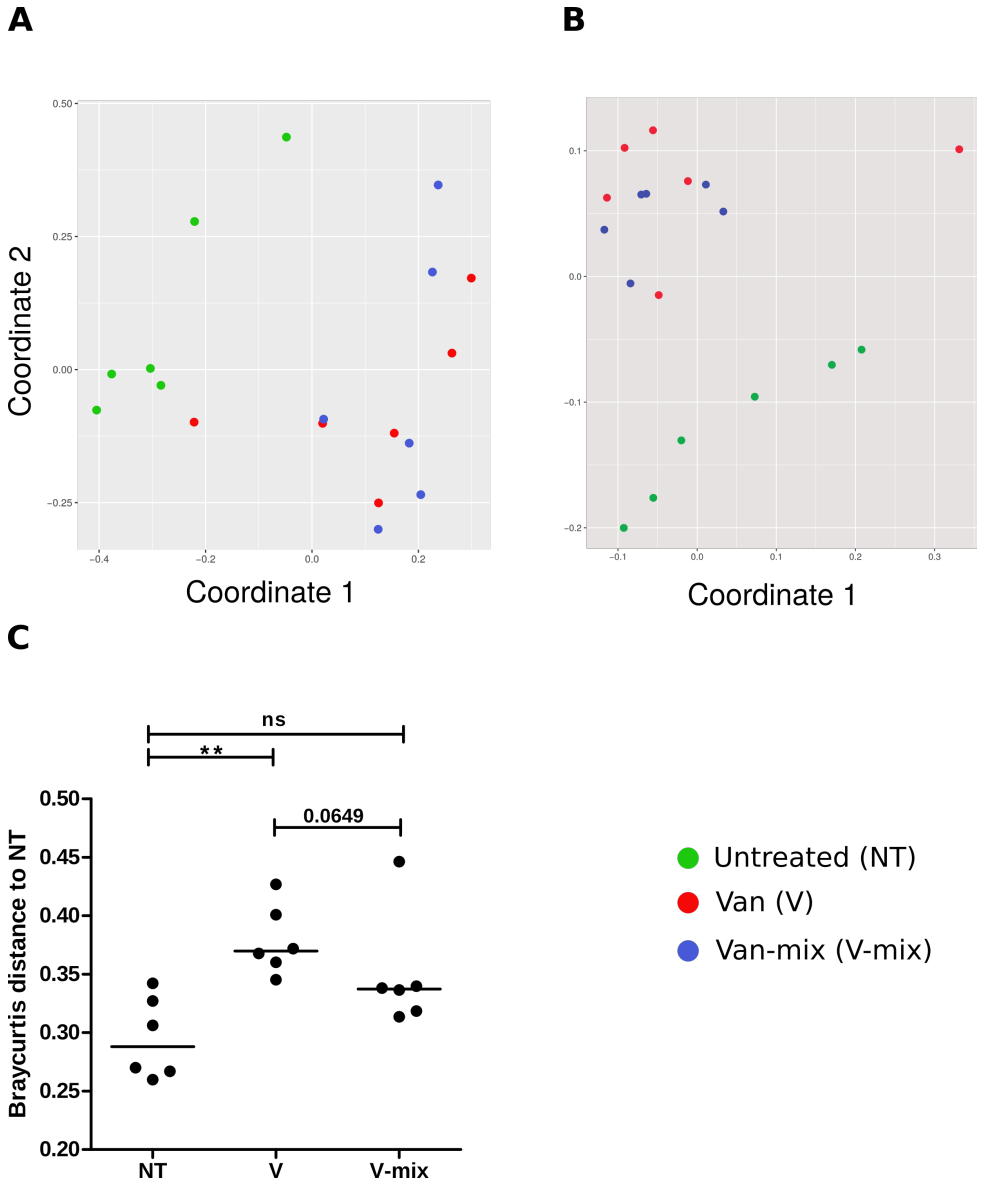


Figure 3.43: Separation of samples according to their metatranscriptomic profile. Mice were treated with vancomycin. After the antibiotic treatment withdrawal they received either PBS (Van) or the protective bacterial mix (Van-mix). Two-weeks after antibiotic cessation a cecal sample was collected and processed for metatranscriptomic studies. A group of untreated mice was used as control. (A) Principal coordinate analysis performed on the Braycurtis distance matrix of all the genes present in at least 5 out of 6 samples of a group. (B) Principal coordinate analysis performed on the Braycurtis distance matrix of genes that could be annotated with KEGG database and present in at least 5 out of 6 samples of a group. (C) Average Braycurtis distance to untreated samples. The distance comparing the untreated (NT) samples between them is shown for comparison. It is the baseline distance due to the intra-group variation. Mann-Whitney test. **: p-value<0.01, ns: not significant, values near the threshold of significance are written.

C) (the three groups are different, adonis test with 1000 permutations, p.value< 0.01). Interestingly, although the first two principal coordinates of the analysis did not separate the mice treated with antibiotics that reveived PBS from those that received the bacterial mix, the overall metatranscriptome was more similar between untreated mice and mice that received the bacterial mix, as compared to those that received PBS after antibiotic treatment (lower Bray-curtis distance values between untreated mice and mice that received the bacterial mix as compared to mice that received PBS)(Figure 3.43 C). This last result suggest that the bacterial mix could restore some of the bacterial functions lost after antibiotic administration.

Additionally, the Bray-curtis distance between the untreated mice group and both treated groups that received the bacterial mix or PBS is lower than 0.5 (Figure 3.43 C), indicating that these groups share an important part of the meta-transcriptome with the untreated mice (the Bray-curtis distance can take a value between 0 and 1, 0 indicating that samples are identical and one that the samples are totally different).

3.5.3.2 Effect of the bacteria administered on the metatranscriptomic profile.

As we have seen, the complete meta-transcriptome of the different groups share numerous functions (the Braycurtis distance to untreated mice is inferior to 1).

In order to study in more details possible transcriptome differences among the different groups of mice, we first focused on the genes expressed by each of the bacterial isolates administered. For that, we mapped the genes expressed in each sample to the genome of the bacteria administered, as described in material and methods (section 2.9 and 2.13.3).

As expected, the total number of expressed genes that match those encoded in the genome of the bacterial isolates administered was higher in the groups of mice treated with the antibiotics that received the bacterial isolates, as compared to those that just received PBS (Mann-Whitney test, p.value <0.01) (Figure 3.44 A and B). Notably, the number of genes expressed by the bacterial isolates in untreated mice was not significantly different from the treated mice that received the bacterial isolates (Figure 3.44 A and B). We next studied, separately, the level of expression of each particular isolate administered.

The number of genes expressed by *Barnesiella* and *Alistipes* isolates was drastically reduced after the vancomycin treatment and could be restored by the administration of the bacterial mix (Figure 3.44 C). On the opposite, the functional profile of *Oscillibacter* and unc. Ruminococcaceae is similar in the three groups (Figure 3.44 C). A higher number of functions corresponding to *Oscillibacter* were detected, which could be explained by the bigger size of its genome (4068 ORFs detected whereas the genome of *Barnesiella*, *Alistipes* and the unclassified Ruminococacceae contain 2796, 1994 and 1867 ORFs respectively). Unc. Ruminococcaceae presented the lowest number of genes expressed. In total, only 73% and 43% of the genes encoded by of *Oscillibacter* and unc. Ruminococcaceae were detected to be expressed accross all the samples whereas 87% and 88% of the genes encoded by *Barnesiella* and *Alistipes* were detected to be expressed. It is possible that in reason of the lower abundance of *Oscillibacter* and unc. Ruminococcaceae, only their highest expressed functions could be detected. Indeed, among the samples included in the metatranscriptomic study, *Oscillibacter* represented at maximum the 4.5% of the microbiota of a sample, unc. Ruminococcaceae the 3.0% whereas *Barnesiella* and *Alistipes* represented more than 30%.

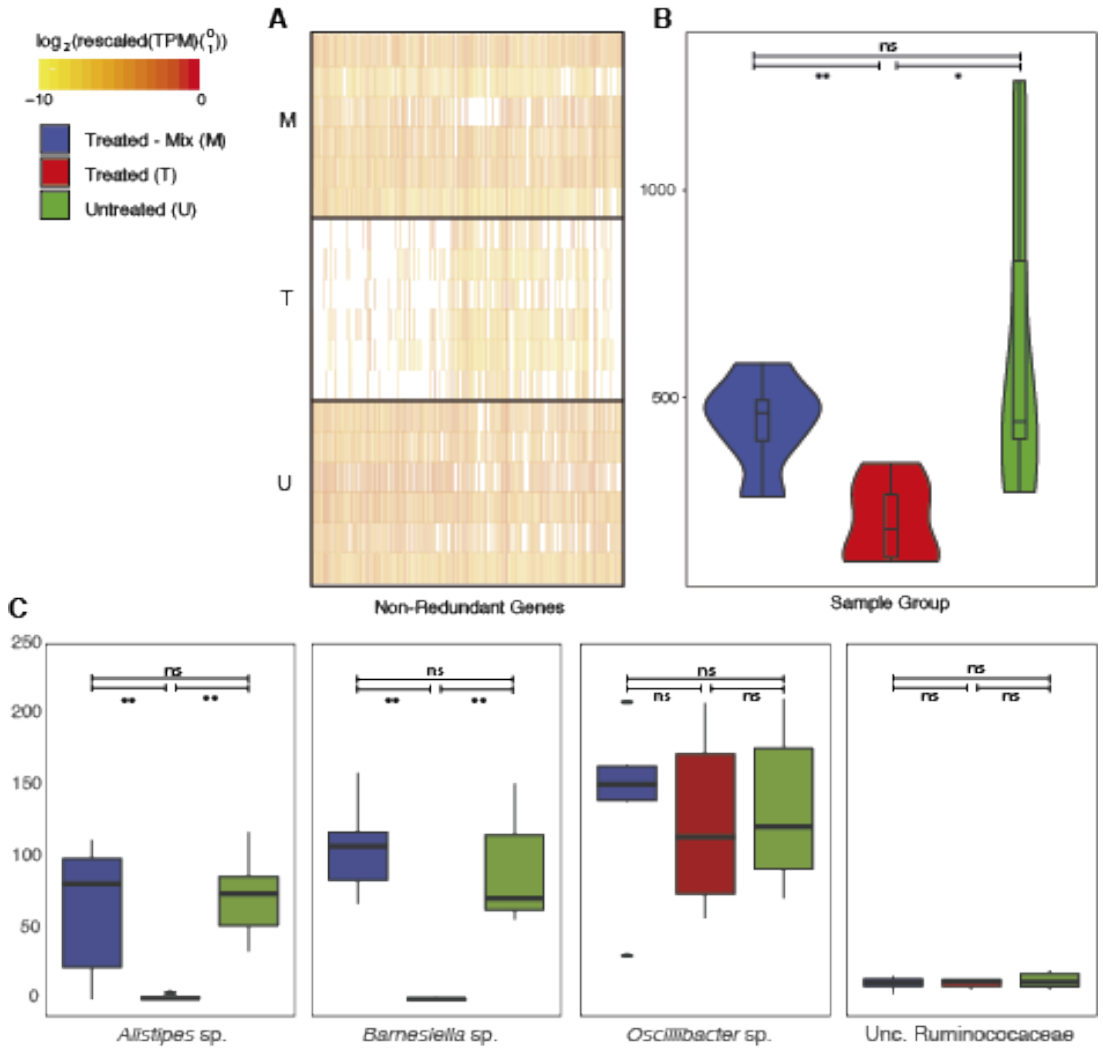


Figure 3.44: Separation of samples according to their metatranscriptomic profile. Mice were treated with vancomycin. After the antibiotic treatment withdrawal they received either PBS (Van) or the protective bacterial mix (Van-mix). Two-weeks after antibiotic cessation a cecal sample was collected and processed for metatranscriptomic studies. A group of untreated mice (Untreated) was also analyzed. (A) Level of expression of bacterial genes in cecal samples from mice. Only the non-redundant genes codified by the bacteria administered were taken into account. (B) number of genes codified by the bacteria administered that were found to be expressed in the cecal samples analyzed. (C) boxplot representing the number of genes expressed by the bacteria specified in each group of samples. For B and C, boxes represent the interquartile range with the IC95, and the line represent the media.

Thus, the probiotics administered restore the expression of specific nr genes. The restoration of the genes depended mainly of the isolates belonging to the genus *Barnesiella* and *Alistipes*. In contrast, the isolates belonging to the taxa *Oscillibacter* and the unc. Ruminococcaceae do not seem to exert an important influence on the gene expression profile of the different groups.

3.5.3.3 Bacterial functions that differentiate the group of mice that received the bacterial mix compare to those that received PBS.

In an attempt to identify mechanisms by which the inoculated bacteria could confer colonization resistance to VRE, we compared the specific functions identified in the metatranscriptome of the group of mice treated with antibiotics with the ones identified in the group of mice that besides antibiotic treatment was inoculated with the protective bacteria. Although the most likely scenario is that a function expressed by an administered protective bacteria is key for conferring protection, it is possible that the inoculated bacteria will influence on the expression of other commensal bacteria which will promote resistance. Moreover, as we described in 3.5.2, the abundance of others bacteria was also alterered upon the administration of the bacterial mix. For this reason, we expand our metatranscriptomic analysis to all the genes identified in the metatranscriptome, irrespectively if they were encoded by the inoculated bacteria.

For this analysis also, we considered only the prevalent genes (those found to be expressed in at least five out of the six samples in at least one group). Applying this parameter, we take into account only the functions that are really representative of each group of mice.

From the selected genes, we determined the ones that were significantly altered comparing the treated group respect to both the untreated group and the mice treated with antibiotics that received the protective bacteria (FDR adjusted p.value<0.1). From a total of 74658 genes analyzed, 11271 were significantly different between these groups of mice. Only 167 nr genes were consistently increased both in the untreated group and the group that received the bacterial mix, as compared to the treated mice that received PBS (wilcoxon test p value<0.05, FDR adjusted p.value<0.1).

Of this 167 nr genes, 33 could be annotated with the KEGG database to infer their functions (as described in 2.13.3), Doing so, some unique genes mapped to the same KO (Kegg Orthology). Thus, we summed their TPM and finally obtained 28 KOs that were not present in mice treated with vancomycin that received PBS but were expressed both in untreated mice and in the mice that received the bacterial mix (Figure 3.45).

Of all the KOs identified, four are implicated in the bacterial chemotaxis. They were annotated as a chemotaxis system K03407 and K03408; cheA-cheW), a serine sensor receptor (K05874; tsr), an aerotaxis receptor (K03778; aer) and the flagellin (K02406; fliC).

In addition, three genes were identified to be involved in the membrane transport belonged to the ABC transporters family (K10188; LacE for the acquisition of the carbohydrates lactose/L-arabinose; K10240; CebE for the acquisition of the carbohydrate cellobiose and K17208; IbpA for the the acquisition of myo-Inositol) and two genes belong to the phospho-transferase system (PTS) (K02745; AgaV and K17466; DgaC that are implicated in the assimilation of the carbohydrates N-Acetyl-galactosamine and D-

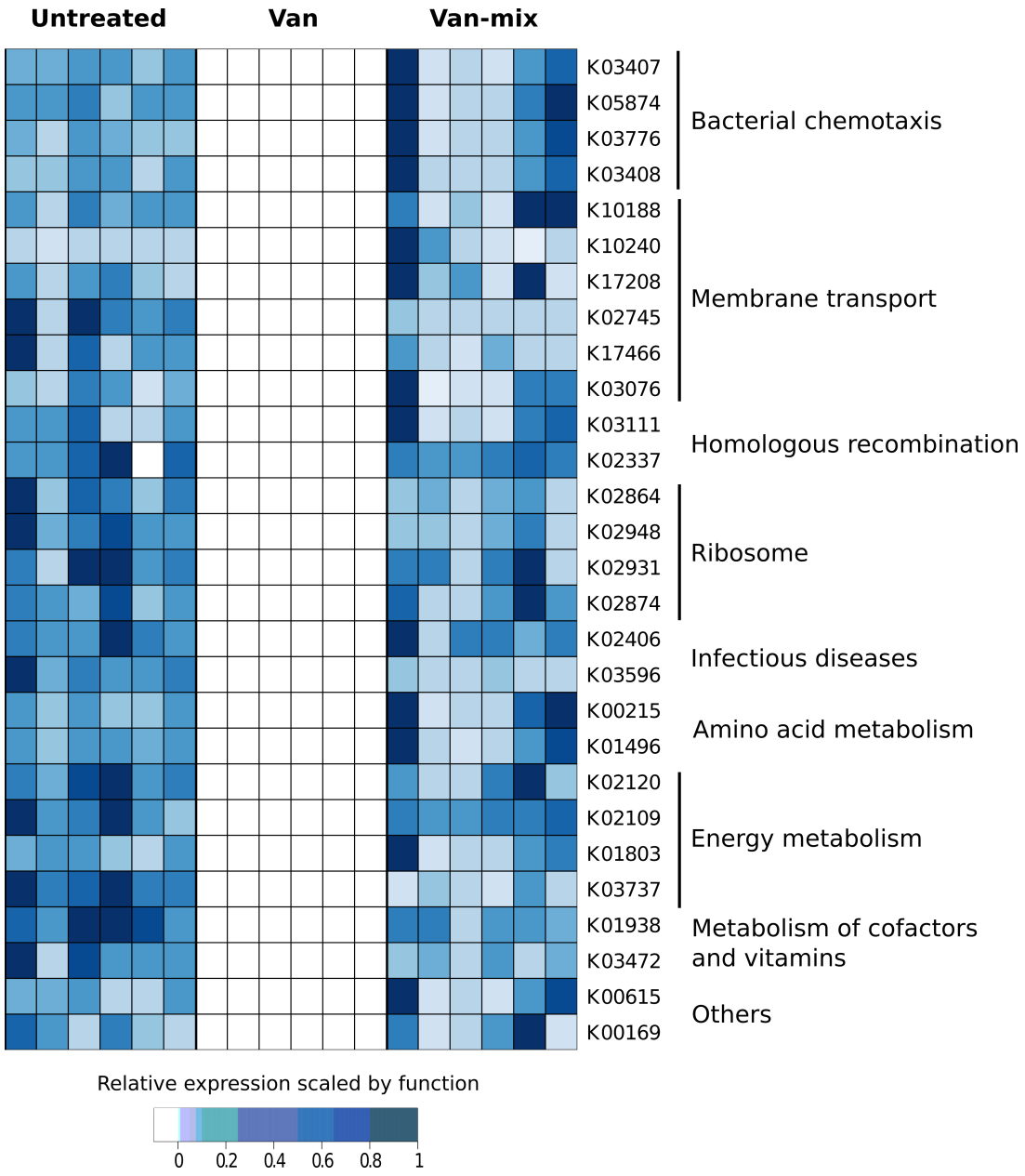


Figure 3.45: Bacterial KOs whose expression, lost after vancomycin treatment, was recovered upon the inoculation of the protective bacteria. Mice were treated with vancomycin for one week, one group received a bacterial mix for three days, while the other group received PBS. The cecal samples used to perform the metatranscriptome analysis were collected two weeks after the withdrawal of the treatment. Heatmap representing the expression of the functions whose expression was significantly different in the treated group respect to Only the nr genes whose expression was altered by the vancomycin treatment and could be restored by the administration of the bacterial mix are shown. Theses genes were mapped to the KEGG database using HMMSearch as described in the section 2.13.3. The TPM of the nr genes that mapped to the same KO were combined (the differences were still signifiant, adjusted p.value<0.01). In order to facilitate the comparison of the expression of the different functions between groups, for each function, the data was normalized between 0 and 1, being 0 the least expressed sample and 1 the most expressed one.

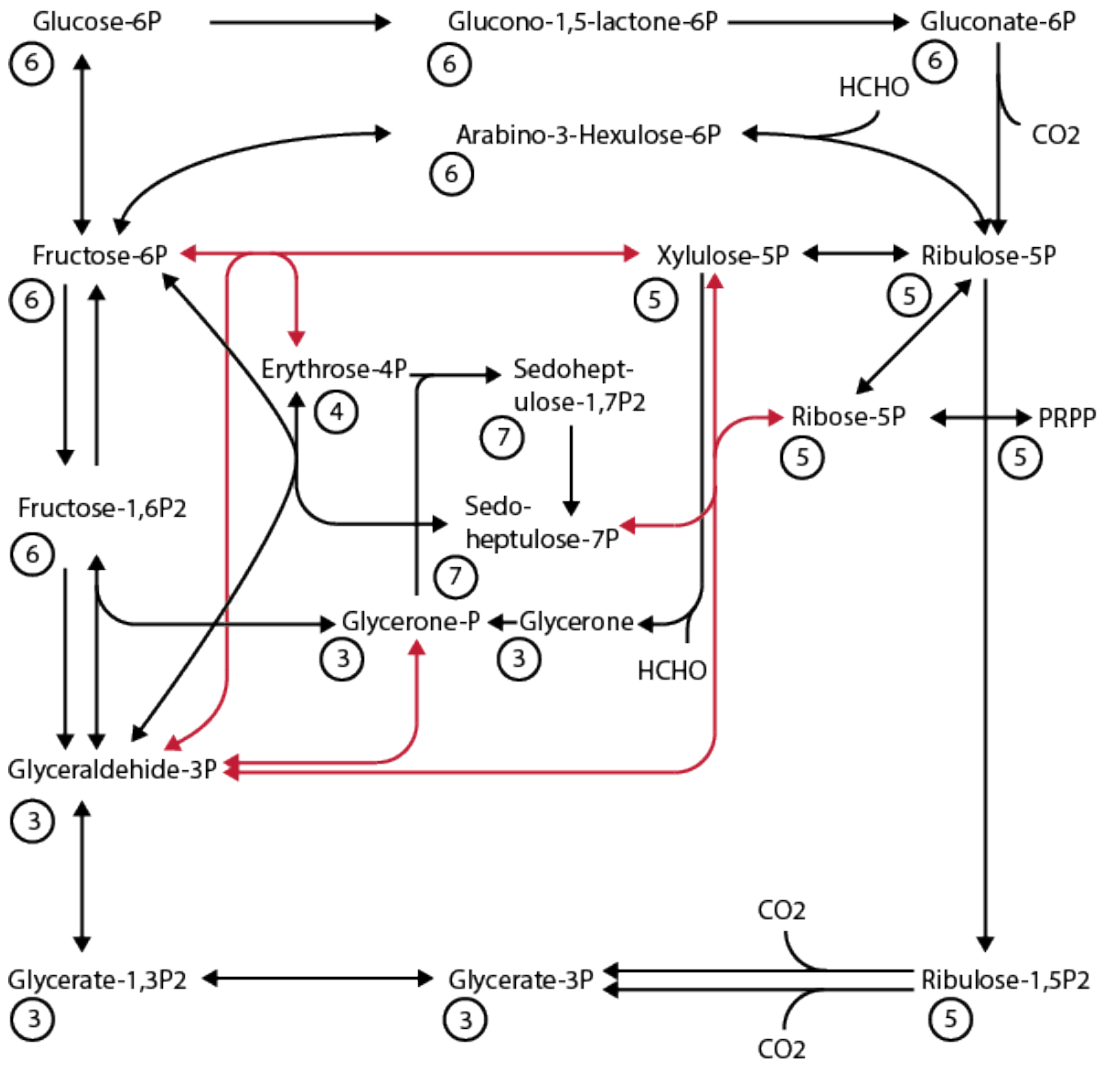


Figure 3.46: Functions implicated in the carbon metabolism lost after vancomycin treatment and restored upon administration of the bacterial mix. Subgraph of the KEGG carbon metabolism pathway. The functions that were significantly increased in the group that received the bacterial mix in comparison with the treated group that received PBS are indicated in red (K00169, K00615, K01803, K01938, K03737).

Glucosaminate or N-Acetyl-glucosamine) [256]. Moreover, one KO corresponding to the bacterial secretion system type 2 was also increased (K03076; secY).

Interestingly, we also detected an increase in different KOs related to the metabolism of carbohydrates (Figure 3.46). These functions are mainly related to the pentose phosphate pathway (PPP), implicated in the metabolization of different saccharides.

Two of the KOs over-expressed represent important steps related with the synthesis of the histidine (K01496) and O-lysine (K00215). Other proteins detected are implicated in the homologous recombination or are part of the ribosome. Two KOs were also implicated in the metabolism of cofactors and vitamins, specifically, of the vitamin B6 (K03472) and of folate (K01938).

On the other hand, the proteins porA (K00169) and por (K03737), which were found to be absent in treated mice and recovered in mice that received the protective bacteria, are implicated in the butanoate and the propanoate metabolism.

In summary, several KOs, which could be involved in the colonization resistance against VRE (e.g. transporters of carbohydrates that could diminish the level of specific nutrients for VRE) were recovered upon inoculation of the protective bacteria.

3.5.4 Metabolomic study

Taking into account that several bacterial funtions were recovered upon commensal bacterial inoculation, we next decided to study how these functions could modify the gut metabolite landscape which could affect VRE intestinal colonization (e.g. depletion of specific sugars require for the growth of VRE or increase in inhibitory molecules). For this reason, we performed a metabolomic study using the cecum filtrate of the mice analyzed by RNA-seq. This analysis could allow us to determine the levels of nutrients (e.g. carbonhydrates) or inhibitory substances (e.g. SCFA) that may be involved in the protection against VRE colonization. In the present metabolomic study, performed by nuclear magnetic resonance (NMR), the most difficult step is the assignation of metabolites to each peaks given by the NMR spectra. For this reason, a statistic analysis is first performed on the spectra to determine which peaks are different between the groups. Then, only the selected peaks are further studied for the identification of the metabolite that they represent. These steps were performed as described in the section of material and methods 2.11. Following, we will describe the identified peaks and assigned metabolites, whose abundance differ between mice treated with antibiotics and those that in addition received the protective bacteria.

3.5.4.1 Carbohydrates, end products of fermentation and bile acids

When analyzing the metatranscriptomic profile, we have already seen differences in the expression of genes implicated in the carbohydrates uptake and metabolism. Thus, we first focused our attention on the concentration in carbohydrates in the mice cecum. Most of the RMN spectra present in the region corresponding to the oligosaccharides (3.4-4.25ppm) could not be attributed to specific carbohydrates (Figure 3.47). However, there is a visible increase of this region in the treated group, which suggest an increase in oligossacharides available after the administration of the antibiotic, as documented by Zhao *et al.* [257]. We cannot discard the presence of additional signals corresponding to

other molecules in this region, thus the integration of the corresponding area would be erroneous.

Interestingly, it was possible to identify one of the carbohydrates, the cellobiose (Figure 3.48 A). Different peaks were attributed to the cellobiose. Although the relative abundance was not similar in all the cases, its concentration was the highest in the treated group and the lowest in the untreated group. The group that received the bacterial mix (treated-mix), presented intermediate levels. This result is consistent with an increase in available oligosaccharides in the treated group and a diminution in the group that received the bacterial mix.

Secondly, we observed an increase in phenol, a product of the fermentation of aromatic amino acids, in the untreated group and the treated group that received the bacterial mix as compared to the treated group that received PBS (Figure 3.48 B).

Additionally, we also detected that the total amount of bile acids was lower upon the treatment and partially restored in the group that received the bacterial mix (Figure 3.48 C). Unfortunately, it was not possible to distinguish between primary and secondary bile acids, which could influence differently the intestinal colonization by pathogens.

The fermentation of the carbohydrates and of the amino acids results in the production of SCFAs. Thus, a higher level of oligosaccharides in the intestine, not fermented by the microbiota, should result in a decrease in the concentration of SCFAs. Concordant with the increase in oligosaccharides that was detected in the mice treated with vancomycin, the relative abundance of most of the SCFAs was decreased in this group (Figure 3.48 D). The normally high abundant SCFAs butyrate and propionate were diminished upon the antibiotic treatment, as well as the less abundant SCFAs 2-methylbutyrate and valerate.

On the opposite, the group of mice treated with vancomycin presented the highest level of lactate, probably related with the increase of the lactate producer *Lactobacillus*.

Importantly, the administration of the bacterial mix restored partially the relative abundance of the different SCFAs, decreasing the relative abundance of lactate and increasing the relative abundance of the other ones.

3.5.4.2 Amino acids and metabolites containing nitrogen

The NMR allowed the relative quantification of numerous amino acids (Figure 3.49). The relative abundance of most of these aminoacids was increased in the group of mice treated with vancomycin (i.e proline, leucine, serine, threonine) (Figure 3.49 A) and the relative abundance of one aminoacid, the phenylalanine, was decreased (Figure 3.49 B). Inoculation of the bacterial mix restored partially the levels of these aminoacids.

Finally, the relative abundance of trimethylamine was lower during the treatment and the administration of the bacterial mix restored the levels observed in the untreated group (Figure 3.49 C).

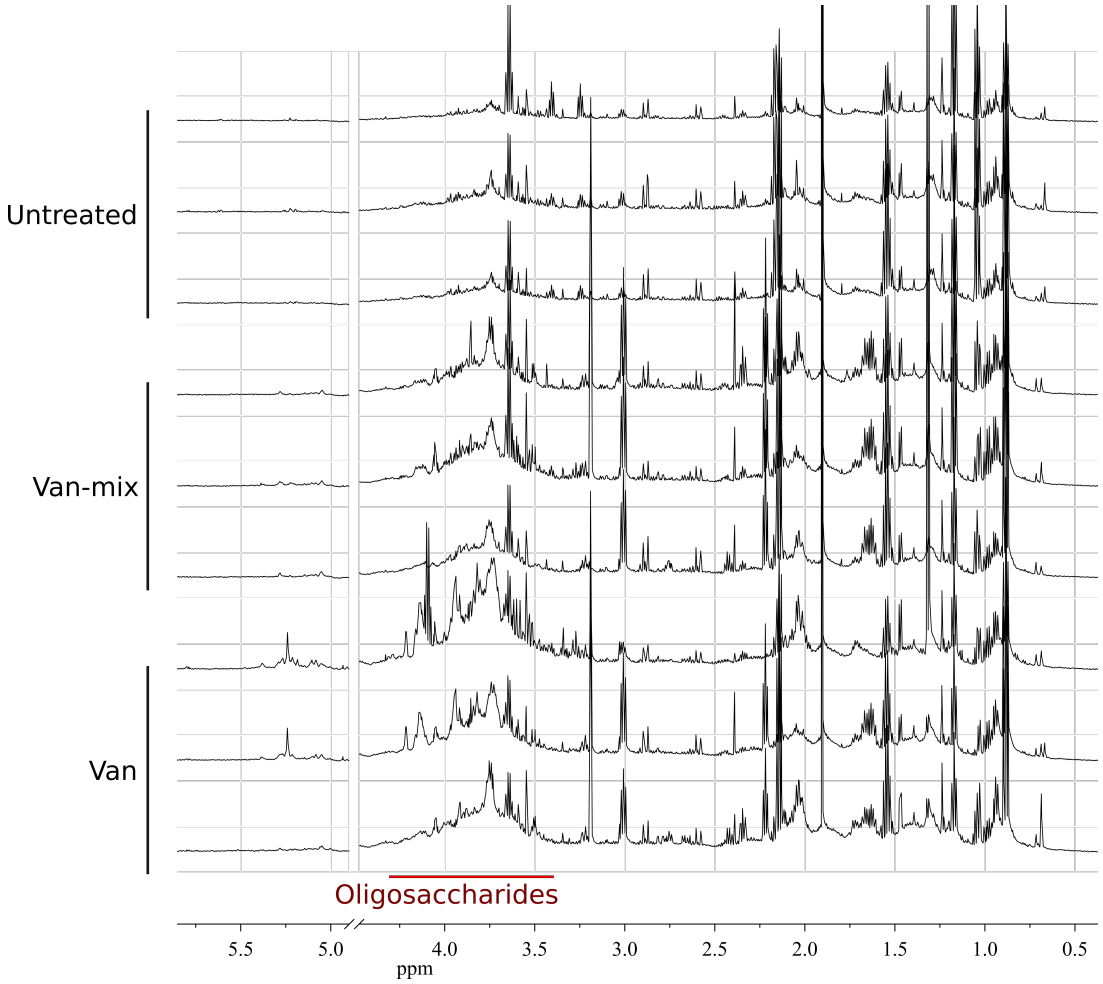


Figure 3.47: The region of the RMN spectra corresponding to the oligosaccharides present different patterns in the three analyzed groups. Mice were treated with vancomycin for one week (Van), then one group received a bacterial mix for three days (Van-Mix). The cecal samples used to perform the metabolome study were collected two weeks after the withdrawal of the treatment. To be able to assess the level of the metabolites in healthy conditions, the cecal samples of mice without treatment (Untreated) that were accomodated in the same conditions and for the same period as the two other groups of mice were also collected. RMN spectra of three representative samples by group. The region corresponding to the oligosaccharides is indicated in red.

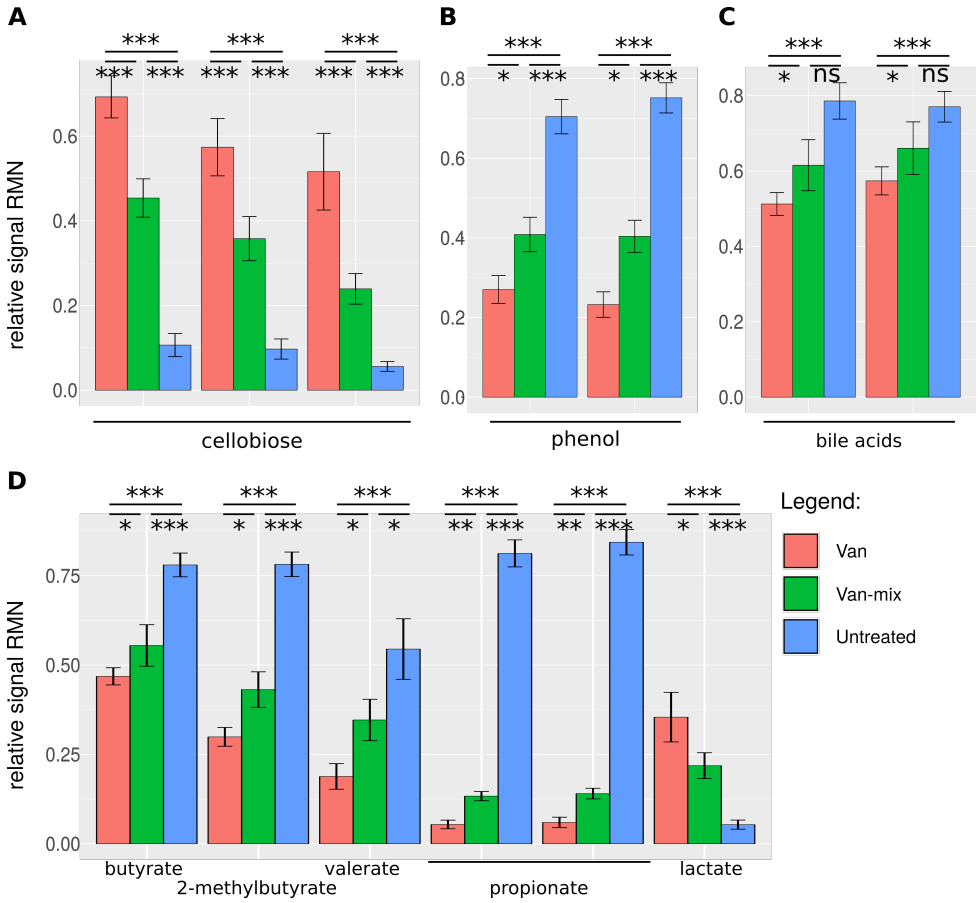


Figure 3.48: Relative abundance of metabolites in the cecum of untreated mice and treated mice that received either PBS or the bacterial mix. Mice were treated with vancomycin for one week (Van), then one group received a bacterial mix for three days (Van-mix). The cecal samples used to perform the metabolome study were collected two weeks after the withdrawal of the treatment. To be able to assess the level of the metabolites in healthy conditions, the cecal samples of mice without treatment (Untreated) that were accomodated in the same conditions and for the same period as the two other groups of mice were also collected. (A) Cellobiose. (B) Phenol. (C) Bile acids. It was not possible to distinguish between primary and secondary bile acids. (D) Short chain fatty acid (SCFAs). Relative abundance of butyrate, 2-methylbutyrate, valerate, propionate and lactate. When several peaks corresponding to a unique metabolite were identified, the result corresponding to the different peaks are represented. The relative abundance of the different metabolites was determined by NMR, and was normalized between 0 and 1 for the representation, being 0 the least expressed sample and 1 the most expressed one. Only the metabolites whose abundance differed between the Van group and the Van-Mix are represented (wilcoxon p.value<0.05, adjusted p.value<0.1). Wilcoxon test, ns not significant, * p.value<0.05, ** p.value<0.01, *** p.value<0.001.

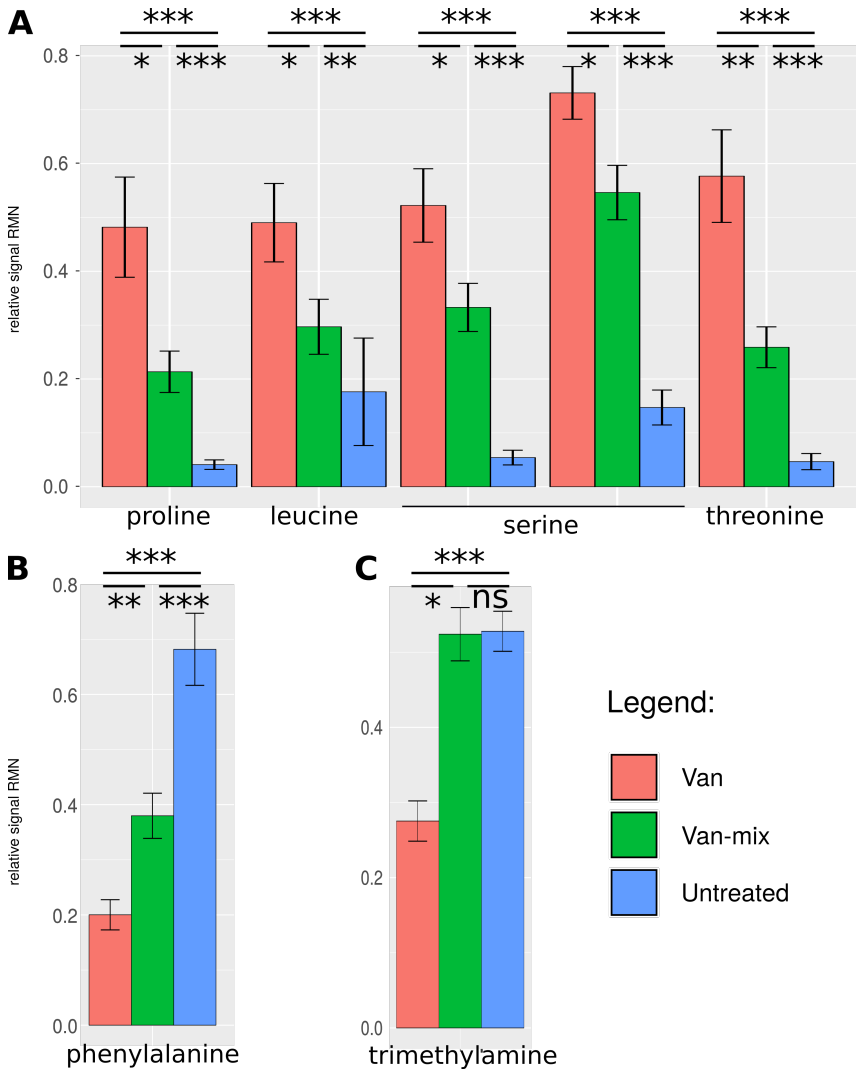


Figure 3.49: Relative abundance of metabolites with nitrogen in the cecum of untreated mice and treated mice that received either PBS or the bacterial mix. Mice were treated with vancomycin for one week (Van), then one group received a bacterial mix for three days (Van-Mix). The cecal samples used to perform the metabolome study were collected two weeks after the withdrawal of the treatment. To be able to assess the level of the metabolites in healthy conditions, the cecal samples of mice without treatment (Untreated) that were accomodated in the same conditions and for the same period as the two other groups of mice were also collected. (A) Relative abundance of the amino acids increased in the group of mice treated with vancomycin. (B) Relative abundance of the amino acid decrease in the group of mice treated with vancomycin (c) Relative abundance of trimethylamine. When several peaks corresponding to a unique metabolite were identified, the result corresponding to the different peaks are represented. The relative abundance of the different metabolites was determined by NMR, and was normalized between 0 and 1 for the representation, being 0 the least expressed sample and 1 the most expressed one. Only the metabolites whose abundance differed between the Van group and the Van-Mix are represented (wilcoxon p.value<0.05, adjusted p.value<0.1). Wilcoxon test, ns not significant, * p.value<0.05, ** p.value<0.01, *** p.value<0.001.

3.6 Study of the transcriptome of VRE *in vivo*

The previous results have demonstrate that the inoculation of the protective bacteria restore some bacterial functions lost upon vancomycin treatment and subsequently alter the metabolic landscape of the murine intestinal tract. These results have suggested possible mechanisms by which the protective bacteria could confer resistance (e.g. depletion of specific carbohydrates or amino acids). In order to identify mechanisms of protection, besides understanding which genes are expressed by the commensals, it will be important to study the genes expressed by the pathogen *in vivo*, which may give us some clues about how the commensal counteract the ability of the pathogen to colonize the gut.

To determine which genes are expressed by VRE during the intestinal colonization, we decided to sequence its transcriptome *in vivo*. The first idea was to sequence the transcriptome of VRE grown in the same conditions as when we tested the probiotics, that is to say, infecting treated mice that were first allowed to recover for two weeks after vancomycin treatment. However, VRE represents a very low fraction of the microbiota in this condition and the RNA quantity from VRE that could be retrieved would not be sufficient to sequence its transcriptome. For this reason, we used as an approximation the mice model without recovery, administering VRE while the mice are being treated with vancomycin. In this case, VRE represents more than one percent of the microbiota. Therefore, we could sequence the meta-transcriptome of cecal samples from mice colonized with VRE, and determine the specific VRE transcriptome retrieving the sequences that match with its genome.

Although we were going to analyze three samples from mice colonized with VRE, only one sample gave enough RNA quantity for sequencing. Therefore, the results described in this section were originated from a single mouse colonized with VRE. Replicates should be done in order to confirm the results that we present here. The sequences were analyzed and mapped as described in the section 2.14. The analyzed sample contained 14.206.970 sequences, of which 1.178.624 (8.3%) mapped to the VRE genome.

2718 out of the 2827 ORFs encoded in the VRE genome were found to be expressed *in vivo* and only 789 could be annotated with KEGG database, which represents 526 unique KOs.

Among the one percent most expressed genes by VRE *in vivo*, only 6 could be annotated with KEGG database. Interestingly, one of these gene codify for a D-Ala-D-Ala dipeptidase (K08641, vanX) implicated in the resistance to vancomycin. The cecal sample analysed was collected from a mouse under vancomycin treatment to promote the expansion of the pathogen and sequence its transcriptome. The high expression of a gene of resistance to vancomycin indicate that the approximation used to determine the VRE transcriptome *in vivo* was effective. The others genes codify for a superoxide dismutase (K04564), a protein of the ribosome (K02970) and three enzymes implicated in carbon metabolism (i.e. glyoxylate reductase (K00015), aspartate carbamoyltransferase (K00608) and ribose-5-phosphate isomerase B (K01808)). This last enzyme is part of the pentose phosphate pathway, which is recovered upon the administration of the bacterial mix (Figure 3.46).

As we have previously showned that the protective bacteria administered may be competing for the use of specific oligosaccharides, we determined which PTS transporters, ABC transporters and two-component systems VRE expresses *in vivo*. This is a first

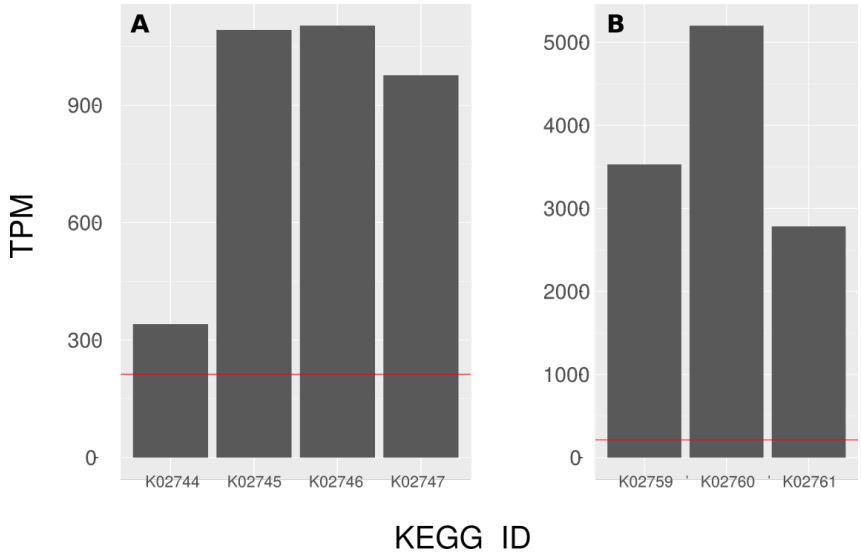


Figure 3.50: VRE *in vivo* expression of the PTS system subunits specific for the N-acetylgalactosamine and the cellobiose internalization. (A) Expression of the PTS system, N-acetylgalactosamine-specific. K02744: PTS-Aga-EIIA; K02745: PTS-Aga-EIIB; K02746: PTS-Aga-EIIC; K02747: PTS-Aga-EIID. (B) Expression of the PTS system, cellobiose-specific. K02759 PTS-Cel-EIIA; K02760 PTS-Cel-EIIB; K02761 PTS-Cel-EIIC. Both transporters are highly expressed. For comparison the median TPM value of all the genes annotated with KEGG in VRE is indicated with a red line (213 TPM).

approximation to determine which saccharides VRE is using *in vivo*. VRE was expressing 13 KOs of a two-component system, two of which were implicated in the internalization of malate (K00027, ME2 and K11616, maeN). In the category ABC transporter, VRE expressed 8 KOs. They were mainly implicated in the internalization of metals (K02012, afuA and K02015, ABC.FEV.P for the iron, K02008, cbiQ, for the cobalt and K02020, modA, for the molybdate). Moreover, one ABC transporter was implicated in the absorption of the biotin (K03523, bioY) and two for the absorption of amino acids (K01998, livM for the branched-chain amino acids and K02001, proW, a glycine betaine/proline transporter). For the internalization of amino acids, VRE also expressed an arginine/ornithine permease (K02205 rocE).

In the category of the PTS transporters, VRE expressed 13 different KOs. Interestingly, the complete systems for the internalization of N-acetylgalactosamine (4 subunits) and cellobiose (3 subunits) were expressed (Figure 3.50).

3.7 Determination of the nutritional requirements of VRE

The results obtained above indicate that the protective commensal bacteria can restore specific bacterial functions involved in the uptake of sugars including N-acetylgalactosamine or cellobiose. Moreover, we were able to demonstrate that the expression of this functions impact the metabolites and nutrients available in the intestinal tract (i.e. depletion of

cellobiose). In addition, we have shown that VRE highly express *in vivo* PTS systems involved in the uptake of similar sugars (i.e. cellobiose and N-acetyl galactosamine) suggesting that these sugars may be utilized by VRE as carbon source and its reduction after administration of the protective bacteria could impede VRE intestinal colonization.

To further explore the hypothesis of a nutritional competition between the bacteria administered and VRE as a mechanism by which commensal bacteria confer protection, we determined on which nutrients VRE could grow most efficiently in anaerobic conditions. For this, we determined the growth of VRE in minimal medium complemented with a unique source of carbon as described in material and methods section 2.15.

3.7.1 Verification of anaerobic condition

To determine the capacity of VRE ATCC700221 to grow on different nutrients in anaerobic conditions, we used Biolog plates PM1 and PM2a (carbon sources), which are 96 well plates where each well contains a different and unique source of carbon. This plates can be incubated in a Biolog OmniLog phenotype microarray system, which allow the analysis of numerous plates at the same time.

As we did not have a Biolog OmniLog phenotype microarray system in the laboratory, the device usually used for this kind of experiments, we measured the bacterial growth in a plate reader Tecan Infinite F200, which allow the incubation at a controlled temperature and the measure of the absorbance of a unique plate.

In contrast with the Biolog system, the space where the array is introduced is smaller, it is then impossible to enclose the array in a plastic bag with an anaerobic atmosphere generator. Therefore, in order to maintain an anaerobic atmosphere, we used the method applied in the API strip from Biomérieux : we covered the liquid medium with sterile mineral oil. To check the maintenance of the anaerobic condition, we grew a strictly anaerobic bacteria, *Barnesiella* (Figure 3.51). *Barnesiella* was able to growth in the described conditions, demonstrating that we could utilize the microarrays from Biolog in anaerobic conditions using our Tecan instrument to measure the absorbance over time.

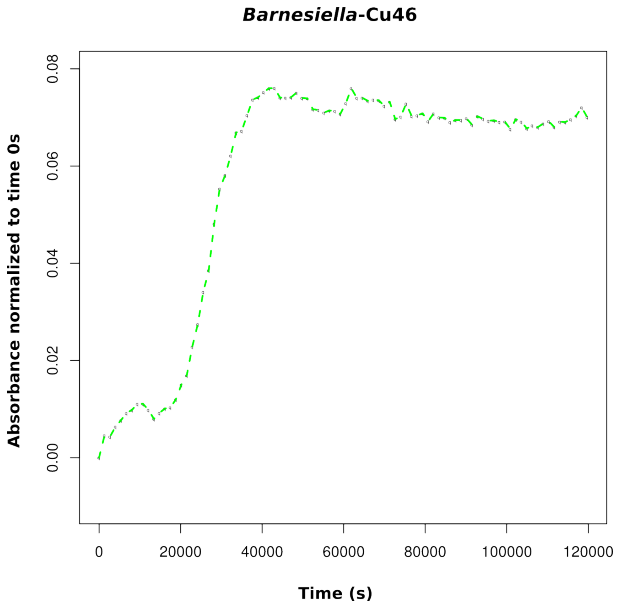


Figure 3.51: Growth curve of the anaerobe *Barnesiella*. We measured the growth of the anaerobe *Barnesiella* CU46 in the conditions selected to test the metabolic requirements of VRE in anaerobiosis.

3.7.2 VRE growth on unique carbon sources

After 24h of incubation at 37ºC in anaerobic conditions, VRE grown significantly with 73 of the 192 carbon sources tested.

For these 73 carbon sources, the number of CFUs had duplicated respect to the control (fold change superior or equal to 2). Focusing on the carbon sources that promoted the highest growth of VRE, only 30 carbon sources produced an increase in VRE CFUs by at least 5 times respect to the control (without carbon source). The range goes from a fold change of 5.3 for malic acid to 47.9 in the case of D-galactose (Figure 3.52). Only 11 metabolites promoted a 20-fold growth of VRE or superior (i.e: in decreasing order, D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-trehalose, alpha-cyclodextrin, D-mannose, gentibiose, maltose, D-cellobiose, D-mannitol and D-ribose). As one can appreciate, two of the most important carbon sources for VRE growth are two of the carbohydrates (cellobiose and N-acetyl-D-galactosamine) that the protective bacteria could be depleting in the gut (expressing ABC transporters for internalizing these sugars). Moreover, as we have previously shown in the metabolome analysis, cellobiose levels are reduced after protective commensal bacterial inoculation, suggesting a possible role of cellobiose depletion in VRE growth inhibition by the protective commensal bacteria. Interestingly, VRE is able to use also the amino acid D-serine as a unique carbon source, one aminoacid that was reduced upon administration of the protective commensal bacteria.

The last objectives of the present work were to identify the bacteria that could perform a protective action against VRE intestinal colonization and to define the mechanism of this protection. The analysis of (1) the metatranscriptomic and metabolomic differences existing between the treated group that received the bacterial mix of PBS and (2) the determination of VRE requirements, performed by the study of its transcriptome during

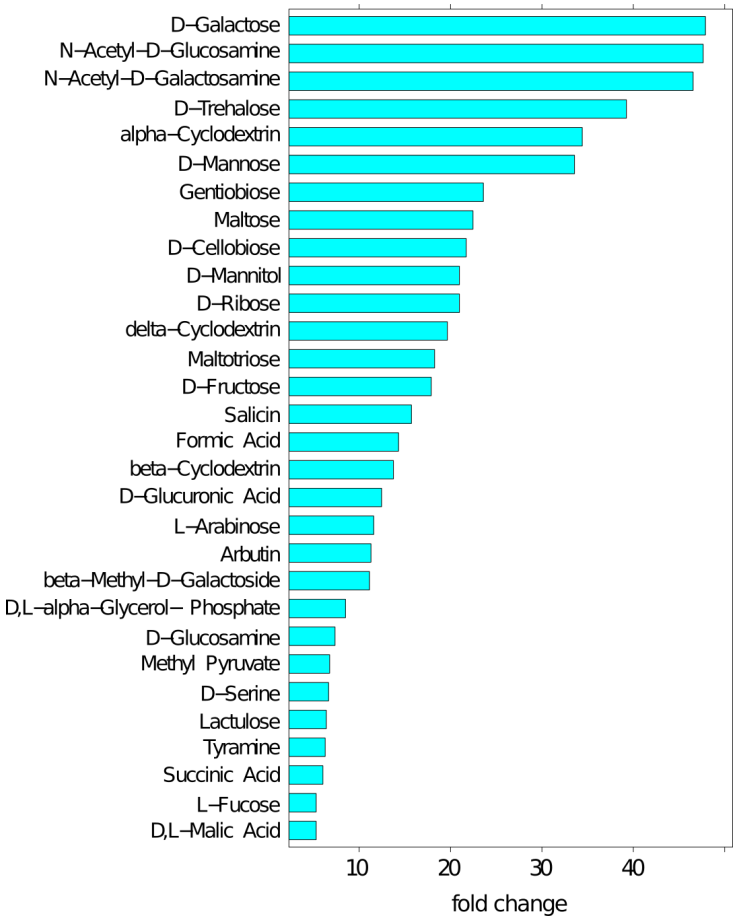


Figure 3.52: Carbohydrates sources that allowed the highest growth of VRE *in vitro*. The inoculate and the suspension of the different wells incubated for 24h at 37ºC was grown on BHI agar to determine VRE growth respect to the inoculate. VRE growth is expressed as the fold change (number of CFUs) respect to control (no growth) in the presence of an unique carbon source.

the infection as well as studying the influence of different carbon sources on its growth, allowed the formulation of several hypothetical mechanisms of protection.

The main mechanisms retained and to be tested are (I) the nutritional competition for oligosaccharides, especially for cellobiose and N-Acetyl-galactosamine, (II) the nutritional competition for aminoacids, especially for threonine, serine, proline and leucine, (III) a possible nutritional competition for choline, betaine, (IV) the possible inhibition of biofilm formation-adhesion to intestinal epithelium by phenol or the liberation of phenol compounds with an inhibitory activity against VRE, (V) the inhibitory activity of the SCFAs whose levels were partially restaured upon the baterial mix administration, especially valerate. Therefore, we will discuss further these mechanisms in the discussion section and some of the experiments that we will perform to test these possible mechanisms of protection.

4. Discussion

4.1 Short- and long-term effects of oral vancomycin on the human intestinal microbiota.

In the first part of the present work, we have investigated, using a high-throughput sequencing approach, both the short and long-term impact of vancomycin on the human intestinal microbiota. Our results have revealed that vancomycin depletes most bacterial OTUs found in the intestinal tract, including all detected baseline OTUs from the phylum Bacteroidetes. In contrast, vancomycin did not alter the abundance of the Firmicutes phylum. The obtained results are somehow paradoxical since vancomycin has a theoretical spectrum against gram-positive bacteria. A priori, the depletion of the Firmicutes phylum (gram-positive) and expansion of the Bacteroidetes phylum (gram-negative), besides the expansion of the other two gram-negative phyla (Proteobacteria and Fusobacteria), were expected to occur. Consistent with its effect against gram-positive bacteria, the abundance of most of the identified Firmicutes genera diminished during vancomycin treatment. However, certain Firmicutes significantly increased, which could compensate for the loss of the rest. For example, an increase of specific *Lactobacillus* OTUs occurred during treatment, perhaps due to a known resistance to vancomycin of certain *Lactobacillus* strains [258]. As expected, the phyla Proteobacteria and Fusobacteria increased after the treatment.

Specifically, an expansion of the genera *Escherichia-Shigella*, *Klebsiella* (Proteobacteria phyla) and *Fusobacterium* (all gram-negative bacteria and highly resistant to vancomycin (Minimum inhibitory concentration 50, MIC50=128-1024 µg/ml) [259–263] was observed during vancomycin treatment. However, bacteria belonging to the Bacteroidetes phylum did not expand but were completely depleted by vancomycin treatment. Bacteria belonging to this phylum are also resistant to vancomycin, but typically to a lower extent (MIC50=4-128 µg/ml) (Figure 4.1). It is well established that vancomycin is not absorbed in the intestine, reaching very high concentrations in feces when administered orally (500 µg/g) [264]. Thus, it is conceivable that the opposed directional changes observed in the gram-negative phyla Bacteroidetes as compared to Fusobacterium and Proteobacteria were mainly due to their different sensitivity to vancomycin. Notably, the *C. difficile*'s vancomycin MIC90 range is typically low (0.5-4 µg/ml), while the concentrations achieved by vancomycin in feces to treat CDI widely exceed its MIC90. In the future, it will be important to test if lower doses of oral vancomycin could still maintain efficacy while decreasing the impact on the Bacteroidetes phylum, whose significance in maintaining a healthy state has been extensively documented [265].

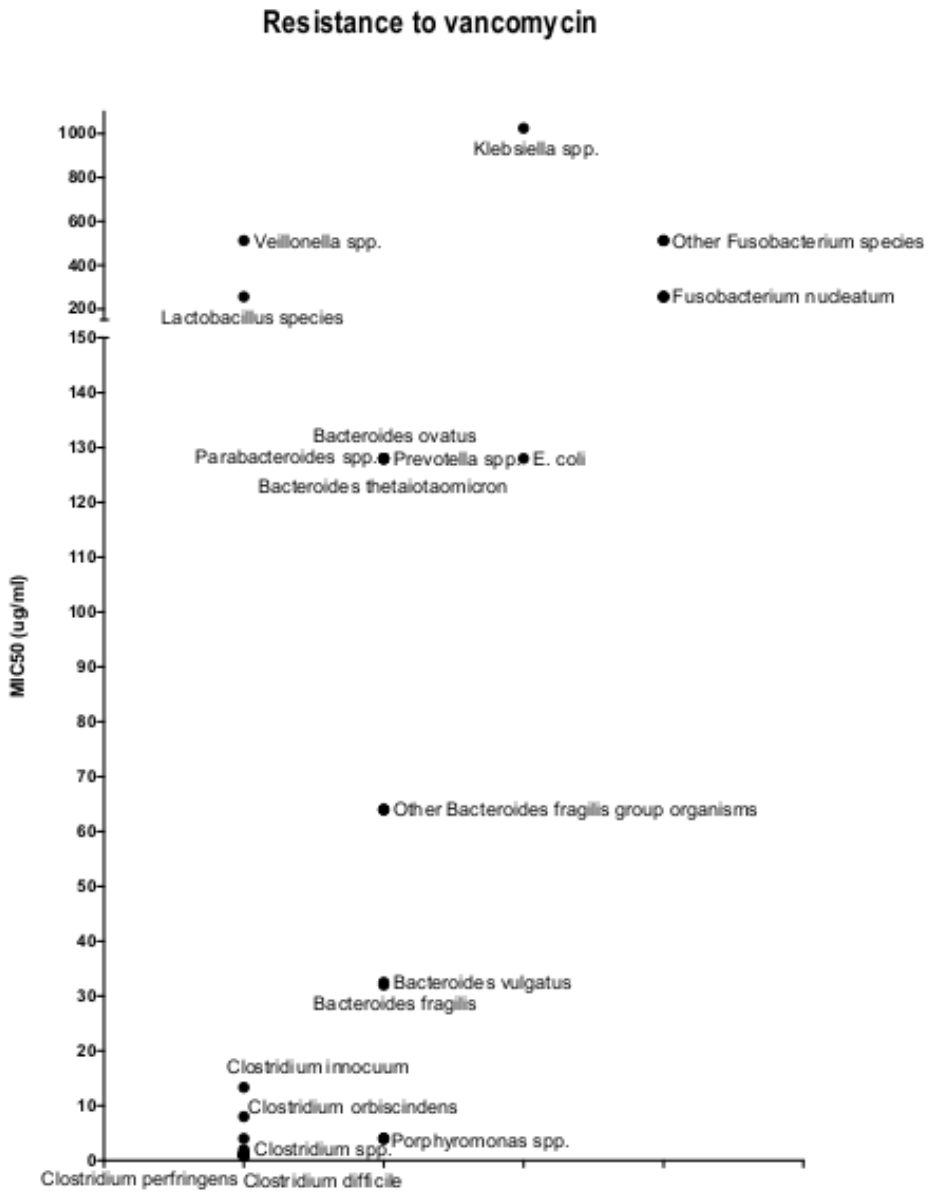


Figure 4.1: Vancomycin MIC50 of different commensal species MIC50 data for the different isolates was obtained from previous studies [259–263]. If the MIC50 for the same type of bacterium was analyzed in more than one study, the average of the MIC50 obtained for that particular organism in the different studies was calculated. Bacteria are sorted from left to right according to their phyla classification (Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria).

Our results showing Bacteroidetes depletion and a concomitant expansion of Proteobacteria are in agreement with previous studies utilizing lower resolution techniques to study the effects of vancomycin in CDI patients [266, 267]. Importantly, our high-throughput sequencing approach allowed us to define that essentially all highly prevalent OTUs belonging to the Bacteroidetes phylum were affected by vancomycin. Consistent with our results but using a microarray lower throughput approach, Vrieze *and co-workers* also observed a decrease in the levels of *Faecalibacterium* and *Ruminococcus* [268].

We acknowledge several limitations of our study. First, we analyzed the effects of vancomycin on patients with rheumatoid arthritis (RA), a population that demonstrates microbiota features usually absent in normal healthy subjects (i.e. higher levels of *Prevotella copri*) [212]. Therefore, it is possible that different changes could be observed in a healthy human population. Nevertheless, the fact that a) similar changes were observed in healthy mice, and b) comparable alterations at the phylogeny level had been identified using lower resolution techniques, suggest that vancomycin effects are expected to be similar in healthy human subjects. Importantly, our study was performed in a human population that had not recently received other antibiotic treatments. This is in contrast to other studies performed in CDI patients, where most patients had received other antibiotics before vancomycin administration. This has allowed us to define the long-term effects of vancomycin administration in the absence of confounding factors such as exposure to other antibiotics. Besides vancomycin treatment, RA patients did also receive methotrexate. For this reason, as a control group, we enrolled patients with RA that did not receive vancomycin but were treated with methotrexate from the very beginning of the study. We did not observe any significant intestinal microbiota changes in any of the analyzed time-points in this control group, suggesting that microbiota perturbations observed in the vancomycin-treated group were solely induced by vancomycin. Similarly to our results, a recent study showed that methotrexate has a minor effect on the fecal microbiome composition [269]. A second limitation of our study may be the low number of participants included. Nevertheless, due to the drastic changes induced by vancomycin in the microbiota composition, even with this small number of participants, we were able to detect statistically significant changes upon vancomycin administration. Thus the inclusion of further participants would have not substantially changed the conclusions of the study.

Beyond analyzing the short-term effects of vancomycin, we also examined its long-lasting consequences on the intestinal microbiota. Despite the drastic microbiota disruption observed in the treated patients as a group, 22 weeks after cessation we could only identify changes in 2 OTUs, probably due to the variable microbiota recovery among patients. Similarly, a recent study identified significant changes in just 2 OTUs, 4 months after stopping either ciprofloxacin or clindamycin treatment [270]. In that study, only changes in the microbiota common to all subjects were analyzed. Here, in contrast, we further analyzed the extent to which each subject was able to recover the baseline microbiota. Interestingly, we found a wide inter-individual variability in microbiota recovery after vancomycin cessation. This result is highly relevant because a subset of CDI patients develop secondary infections after vancomycin therapy, including VRE infections [271–273]. Although the reasons for developing these secondary infections are not completely understood, it has been proposed that alterations of the microbiota after vancomycin treatment may enhance intestinal colonization by bacterial pathogens and

subsequent infections. This could have direct clinical implications, as a different microbiota recovery rate upon oral vancomycin therapy in hospital settings (i.e., CDI treatment) could ultimately influence the susceptibility to intestinal colonization by pathogens such as VRE. In fact, the results obtained in mice showing a significant negative correlation between the microbiota recovery rate and the intestinal VRE levels strongly support this hypothesis.

Altogether, our results demonstrate the negative long-term consequences of oral vancomycin administration, an antibiotic highly associated with secondary VRE infections. In addition, our results highlight the potential value of monitoring microbiota dynamics on each patient before and after antibiotic administration. Microbiota tracking could lead to the identification of patients at higher risk of suffering the collateral negative effects of vancomycin (i.e. infections) and eventually inform the potential need for microbiota restoration through fecal transplantation or probiotic administration.

4.2 Study of the effect of antibiotics of different spectrum on the gut microbiota using a mouse model.

4.2.1 Characteristics of the taxa altered by several treatments

It has been previously described that antibiotics, by disrupting the gut microbiota, annihilate the colonization resistance normally proportionated by a healthy gut microbiota [17,112,251]. This colonization resistance to the infection by VRE can be restored through a fecal transplant [112]. Thus, commensal bacteria have a key role in conferring protection against VRE intestinal colonization.

In the first part of this thesis, we have investigated the effects of a specific antibiotic, vancomycin, on the gut microbiota. We were also able to establish a relationship between the rate of recovery of the microbiota and the intestinal VRE colonization capacity.

However, we did not study how other antibiotics would affect the microbiota and the VRE colonization capacity nor which commensal bacterial species not recovered after the vancomycin administration were crucial for conferring the protection.

For this reason, we studied how antibiotics of various spectrum (ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin, in addition to vancomycin) affect the gut microbiota and promote VRE intestinal colonization. We are conscious that to infer the effect of different antibiotics on the microbiota as well as their recovery, it would have been better to collect samples pretreatment, during the treatment and during the recovery from the same mice for the different treatments tested. However, the study was designed with the main objective of analyzing the effect of the different antibiotics on VRE colonization levels. For this reason, we had to use different animals for studying changes in the microbiota during antibiotic treatment and upon recovery from antibiotic treatment.

Our results have shown that, as expected, different antibiotics of different spectrum have a different effect on the microbiota composition, being clindamycin and vancomycin the ones that caused more alterations. All the antibiotics tested caused a decrease in microbial richness and all but ciprofloxacin also caused a decrease in the biomass and diversity. However, the decrease observed was clearly different depending on the antibiotic (Figure 3.12).

In this section of the discussion, I will first describe the taxa commonly altered by the antibiotic treatments and I will review the properties of these bacteria that could influence the colonization resistance against VRE.

Interestingly, numerous genera diminished upon administration of any of the antibiotics tested (i.e. *Dorea*, *Turicibacter*, *Ruminococcus*, *Allobaculum*, *Butyricicoccus*, *Coprobacillus*, *Holdemania*, *Anaerovorax* and *Acetanaerobacterium*). This could suggest a broad sensitivity to all the antibiotics tested or that these bacteria rely on a syntrophic relationship with other members of the microbiota. The existence of syntrophic relationship among members of the gut microbiota has indeed been demonstrated [274]. Thus it is possible that after administration of the different treatments, these syntrophic bacteria would lose part of their syntrophic network and thus their capacity to grow. Further studies should be performed in order to demonstrate the mechanisms by which the abundance of these particular genera diminish upon administration of all the antibiotics tested.

Observing the changes at the phylum level, one of the most abundant one, the phyla Firmicutes, presented fewer alterations, probably because this phyla is composed by different genera, and the expansion of some genera compensate for the reduction of other genera (Figure 3.15). Indeed, we already saw in the first part of the present work that the administration of vancomycin do not alter the phylum Firmicutes, which seemed quite surprising knowing that this antibiotic affects the gram positive bacteria (most of the Firmicutes). As we already mentioned, most of the genera from the phylum Firmicutes were indeed diminished upon the administration of vancomycin but the expansion of at least one abundant genus, *Lactobacillus*, compensated it. Considering the other antibiotics tested, very few genera from the phylum Firmicutes increased after their administration. The two most remarkable ones were *Enterococcus*, which increased after the administration of β-lactams or clindamycin and *Lactobacillus*, one of the most abundant genus of the phylum Firmicutes, which increased after the administration of vancomycin. In addition, we also detected an increase of *Anaerostipes* (another genera belonging to the phylum Firmicutes) in the case of ceftriaxone and clindamycin treatment. At the same time, we detected an important decrease of *Muscispirillum* and *Akkermansia* after the administration of these two antibiotics. In contrast, these taxa are increased after the treatment with vancomycin. These bacteria are related with the inflammation and the high turn-over of the mucus layer. Knoop *et al.* [275] have indeed shown in mice that a single dosis of vancomycin promoted intestinal inflammation. Therefore, it would be interesting to verify if these bacteria promote an inflammatory environment in the mice that have undergone the vancomycin treatment in comparison with the other two treatments. The inflammation can boost the growth of Proteobacteria [276] and would explain the increase in Proteobacteria observe during the vancomycin treatment. In addition, as we will comment later, Proteobacteria have been associated with an increased intestinal colonization by VRE.

The effect of antibiotics on the phylum Bacteroidetes was highly variable both considering the antibiotic administered and the genus affected. For example, the genus *Bacteroides* increased after administration of most of the antibiotics, meanwhile *Alistipes*, *Prevotella* and *Odoribacter* usually decreased.

Bacteroides was able to reach high levels after administration of most of the antibiotic treatments tested, even when these antibiotics should be active against gram negative bacteria (i.e: neomycin, ceftriaxone, ampicillin). Moreover, we also observed an higher

intra-genus richness after the administration of neomycin, ceftriaxone or ampicillin (Figure 3.18). Probably, very low abundant *Bacteroides*, not detectable in untreated mice, expanded to reach detectable levels (Figure 3.16). This result is not that surprising knowing that specific strains from this genus are resistant to the antibiotic administered, for example by producing β-lactamases [277]. On the other hand, this genus presents an extended metabolic capacity, especially for the use of carbohydrate substrates [278]. Possibly, due to its wide metabolic capacity, this genus does not rely on the interaction with other genus from the microbiota to survive. Thus, upon any dysbiosis, it may be able to take advantage of the nutrients newly available. This advantage, however, is lost after antibiotic cessation since this genus was not increased two weeks after the recovery from the therapy. Interestingly, when we studied the *Bacteroides* genus at the OTU level, different OTUs expanded after different antibiotic treatments. For example, in the case of ciprofloxacin and clindamycin treatment, few OTUs were detected within these genera and indeed only one OTU of the genus represented the majority of the genus *Bacteroides* (98 % and 99%, respectively) in mice treated with theses antibiotics. Notably, the OTU that expanded was different after clindamycin treatment and after ciprofloxacin treatment suggesting that different resistance to different antibiotics or a difference in metabolism which would influence their fitness in the presence of the antibiotic-altered bacterial community. (Figure 3.16). If the first hypothesis is correct (resistance to different antibiotics), this result would suggest that certain *Bacteroides* OTUs, present in the murine microbiota, are resistant to specifics antibiotics, and there is no a superbug that is resistant to everything. Nevertheless, it seems that, except for vancomycin, the mouse microbiota always contained some *Bacteroides* OTUs, resistant to the antibiotic administered, that could expand during antibiotic therapy (Figure 3.16).

Consistent with our results, Jernberg *et al.* [178] described an important loss of diversity within the genus *Bacteroides* following a 7-day treatment with clindamycin. They determined that the dominant *Bacteroides* after the treatment, *B. thetaiotaomicron*, had acquired some resistance to clindamycin. Surprisingly, they highlighted that other resistant *Bacteroides* did not expand during the treatment. Thus, the *Bacteroides* that expands must probably be both resistant to the treatment administered and present specific characteristics necessary for its survival in the altered bacterial community. A similar selection of *Bacteroides* clindamycin-resistant strains could be occuring in the microbiota of the mice that we have studied. Indeed, our results indicated that a single OTU represented the 99% of the *Bacteroides* genus identified in clindamycin treated mice, while other few *Bacteroides* OTUs could also be identified but their relative abundance was very low (<0.4% of the microbiota).

It is noteworthy that the presence of the genus *Bacteroides* in higher abundance defines the human enterotype type 1 [141]. Later, this enterotype has been related with a diet rich in protein and animal fat, such as the well-characterized western diet [279], and has been demonstrated to be more prevalent in the western society [280]. So far, the difference in the prevalence of the enterotype associated with *Bacteroides* has been attributed to difference in the diet. In the light of the present results, it is possible that the high use of antibiotics in the western society could have contributed to the high prevalence of *Bacteroides* in this population.

In mice also the existence of this enterotype has been confirmed [281, 282]. However, in mice only two types of microbiota were detected in contrast to the three enterotypes

found in humans. The second enterotype found in mice is characterized by a higher abundance of Ruminococcaceae or Lachnospiraceae, as seen in the human enterotype type 3 [141]. Interestingly, we observed that the microbiota post-recovery of mice treated with ciprofloxacin, ceftriaxone or clindamycin was also dominated by the taxa Lachnospiraceae.

Finally, the equivalent of the human-enterotype type 2, defined by an increase in *Prevotella*, was not detected in the studies conduced in mice [141, 281, 282]. The authors hypothesized that the reduce sized of their sample could impair the detection of this cluster as it is the least prevalent one [281]. Moreover, the experiments in laboratory were conduced administrating a similar diet to all the mice, which could diminish the diversity [282]. We were able to identify *Prevotella* in the untreated mice analyzed in our study. Neverteless, *Prevotella* was not a dominant member of the murine microbiota. In humans, *Prevotella* is usually associated with communities from rural areas, with an undisturbed microbiota. Interestingly, the taxa *Prevotella* was totally depleted after most of the antibiotics treatments (i.e. ciprofloxacin, ceftriaxone, clindamycin and vancomycin) in mice.

These results suggest that the antibiotic consumption could influence the prevalence of specific enterotypes, meaning the dominance of the microbiota by a specific genus. Nevertheless, further studies should be performed to validate this hypothesis.

Apart from the alterations in the phylum Bacteroidetes, we identified that other phyla, which could constitute a lower proportion of the microbiota, including the phyla Actinobacteria and TM7, were essentially diminished upon any antibiotic administration. These taxa could be susceptible to the diffrents antibiotics tested or rely on a high symbiotic relationship with the microbiota, explaining why these bacteria are diminished no matter how the microbiota is altered.

Among the phylum Actinobacteria, the genus *Bifidobacterium* is used as a probiotic for its immunomodulatory activity as it diminishes the inflammatory status in the gut through TLR2 recognition [283]. Thus, the diminution of this genus abundance (which was observed upon administration of all the antibiotics tested) could have repercussions on the inflammatory status of the host. It was demonstrated in mice that numerous antibiotic treatments (among which vancomycin and ampicillin) promote intestinal inflammation [275]. Future studies should elucidate if the antibiotic driven diminution of the taxa *Bifidobacterium*, along with alteration of others taxa such as *Akkermansia* or *Muscispirillum*, could be responsible for an enhanced inflammatory status in the gut.

The phylum Verrucomicrobia is usually increased after administration of antibiotics. The unique member of the Verrucomicrobia significantly altered, *Akkermansia*, is a mucin degrader normally present in the human intestine [284]. Being located at the interface between the intestinal lumen and epithelium, *Akkermansia* influences both the microbiota and the host [286]. This bacteria activates the TLR2 and TLR4 (which reduces the diabetes incidence) [287, 288] and improves the metabolic disorders associated with high-fat induced obesity [289]. When degrading the mucus, *Akkermansia* produces acetate, propionate and releases oligosaccharides. Being in direct vicinity with the host epithelium, the SCFAs produced (i.e. acetate and propionate) can also influence the immune system and metabolic pathways of the host [286]. On the other hand, *Akkermansia* is associated with an hyperplasia of the goblets cells and an increased number of intestinal tumors [285]. Futhermore the oligosaccharides released upon the mucus degradation can stimulate the growth of bacteria that colonize close to the mucus layer [286]. Future

studies should elucidata how changes in *Akkermansia*, upon antibiotic treatment, could influence metabolites in the gut, colonization of the mucus layer by other bacteria and the immune system.

Finally, the phylum Proteobacteria is increased both during and two weeks after the treatment with clindamycin and vancomycin whereas it is decreased during the treatment with ciprofloxacin. It was already described that fluoroquinolones such as ciprofloxacin diminish the level of Proteobacteria in gut microbiota [113]. Similarly, the increase in Proteobacteria after the administration of vancomycin and clindamycin had been previously documented [179, 288].

The increase of the abundance in Proteobacteria upon vancomycin or clindamycin treatment could be clinically relevant. Indeed, it has been demonstrated that domination by members of the Proteobacteria phylum increased the risk of bacteremia with aerobic gram-negative bacilli [113]. Moreover, in a study comparing untreated mice with the same mucus thickness but with different penetrability, it was seen that mice with a penetrable mucus layer had higher levels of Proteobacteria and TM7 bacteria in the distal colon mucus [293]. Moreover, in one study at least, it was demonstrated that the co-colonization of mice with *Klebsiella*, a member of the Proteobacteria phylum, and VRE promoted the dissemination of VRE to the MLNs [214]. Thus, it could be interesting in future studies to define if those antibiotics that promote the increase of Proteobacteria could promote the dissemination of bacteria to the bloodstream taking into account also the presence of other bacteria such as *Akkermansia* or *Muscispirillum* that influence the mucus layer.

4.2.2 Specific effect of each antibiotic treatment on the gut microbiota.

I have discussed the principal bacteria whose abundance was altered during an antibiotic treatment. Now, I will discuss the different alterations associated with each treatment.

Neomycin. In the case of neomycin treatment, this antibiotic was administered in combination with other antibiotics in the different publications that investigated the gut microbiota alterations. Thus, we have no comparison for the alterations we have observed. Of note, this aminoglycoside presents a broad spectrum against the aerobic bacteria and is the unique antibiotic tested that target only aerobic bacteria. As the main part of the microbiota are anaerobic bacteria, it is not surprising that it caused the smallest alterations and that the microbiota observed after a two-week recovery was not significantly different from the untreated one.

Beta-lactams : ampicillin and ceftriaxone. Interestingly, the two β-lactams, ceftriaxone and ampicillin presented very similar alterations during the treatment (Figure 3.15).

Ubeda *et. al.* [112] analyzed the alterations of the microbiota during the treatment with ampicillin and two weeks after its withdrawal. Although the microbiota pretreatment was different from the one present in our mice, after a two-week recovery, we also observed a recovery of taxa from the phylum Firmicutes. Similarly, Raymond *et. al.* [294] saw that, administering a cephalosporin of second-generation in healthy humans, the most

consistent effect was an increase of Lachnospiraceae, as we have also observed. In our study, we used a cephalosporin of third-generation, ceftriaxone. The spectrum of action is quite similar except that the third generation presents an increased resistance to β-lactamases and thus can be more active against specific gram negative (i.e. clinical *E. coli* or *Klebsiella* isolates) [295].

Ciprofloxacin and Clindamycin. In a study performed on healthy humans, Rashid *et. al.* [270] investigated the alterations produced by ciprofloxacin and clindamycin.

In the study by Rashid *et. al.*, ciprofloxacin and clindamycin caused a diminution of anaerobic gram positive bacteria post-treatment (which are mainly Firmicutes), although the diminution was more important upon the administration of clindamycin, as we also detected with our analysis. In particular, they found that ciprofloxacin diminished the genus *Faecalibacterium* and unclassified Ruminococcaceae. Clindamycin diminished the abundance of several genus of the family lachnospiraceae (*Coprococcus*, *Roseburia*, *Lachnospira*, *Dorea*, unclassified Lachnospiraceae) and of *Ruminococcus*, as we also detected.

The gram negative aerobic bacteria (which are mainly Proteobacteria) were importantly drastically diminished upon ciprofloxacin treatment, as detected in our study. After a few days, their level increased. We also detected an increase in the Proteobacteria letting the microbiota recover for two weeks after the administration of clindamycin.

In the study by Rashid *et. al.*, the gram positive aerobic bacteria (which mainly include *Enterococcus* and *Lactobacillus*) were increased upon recovery from both antibiotics. Consistently with their results, we observed an increase in *Enterococcus*, both during the administration of clindamycin and after a two-week recovery.

The gram negative anaerobic (which are mainly Bacteroidetes), were unaffected by any of these antibiotics in their study. We observe the same result at the phylum level. However, as we mentioned before, the abundance of the Bacteroidetes is drastically altered when looking at the genus level.

In that particular study, Rashid *et. al.* performed a q-PCR to determine the abundance of some precise taxa that were altered. Using this approach, they found that the genus *Bacteroides* was increased whereas the genus *Alistipes* was decreased after the administration of ciprofloxacin. We found a similar result. However, our high-throughput sequencing approach allowed us to define that ciprofloxacin caused a drastic diminution in the diversity within the genus *Bacteroides* and that the increase in abundance was associated with the expansion of mainly one OTU of this genus. In a study conduced on three subjects, Dethlefsen *et al.* [173] already detected that the abundance of the genus *Bacteroides* was not altered but that the relative abundance of the different OTUs varied upon the administration of ciprofloxacin.

The ciprofloxacin treatment caused a number of alterations, detected at the phylum and the genus level, similar to the neomycin, ampicillin or ceftriaxone treatment. However, the ciprofloxacin group was far more different from the untreated mice than were the neomycin, ampicillin or ceftriaxone treated groups (Figure 3.13). This may be explained by the important alterations of the intra-genus diversity following the ciprofloxacin treatment.

We also observed an alteration of the intra-genus diversity following the clindamycin treatment. In agreement with our results, Buffie *et al.* already described in mice that a single dose of clindamycin caused a loss of 90% of the normal microbiota of the ce-

cum [179]. In this study, the bacteria present the day following the clindamycin administration were, by order of abundance, Enterobacteriaceae, *Lactobacillus*, *Enterococcus*, *Turicibacter* and a Tenericutes. As mentioned before, we also observed a drop in the bacterial diversity and an increase in the abundance of Proteobacteria and *Enterococcus*. Nonetheless, in our model, the most abundant bacteria during the antibiotic treatment were *Bacteroides* and *Barnesiella* followed by Enterobacteriaceae. This difference could be due to the different initial microbiota present in the mice. Interestingly, two weeks after the withdrawal of the antibiotic treatment, our results agree on that the dominant bacteria is a Lachnospiraceae (*Blautia* in their study and an unclassified lachnospiraceae in ours). Similarly the abundance of the Enterobacteriaceae and the taxa *Lactobacillus* and *Enterococcus* were increased in both studies. Thus, although the administration of clindamycin resulted in a different microbial composition during treatment, in both studies the microbiota adopted a similar structure upon two-weeks of recovery.

Vancomycin. Finally, the highest alterations were observed after the administration of vancomycin. During the treatment and upon recovery, bacteria normally very low abundant in untreated mice dominated the microbiota.

The alterations we detected are consistent with the ones described in humans in the first part of this work and by others authors [175, 296]. We also observed an important depletion of the phylum Bacteroidetes and of most of the Firmicutes except *Lactobacillus* whereas the phylum Proteobacteria increased.

A study focused on the *C. difficile* colonization capacity after antibiotic treatments also explored the effect of vancomycin on the mouse gut microbiota [175]. They described similar alterations as we observed (an important decrease of the phylum Bacteroidetes and of most of the Firmicutes whereas *Akkermansia*, *Lactobacillus* and Proteobacteria expanded). Interestingly, their results vary according to the microbiota that was present before treatment. For example, in a repetition of this experiment, they detected an expansion mainly of *Klebsiella*, instead of *Akkermansia* and *Lactobacillus*.

Thus, the alterations associated with the vancomycin treatment can vary sligtly according to the baseline microbiota as we discussed before for clindamycin treatment.

In summary, our results, obtained in mice, show similar changes as the ones described by other authors in humans (specifically clindamycin, ciprofloxacin and vancomycin). Thus, our results indicate that at least for these antibiotics mice could represent a good model to study the effects of antibiotics on the microbiota and subsequent impact on health.

4.3 New microbiota state upon antibiotic cessation

Upon neomycin treatment, the microbiota recovered and almost did not differ from that of untreated mice. On the other hand, upon vancomycin treatment, the microbiota slighly recover but basically resemble to the one observed during vancomycin treatment. Interestingly, ceftriaxone and clindamycin treatment caused the aggregation of a different bacterial community upon the two-week recovery period.

Ceftriaxone promoted a drastic diminution in the total bacterial load (biomass) during the treatment (see 3.12 A). To what extent the bacteria detected by high-throughput

sequencing upon ceftriaxone treatment are alive, dead or even come from an external sources is unknown. It is possible that only a few of the bateria detected were alive commensals and the expansion of these few bacteria would promote the development of a new microbiota state.

In the experiment of Raymond *et. al.* [294] that we previously mentioned, the authors also observed an variable alterations of the microbiota during the treatment with cefprozil, a second generation cephalosporin, and after its withdrawal. Especially, some patients also showed a total remodeling of their microbiota community after a recovery period [294].

The establishment of an alternative state after the treatment by clindamycin (Figure 3.22 C) is not surprising if we take into account that the stability of the gut microbiota depends on the diversity of the community, and we have seen that during the treatment only five OTUs accounted for 97% of the clindamycin-treated microbiota (see 3.2.3). After the withdrawal of the antibiotic, there could be a reshape of the bacterial community. As the alteration experimented was so extreme, there could be an establishment of a new community instead of resilience to the untreated state. The onset of a totally different microbial community following the clindamycin treatment was also evident in the study of Buffie *et al.* [179], even if the experiment was not designed with the aim to explore the alteration caused this antibiotic. Nevertheless, although our results suggest the possibility of new microbiota states upon antibiotic recovery, additional experiments such as monitoring the changes in the microbiota of the same mouse during and after recovery should be performed to confirm our results. If these results are confirmed in future studies, an interesting line of research would be to try to understand how these different microbiota states are developed, to what extent are they randomly generated or to what extent they depend on the changes observed during antibiotic treatment or on the baseline microbiota before treatment.

4.4 Influence of antibiotics on VRE colonization

The alteration of the microbiota caused by an antibiotic treatment (i.e. vancomycin) is associated with an increased susceptibility to secondary infections, as we have demonstrated in the first part of this work.

Using another pathogen, other groups already assessed the effect of precise antibiotics on the susceptibility to secondary infections. For example, a single dose of clindamycin caused a sustained susceptibility to *C. difficile* infection [179].

Because the microbiota is essential to prevent the colonization by this opportunistic pathogen, fecal transplantation has been used with success for the treatment of recurrent CDI in order to restore the colonization resistance against this pathogen. Nevertheless, the fecal transfer approach suppose several difficulties, including the possible transmission of diseases in which the microbiota is involved. For this reason, other solutions have been investigated, such as the administration of specific commensal bacteria. For example, it has been demonstrated that the administration of 4 bacteria in a mouse model is sufficient to diminish significantly the colonization by *C. difficile* [189]. This study demonstrate that it is possible to restore the colonization resistance by administration of specific commensal bacteria. Moreover, it has been demonstrated that the administration of a specific fraction of the microbiota, cultivated in columbia blood agar media, was sufficient to achieve the

restoration of the resistance to the colonization by VRE whereas the administration of the aerobic fraction was not [211]. Thus specific bacteria are implicated in the colonization resistance and they are cultivable.

In order to define bacteria that could restore the colonization resistance against VRE, we studied the relation between several antibiotic-driven microbiota alterations and the capacity of VRE to colonize the intestinal tract.

Infecting mice receiving a treatment with any of the studied antibiotics (i.e. ciprofloxacin, neomycin, ceftriaxone, ampicillin and clindamycin or vancomycin) lead to a significant colonization by VRE (Figure 3.25), although the levels of colonization were different depending on the antibiotic received. After a two-week recovery, for all antibiotics, the VRE colonization levels were lower, suggesting that the recovery of important microbial taxa and subsequent colonization resistance occurred after antibiotic cessation.

As expected, the more the changes an antibiotic produced in the microbiota, the more the colonization resistance was abrogated. Neomycin and ciprofloxacin were the antibiotics that produced less alterations in the microbiota diversity (as measured by the shannon index), and less alterations on the different genera analyzed. These two antibiotics allowed low levels of VRE colonization. However, vancomycin and clindamycin, which promoted the highest decrease in the overall microbiota diversity and richness, were the ones that allowed a higher colonization by VRE.

Previous studies also reported the effect of certain antibiotics on the ability of VRE to colonize the intestinal tract.

It was already known that vancomycin and treatments against anaerobic bacteria (ie.e.metronidazol) promoted high levels of intestinal colonization by VRE [204], although the microbiota alterations associated with the loss of colonization resistance were not described. In 2015, a study analyzed the alterations of the microbiota associated with these two treatments, principally in relation with *Clostridium difficile* colonization capacity [175]. Moreover, they verified that in their model vancomycin but not metronidazol allowed the colonization with high levels of VRE [175]. However, in this article, the authors did not try to determine which bacteria of the commensal microbiota were responsible for the colonization resistance against VRE.

The antibiotic treatment that we tested that targeted the anaerobic bacteria, clindamycin, enabled VRE to colonize the intestine at high levels. Clindamycin administered orally has been shown to increase the VRE burden in a patient previously colonized with VRE [106, 251]. However, the specific alterations of the microbiota related to the loss of colonization resistance were not investigated in this study.

In the description of a model to infer microbial community ecology, applied to *C. difficile* infection, the authors described a sub-network of interactions in which they proposed that *Coprobacillus* promoted the expansion of *Akkermansia* and *Blautia* and that these three bacteria would inhibit *Enterococcus* growth [297]. Upon a treatment with clindamycin, the three bacteria that inhibit *Enterococcus* growth would be depleted, then *Enterococcus* could grow and promote *C. difficile* colonization. *Akkermansia* is an important taxa for the intestinal homeostasis, and possibly its combination with *Coprobacillus* and *Blautia* inhibits *Enterococcus* growth. However, we have detected a positive association between *Akkermansia* and VRE when we analyzed the combined data obtained from all the antibiotics tested, suggesting that the negative interaction between *Akkermansia* and *Enterococcus* may occur exclusively in the case of the clindamycin treatment.

Subcutaneous administration of ceftriaxone, which is secreted into human bile at high concentrations, was demonstrated to promote a high colonization with VRE, as we also detected in our model [106, 298, 299]. In the case of ceftriaxone, we mentioned that the recovered microbiota can be very variable and, consequently, also the VRE colonization capacity.

The administration of ciprofloxacin in colonized patients decreased the VRE burden although the VRE isolates proceeding from these patients were highly resistant to ciprofloxacin *in vitro* [106, 251]. Consistent with these results, ciprofloxacin did not promote high levels of VRE colonization in our mouse model.

In summary, the impact on the VRE colonization capacity of the different antibiotics administered was already known. However, most of theses studies did not investigate the relation between the loss of colonization resistance with the alterations in the gut microbiota which we indeed performed and will be discussed in the next section.

4.4.1 Relation between the microbiota alterations and VRE colonization

To determine which changes in the microbiota could be associated with the higher capability of VRE to colonize the intestine and therefore to identify commensal bacterial candidates that could have a role in the protection against VRE colonization, we performed a spearman correlation test between the VRE load and the relative abundance of the different commensal bacteria. Using this approach, we determined that unclassified Ruminococcaceae, unclassified Lachnospiraceae, *Oscillibacter*, unclassified Porphyromonadaceae, unclassified Bacteroidetes, *Allobaculum*, *Barnesiella* and *Alistipes* were negatively associated with VRE (Table 3.2).

As we mentioned in the methodology, we performed this analysis first using the 454 platform to evaluate changes in the microbiota composition. This analysis has several limitations. First, the number of sequences achieved using 454 Roche sequencing is relatively low in comparison with the actual output achievable with illumina, so we could focus our analysis only in commensal bacteria with high abundance. Nevertheless, we resequenced the samples with illumina and verified that the commensal bacteria identified by 454 to be associated with the resistance against VRE, were also identified by illumina sequencing. In our model, the bacteria finally selected and tested achieved an important diminution of VRE load. Thus, the methodology followed was useful. Nevertheless, VRE colonization was not completely abrogated in some mice that received the bacterial mix. Thus, it is likely that other bacteria that we did not test could have a role in protection againste VRE. In addition, the analysis that we performed to select the protective commensal bacteria allows to detect the existence of a relation between a unique bacterial taxa and the level of VRE. Thus, we are not taking into account the interactions between several bacteria and we could be overlooking a synergistic effect.

Of notes, we were surprised that ampicillin caused few alterations during the treatment but still allowed an important colonization by VRE whereas upon recovery, even without a significant recovery of the microbiota, the VRE colonization capacity diminished so much. We then determined which genus were significantly increased upon the recovery period to check whether it was one of the bacteria selected. Among the few genus that increased upon the recovery period, *Ruminococcus* and *Dorea* were the most important

one (increased by 123 and 10 times, wilcoxon test, p.value=0.02 and 0.04). Interestingly, these taxa are member of the family Ruminococcaceae and Lachnospiraceae, that showed a significant negative correlation with VRE, thus confirming the suitability of the bacteria selected.

Interestingly, the strongest negative correlations were obtained with the Firmicutes Unc. Ruminococcaceae and *Oscillibacter*. Among the Bacteroidetes, *Barnesiella* presented the highest negative correlation. This bacteria had already been associated with a protective effect against VRE colonization, confirming the validity of our approximation [211]. From all the commensal bacteria that we identified to be associated with VRE protection, we finally decided not to include in the protective mix the unc. Lachnospiraceae and Allobacullum. Unc. Lachnospiraceae increases in mice after withdrawal of vancomycin treatment (the mouse model to test the protective effect of commensal bacteria). So we thought that adding unc. Lachnospiraceae to the mix would not make any difference in terms of VRE protection. On the other hand, LEfSe analysis did not identified *Allobaculum* to be clearly associated with resistance against VRE. As shown in the result section, removal of *Allobaculum* from the bacterial mix did not reduce the capacity of the mix to confer protection. Thus, we were able to reduce the bacterial mix administered to four bacteria (i.e. *Barnesiella*, *Alistipes*, *Oscillibacter* and a specific unc. Ruminococcaceae isolate).

As just mentioned, from these four bacteria selected, *Barnesiella* had already been associated with the resistance against VRE colonization [211].

The genus *Alistipes* has been associated with a protection against *C. difficile* colonization [301]. *C. difficile* and VRE co-infections can occur in the same patient [205], suggesting that certain microbiota changes may enhance colonization by these two pathogens and therefore that some commensal bacteria important for suppressing *C. difficile* may be also important for suppressing VRE.

Interestingly, Zhao *et. al.* [257] described the gut metabolic profiles associated with the administration of gentamicin and ceftriaxone. Upon administration of these treatments, they highlighted a decrease in *Prevotella*, *Barnesiella* and *Alistipes* (two of the bacteria that we found negatively correlated with VRE) and a concomitant increase in the genus *Enterococcus*. *Enterococcus* was positively correlated with the levels of deoxycholic acid (DCA) (a secondary bile acid) and the oligosaccharides raffinose, stachyose and cellobiose. On the other hand, it was negatively correlated with the SCFAs acetate, propionate, N-butyrate and various amino-acids (alanine, methionine, valine and tyrosine). On the opposite, *Barnesiella*, *Alistipes* and *Prevotella* were negatively correlated with the levels of raffinose and stachyose whereas they were positively correlated with the levels of SCFAs acetate, propionate, N-butyrate and various amino-acids (alanine, valine, lysine and tyrosine). The authors hypothesized that the unaltered microbiota, rich in *Barnesiella*, *Alistipes* and *Prevotella*, was able to catabolyze the oligosaccharides, thus decreasing their concentration. Consequently, the depletion of these three genera during antibiotic treatment liberate certain nutrients sources that could be used by *Enterococcus* to expand (oligosaccharides cellobiose, raffinose and stachyose) whose levels positively correlate with *Enterococcus*. At the same time, the taxa *Barnesiella* and *Alistipes* would ferment the monosaccharides produced as well as some amino-acids to produce the SCFAs. Interestingly SCFA have been shown to inhibit the growth of some pathogens (i.e. *E. coli*) [186]. Thus providing a second mechanism by which the depletion of *Barnesiella*

and *Alistipes* could promote the expansion of the genera *Enterococcus*. As we will discuss below, some of the possible mechanisms of interaction between these different bacteria are in agreement with the possible mechanisms of resistance that we have identified by which the bacterial mix may be suppressing VRE colonization.

The other genus identified, *Oscillibacter* is relatively new, it was described for the first time in 2007 and its whole genome was sequenced in 2012 [302,303]. The most interesting capability in relation with the possible role in VRE inhibition is its capacity to produce the SCFA valeric acid as its main metabolic end product [302, 304]. Thus, for the case of *Oscillibacter* also, the production of SCFA may have a role in its negative association with VRE.

We cannot a priori investigate in the literature what function could be doing the unc. Ruminococcaceae strain administered. Moreover, as we will describe when referring to the metatranscriptomic analysis, we found that it did express a low number of functions in the three groups of mice analyzed (mice treated with vancomycin that received PBS or the bacterial mix and untreated mice). Probably, this bacterium is not the main responsible for the colonization resistance achieved with the administration of the bacterial mix. Moreover, all of the bacteria administered did not colonize in the same proportion. From the four bacteria administered, only *Barnesiella* and *Alistipes* levels were significantly higher in the mice that received the bacterial mix as compared to those that received PBS (Figures 3.40 and 3.41). Consequently, these taxa are probably responsible for a significant part of the metabolomic alterations observed upon administration of the bacterial mix. Importantly, the administration of the bacterial mix did not restore the bacterial diversity (Figure 3.39 D), distinctly from the results obtained by Lawley *et al.* [305]. In their study, Lawley *et al.* [305] were able to eliminate *C. difficile* from colonized mice through the administration of six bacteria. The administration of these bacteria were associated with a restoration of the microbiota diversity, which could be the mechanism of *C. difficile* elimination. In our case, we did not observed a restoration of the diversity, thus it is probable that the bacteria administered are responsable themselves for the restoration of the colonization resistance against VRE. This is the case also for the results published by Buffie *et. al* [189] in which a cocktail containing four bacteria was able to protect against *C. difficile* infection without increasing the microbiota diversity.

On the other hand, we also detected bacteria positively associated with the presence of VRE (i.e. *Escherichia/Shigella*, *Enterococcus*, *Akkermansia*, unclassified Enterobacteriaceae, *Parasutterella*, unclassified Burkholderiales, *Lactobacillus* and unclassified Desulfovibrionaceae) (Table 3.3). These bacteria could be associated positively with VRE colonization just because they could be taking advantage of the dysbiosis for expansion, similarly as with VRE, without influencing VRE levels. These bacteria could therefore be used as a biomarker of the dysbiosis and the associated risk of VRE colonization. Alternatively, these bacteria could promote the colonization by VRE. For example, Caballero *et. al* [214] demonstrated that the co-colonization of VRE and *Klebsiella* (a member of the family Enterobacteriaceae) in mice, slightly increased the VRE intestinal load in comparison with the VRE levels observed in those mice that did not received *Klebsiella*.

The endogenous *Enterococcus* was found to be one of the commensal bacteria to be positively correlated with VRE levels. Contrary to our results, it has been described that different strains of the genus *Enterococcus* can also compete between them. For example, it has been demonstrated that native commensal strains of *Enterococcus faecalis* are

able to selectively kill through a pheromone mechanism the multidrug-resistant *Enterococcus faecalis* V583. The fact that we detected a strong correlation between the level of commensal *Enterococcus* and the VRE colonization capacity (see Table 3.3) suggests that the commensal *Enterococcus* present in our mice does not express a bacteriocin to out-compete VRE and both are probably adapted to the same conditions. Thus, the outgrowth of the commensal *Enterococcus* indicates that the antibiotic treatment and the resulting dysbiosis created the perfect conditions for *Enterococcus* growth (both the endogeneous and the exogeneous). This observation is reinforced by the fact that *Enterococcus* increase during treatments is higher upon administration of antibiotics that allow the highest VRE colonization levels (ceftriaxone, ampicillin, clindamycin) (Figure 3.15), except for vancomycin which is active against the endogeneous *Enterococcus* but not the exogeneous administered VRE.

On the other hand, we also detected a positive correlation between VRE and *Lactobacillus*. This is probably due to the fact that this genus increased in the mice that received the treatments that promote the higher colonization by VRE (i.e.ceftriaxone, clindamycin and vancomycin) (Figure 3.23 and 3.25). Nevertheless, we cannot discard that the endogeneous *Lactobacillus* may be somehow facilitating VRE colonization, which should be further studied in future investigations.

In summary, despite the limitation of the study, we were able to identify and test a mix of four bacteria that were very effective in diminishing the intestinal colonization capabilities of VRE.

Interestingly, a study that was published, while writting this thesis, also identified other commensal bacteria that confer protection against VRE colonization using a mouse model [308]. The bacteria identified belong to the Clostridium cluster XIVa and includes the species *Blautia producta* and *Clostridium bolteae*. Thus different commensal bacteria can confer protection against VRE. Among the bacteria we administered, *Barnesiella* and *Alistipes* colonized with higher levels and are probably responsable for the effect detected, since they restore several bacterial functions, lost after vancomycin treatment, as shown in the metatranscriptomic studies. Especially, *Barnesiella* had already been associated with the resistance against VRE colonization. Results from previous studies suggest that this bacterium could be consuming different oligosaccharides and modify the concentration in aminoacids and SCFAs, which would influence *Enterococcus* colonization capacity. *Oscillibacter* was shown to produce the SCFA valerate, however, its contribution as well as unc. Ruminococcaceae effect are uncertain as their intestinal levels are very low, as compare to *Barnesiella* and *Alistipes*. In addition, as we will discuss later, when we will talk about the transcriptomic studies, their functional contribution to the microbiome is minimal as compare to that one from *Barnesiella* or *Alistipes*.

4.5 Mechanism of protection against VRE colonization associated to the administration of a defined bacterial mix

To investigate the mechanisms by which the bacteria administered inhibit VRE colonization, we first tried to determinate in vitro if any of the bacteria administered produced

a bacteriocin or if we could highlight a nutricional competition. We did not observed any phenotype in the conditions tested, thus we decided to perform metatranscriptomic and metabolomic studies in order to obtain information about the functions expressed by these bacteria *in vivo* and how this functions may have change the metabolic landscape of the gut, subsequently influencing VRE gut colonization.

We observed a clear separation of the metatranscriptomic profile of the untreated mice as compared to those mice that received vancomycin treatment. On the other hand, the overall metatranscriptomic profile was not significantly different among mice treated with antibiotics that received either PBS or the protective bacteria. Interestingly, however, we did find differences in the microbiota structure of these two groups when we studied taxons instead of functions (Figure 3.43 C). It has been demonstrated that the functional profile of the gut microbiota is more conserved than the phylogenetic one [139]. Thus, it is possible that the administration of a few bacteria do not modify the overall metatranscriptome, although it could have a higher influence at the taxonomical level: increase in the phylum Bacteroidetes(*Barnesiella* and *Alistipes*) which is depleted by vancomycin treatment.

As expected by their significantly higher abundance, we found that the number of functions expressed by the *Alistipes* and *Barnesiella* isolates administered was higher in the mice that received the commensal bacteria as compared to those mice that were treated with vancomycin but did not received the protective bacteria. Moreover, the number of functions expressed by these two isolates was similar both in vancomycin treated mice that received the bacterial mix and in untreated mice. This result indicates that the commensal bacteria administered were not only able to colonize the gut after vancomycin treatment but also expressed similar functions as in untreated mice, in the absence of other commensal bacteria (present in untreated and not in vancomycin treated mice). The number of functions expressed by the *Oscillibacter* isolate was not significantly higher in the group that received the commmensal bacteria as compared to the treated mice that received PBS. This is what we expected since the level of the OTU administered did not differ in the these two groups of mice, at least in this particular experiment. On the other hand, the number of functions expressed by the unc. Ruminococcaceae was very low in all groups of mice analyzed. These results indicate that probably the most important bacteria in terms of conferring resistance in our model were *Barnesiella* and *Alistipes*.

Within specific bacterial functions that could be important for conferring colonization resistance, we identified the levels of 33 nr genes that could be annotated with the KEGG database (KO) to be restored or partially restored after the administration of the bacterial mix to treated mice.

Several of these KOs were implicated in the bacterial chemotaxis. Basically, they allow the movement in response to an attractant (such as a nutrient) or a repellent. The methyl-accepting chemotaxis proteins (MCPs) detected, CheW and CheA form a complete bacterial chemotaxis system. In *Clostridium acetobutylicum* this system transmit the movement to the flagellin fliY [312]. In our case, we detected a higher expression of CheW, CheA, and of the flagellin fliC. In *E. coli*, chemotaxis systems have been implicated in the response of attraction to ribose, galactose, aspartate, serine and peptides [313]. Thus, one could hypothesize that the presence of this chemotaxis system could enhance some commensal bacteria to gain access to particular nutrients in the gut. This would be compatible with a mechanism of nutritional competition with VRE, in which the

commensal bacteria could most efficiently reach nutrients, as compared to VRE that does not express this type of chemotaxis system. Nevertheless, it would be necessary to investigate the exact function of the chemotaxis system detected.

On the other hand, the increase of the flagellin fliC after commensal bacterial inoculation, could influence the immune response of the host. It has indeed been demonstrated that the systemic administration of flagellin can restore the murine Reg3γ expression by stimulation of the TLR5 receptors and subsequently enhance the resistance to dense colonization by VRE [207].

Among the KOs identified to be restored by administration of the protective bacteria, the ABC transporters codify for the internalization and use of several saccharides, including the cellobiose. The results obtained by the metabolomic studies are consistent with a decrease of this sugar upon administration of the bacterial mix (Figure 3.48 A). Moreover, we have demonstrated *in vitro* that this is one of the saccharides that promote the highest growth of VRE and that VRE is highly expressing a transporter for internalization of this sugar *in vivo* (Figures 3.52 and 3.50). These results suggest a mechanism by which a nutritional competition for cellobiose upon the administration of the bacterial mix could impair VRE colonization.

In addition, inoculation of the protective bacteria also restored the levels of others sugar transporters that were also depleted after the vancomycin treatment, such as KOs corresponding to the PTS system for the transport and internalization of two sugars, N-Acetyl-galactosamine and N-Acetyl-glucosamine. Notably, N-Acetyl-galactosamine and N-Acetyl-glucosamine are to of the carbon sources that promote higher VRE growth *in vitro*. Thus, consumption of these two sugars upon administration of the protective commensal bacteria could also represent another mechanism by which these bacteria confer resistance against VRE. Interestingly, these two sugars originate from the degradation of the host mucus and commensal bacteria such as *Akkermansia*, which degrades the mucus, could liberate these types of sugars and make them accessible to VRE [314]. Notably, as we shown in figure (Figure 3.15B), an expansion of *Akkermansia* occurs after vancomycin treatment. This bacteria was positively correlated with VRE colonization (Table 3.3). Thus, it is possible that *Akkermansia*, by making available sugars that can be efficiently utilized by VRE, may promote VRE colonization.

Although our results suggest that the protective commensal bacteria may be inhibiting VRE colonization by consuming sugars that are essential for VRE growth, further studies must be performed to validate this hypothesis. One of such studies will be the identification of the levels of sugars in the intestinal tract of the different groups of mice analyzed. We were able to detect the levels of cellobiose, one of the sugars that may be consumed by the protective commensal bacteria. Unfortunately, most of the NMR spectra present in the region corresponding to the oligosaccharides (3.4-4.25ppm) could not be attributed to a specific carbohydrate (Figure 3.47). For this reason, we could not detect the levels of N-acetyl-galactosamine. Nevertheless, a visible increase of this region in the mice that received the antibiotics could be detected, which suggest an increase in oligosaccharides available after the administration of the antibiotic, as documented by Zhao *et al.* [257]. Still, we cannot discard the presence of additional signals corresponding to other molecules in this region. For this reason, we are planning to perform experiments using mass spectrometry (MS) which will allow the identification and cuantification of the different sugars.

Interestingly, we also observed an increase in other KOs related to the use of carbohydrates (Figure 3.46). These functions are mainly related to the pentose phosphate shunt (PPP). The non-oxidative branch of the PPP metabolizes the glycolytic intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate as well as sedoheptulose sugars, yielding ribose 5-phosphate for the synthesis of nucleic acids and sugar phosphate precursors for the synthesis of amino acids. Moreover, the PPP appears to be the only pathway allowing bacteria to utilize sugars such as 3-xylose, 3-ribose, and 3-arabinose [315]. The recovery of the functions corresponding to the main steps of the PPP pathway also support an increased capacity to metabolize oligosaccharides in the group that received the commensal bacteria respect to the mice that just received PBS after vancomycin treatment. Especially, it has been demonstrated in *E. coli* that the saccharide N-Acetyl-galactosamine is metabolized to tagatose 1-6 biphosphate, whose products of degradation can enter the pentose phosphate pathway [316]. Thus, the expression of numerous enzymes from the pentose phosphate pathway in the group of mice that received the bacterial mix is compatible with an increased consumption of the N-Acetyl-galactosamine.

Taken together, the bacterial mix may influence VRE colonization capacity by using saccharides, mainly the cellobiose and the N-Acetyl-galactosamine, and diminishing their availability in the intestinal tract.

Studies performed with prebiotics have already demonstrated that the abundance of different oligosaccharides influence the microbiota composition. For example, it was demonstrated that a mix of cellobiose, raffinose, isomaltulose and an oat $\beta-$glucan hydrolysate, increase the phylum Firmicutes, especially *Lactobacillus* [317], whose levels are positively associated with VRE colonization.

On the other hand, Pultz *et al.* [183] have demonstrated that VRE can grow on an enzymatically degraded mucin and on monosaccharides components of mucins and plants polysaccharides. Curiously, all the VRE tested were able to use the N-Acetyl-glucosamine whereas only one out of nine isolates was able to ferment the N-Acetyl-galactosamine. Thus, the mechanism of competition for this sugar could be strain specific. Interestingly, the differential use of carbohydrates has been suggested as one of the main drivers of the divergent evolution of the different *E. faecium* clades [67]. Especially, the nosocomial clades were found to be enriched in genes coding for the expression of PTS systems and enzymes for the interconversion and metabolism of lactose/cellobiose, glucose, mannose, N-acetylneuraminate, N-acetylmannosamine, and other sialic acids [67]. Therefore, it may be possible to specifically target the clade associated with the nosocomial infections by depleting the specific carbonhydrates utilized by this clade.

The increase in oligosaccharides availability could be responsible for the VRE colonization following the treatment with different antibiotics. After the administration of the vancomycin treatment, as we have demostrated, some oligosaccharides are increased (i.e cellobiose) and probably others that we were not able to identify through NMR. This increase in sugars may be associated to both the expansion of certain mucus degrader bacteria (e.g. *Akkermansia*) and to the extinction of members of the microbiota that utilize sugars.

During the administration of the other antibiotic treatments tested (ciprofloxacin, neomycin, ceftriaxone, ampicillin and clindamycin), we observed an increase of the genus *Bacteroides*. Members of this genus can release simpler carbon sources from the complex carbohydrates of the diet or from the mucus, influencing the microbiota composition [274].

For example, the presence of high levels of *Bacteroides thetaiotaomicron* was associated with an enhanced susceptibility to the infection by *Salmonella typhimurium*, *Clostridium difficile* or *Citrobacter rodentium*, a natural mouse pathogen homologous to the pathogen enterohemorrhagic *Escherichia coli* (EHEC) [181,318]. Thus, the high level of *Bacteroides* detected during several treatments may facilitate the colonization by VRE through an increased availability of simple carbohydrates.

Besides competition for carbohydrates, the metabolomic analysis allow us to consider others alterations of the intestinal environment that could possibly influence the VRE colonization capacity.

The relative abundance of phenol was increased in the group that received the commensal protective bacteria. Phenolic compounds are derived from the microbial catabolism of aromatic amino acids (phenylalanine and tyrosine) and from the degradation of polyphenolic compounds of the diet such as the flavonoids, the anthocyanins or the oleuropein, typically found in the virgin olive oil, to monophenolic compounds [319,320]. Since we detected an increase in aromatic amino acids after admnistration of the protective bacteria, the increase observed in phenol after the administration of the bacterial mix is probably not derived from the metabolism of aromatic amino acids but probably from the metabolization of polyphenolic compounds from the diet. Interestingly, the phenolic compounds can influence the microbiota composition [321]. Especially, the antimicrobial activity of numerous phenolic compounds has been evaluated, identifying compounds highly active both against vancomycin-resistant *Enterococcus faecium* FN-1 and *Enterococcus faecalis* NCTC12201 [322, 323]. Moreover, it has been described that phenol can inhibit the bacterial biofilm formation [324]. VRE produces biofilm, which is clinically relevant as it is responsable for the VRE CLABSIs (Central Line Associated Blood-Stream Infection). Several genetic elements implicated in the VRE biofilm production have been identified (i.e adhesins and pili) [68, 86, 92, 95]. On the other hand, it has been demostrated in a *Vibrio fisheri* model that the proteins involved in the the biofilm formation (i.e. type IV pili) are also implicated in the intestinal colonization [325]. Thus, the increase in phenol could be linked with a diminished VRE colonization capacity through a diminished adhesion to the intestinal epithelium or through an inhibition of VRE growth by specifics phenolic compounds.

The levels of four aminoacids (i.e threonine, serine, proline and leucine) were increased upon the administration of the vancomycin and partially restored in the treated group to which the bacterial mix was administered. Interestingly, the *E. faecium* ATCC 8043 is auxotroph for the threonine [53]. Thus, it would be important to verify whether our strain, VRE ATCC 700221, also imperatively requires this amino acid from the environment, in which case the bacterial mix could be decreasing the VRE colonization through a nutritive competition for this amino acid.

On the other hand, serine is a central amino acid that can be converted to glycine and tryptophan. Moreover, VRE is able to use this aminoacid as unique carbon source, thus a lower availability of serine could affects its growth. We detected an increased expression of a chemotaxis system and of a serine sensor receptor (tsr) in the treated group that received the bacterial mix compared to the treated group that received PBS instead, which supports an increase use of serine and subsequent decreased concentration of serine in this group.

Among the others amino acid whose concentration was diminished in the group that received the commensal bacteria, the proline and leucine are especially interesting. We have seen that VRE was expressing *in vivo* a glycine betaine/proline transporter and a transporter for the internalization of branched-chain amino acids (such as leucine). Thus, the diminished availability of these amino acids may be influencing the growth of VRE *in vivo* and its colonization capacity.

The aromatic aminoacid phenylalanine was increased in the group that received the commensal bacteria, as was the aromatic molecule phenol. Similarly, the relative abundance of trimethylamine was lower during the treatment and the administration of the bacterial mix restored the levels detected in the untreated group (Figure 3.49 C). Trimethylamine is generated from degradation of choline, betaine, and carnitine by members of the gut microbiota [326]. As we have just mentionned, analysing the functions restored upon the administration of the bacterial mix, we detected that a betaine/proline transporter was expressed in the untreated mice and in the vancomycin treated group that received the bacterial mix, but not in the vancomycin treated group that received PBS. Thus, the restoration in the cecal level of trimethylamine could be due to the restoration of the utilization of betaine by the gut microbiota upon the administration of the bacterial mix.

Finally, the administration of the bacterial mix increased the level of the SCFAs butyrate, 2-methyl-butyrate, valerate and, to a less extend, of propionate. SCFAs are metabolic products derived from the bacterial fermentation of the non digestible carbohydrates. The SCFAs have been demonstrated to inhibit the growth of specific pathogens such as *E. coli* strain O157:H7 or to influence the colonization capacity by altering the expression of virulence genes, as seen with *S. typhimurium* pathogenicity island 1 [186,188]. Thus, the increase of the SCFAs may be also contributing to the diminution of the VRE colonization in the group that received the protective bacteria. However, a direct effect of SCFA on VRE growth is unlikely since previous experiments performed in the laboratory did not detected *in vitro* a negative effect of SCFA (i.e. acetate, butyrate and propionate) on VRE growth. Nevertheless, *Oscillibacter*, a known producer of valerate, presented one of the highest negative correlation with VRE levels. Thus, the impact of valerate on the VRE growth, which we have not analyzed yet, should be tested.

In the present state of our study, we reached a significant inhibition of VRE colonization post antibiotic treatment through the administration of four bacterial isolates. The different approaches used to characterize the mechanism of the protection generated several hypotesis that need to be tested and confirmed. Especially, the analysis of the metatranscriptomic data is still in process but, due to the administrative imperative to present this work in the present date, we were not able to include it. For the analysis of the metatranscriptomic data, we still need to determine the contribution of each commensal bacteria administered to the changes observed in the different KEGGs. On the other hand, the statistical analysis was conducted on nr genes. Subsequently we defined the KEEGs of statistically significant nr genes. Alternatively, we are planning to do the analysis the other way around: first define the KEGGs for every nr gene and secondly determine the statistically significant KEEGs among different groups of mice. Nevertheless, the results obtained with our first approach, shown in this thesis, have identified changes in some bacterial functions, which indeed correlate with changes in different metabolites (i.e. increase in the expression of cellobiose transporters and decrease in cellobiose), which have allowed us to generate some hypothesis regarding the mechanisms of protection by

protective commensal bacteria. The definitive demonstration of these mechanisms will require additional experiments as we will discuss below.

From the results obtained to date, the main mechanisms retained and to be tested are (I) the nutritional competition for oligosaccharides, especially for cellobiose and N-Acetyl-galactosamine, (II) the nutritional competition for aminoacids, especially for threonine, serine, proline and leucine, (III) a possible nutritional competition for choline, betaine, (IV) the possible inhibition of biofilm formation/adhesion to intestinal epithelium by phenol or the liberation of phenol compounds with an inhibitory activity against VRE, (V) the inhibitory activity of the SCFAs whose levels were partially restored upon the baterial mix administration, especially valerate.

From the results obtained, the first hypothesis to be tested will be the nutritional competition for the oligosaccharides (I) as it was supported by the different analysis performed (both when we studied the changes in the metatranscriptome and metabolome due to the commensal bacteria administered, and when we studied the VRE transcriptome). We plan to first verify *in vitro* that the bacteria administered can use these oligosaccharides. Next, we will perform a mass spectrometry analysis to determine the concentration of the different oligosaccharides in the untreated mice and the treated mice that received either the bacterial mix or PBS. Knowing the concentration present in the treated mice that received PBS, we will be able to perform an *in vitro* competitive assay between the probiotic(s) that use it and VRE to assess whether their presence can limit the VRE growth through this mechanism.

To test a possible mechanism of nutritional competition for the aminoacids threonine, serine, proline and leucine (II), we will first determine whether VRE ATCC700221 is auxothroph for these aminoacids and if the probiotics are able to use it. Next we will determine the intestinal concentration of these aminoacids and we will also perform an *in vitro* competitive assay between the probiotic(s) that use it and VRE to assess whether their presence can limit the VRE growth through this mechanism.

As we have already mentioned, (V) the inhibitory effect of the SCFAs acetate, butyrate and propionate on VRE growth was already tested *in vitro*. Thus, we will only test how different concentrations of valerate and 2-methybutyrate affect the VRE growth *in vitro*.

The others mechanisms proposed present less support and will be tested secondarily. The possible nutritional competition for choline or betaine (III) was infered from the increased concentration of its product of degradation, trimethylamine, in the treated mice that received the bacterial mix respect to the treated mice that received PBS. Moreover, a transporter for betaine/proline was also increased in the group that received the bacterial mix.

Finally, to assess the possible action of phenol (IV) on VRE intestinal adhesion, we would test the effect of different concentrations of phenol on VRE adhesion to a Caco-2 cells culture (colonocytes).

Conclusions

Study of the effect of oral vancomycin administration on the composition of the human gut microbiota.

(I) Oral vancomycin induces drastic and consistent changes in the human intestinal microbiota, including the depletion of all the operational taxonomical units belonging to the phylum Bacteroidetes.

(II) Upon vancomycin cessation, the microbiota recovery rate varies considerably among subjects, which could influence, as validated in mice, the level of susceptibility to VRE intestinal colonization.

Study of the gut microbiota alterations caused by the administration of different spectrum antibiotics on the VRE intestinal colonization capacity.

(III) Antibiotic treatments of different spectrum (ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin and vancomycin), cause broader alterations in the murine gut microbiota than expected according to their spectrum of action.

(IV) All antibiotics studied (ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin and vancomycin) cause a decrease in murine microbiota richness. Two weeks after the end of the treatment, the microbiota richness only recovers its baseline levels in the case of neomycin treatment. Most of the antibiotics studied (with the exception of ciprofloxacin) also cause a decrease in the murine microbiota diversity and biomass. Two weeks after the withdrawal of the antibiotic treatments, the microbiota diversity does not recover the baseline levels except after neomycin treatment. In contrast, microbiota biomass recovers its baseline levels two weeks after the cessation of any of the antibiotics studied.

(V) All the antibiotic treatments tested in the present work (ciprofloxacin, neomycin, ceftriaxone, ampicillin, clindamycin, vancomycin) cause a decrease in the abundance of the phyla Actinobacteria and TM7 and the genera *Turicibacter*, *Ruminococcus*, Allobaculum and *Coprobacillus* in the fecal murine microbiota.

(VI) The Firmicutes phylum abundance in murine fecal samples is diminished by the treatment with ampicillin, ceftriaxone and clindamycin, whereas vancomycin, whose spectrum of action should target this phylum, do not decrease the abundance of this

phylum both in murine or human fecal samples. However, except for ciprofloxacin, all the antibiotics decrease the bacterial richness within the phylum Firmicutes in fecal samples from mice.

(VII) Bacteroidetes phylum abundance is decreased by vancomycin treatment but not by ciprofloxacin, neomycin ceftriaxone, ampicillin or clindamycin, which indeed induce an increase of the Bacteroidetes genus *Bacteroides*. On the other hand, the bacterial richness within this phylum increases after neomycin treatment and decreases after ciprofloxacin, ceftriaxone, clindamycin and vancomycin. Two weeks after vancomycin, clindamycin and ceftriaxone cessation, mice recover a microbiota with lower abundance of the phylum Bacteroidetes.

(VIII) Vancomycin and clindamycin increase the abundance of the Proteboacteria phyla whereas ciprofloxacin and ceftriaxone treatment decrease the abundance of this phylum in murine fecal samples. Alterations observed within this phylum are detected during the treatment and two weeks after its withdrawal.

(IX) Antibiotic treatments that cause more alterations of the gut microbiota promote higher levels of VRE murine intestinal colonization, being in decreasing order vancomycin, clindamycin, ampicillin, ceftriaxone, neomycin and ciprofloxacin, the treatments that promote the highest VRE intestinal colonization levels. Two weeks after the withdrawal of the treatment, vancomycin, clindamycin and ceftriaxone still promote high levels of intestinal colonization by VRE, ampicillin and ciprofloxacin promote intermediate levels and neomycin, like untreated mice, does not allow VRE intestinal colonization

Study of the protective capacity of commensal isolates against VRE intestinal colonization.

(X) Higher relative abundances of *Alistipes*, *Allobaculum*, *Barnesiella*, *Oscillibacter* and unclassified taxa from the families Lachnospiraceae, Porphyromonadaceae and Ruminococacceae are associated with resistance against VRE intestinal colonization in mice.

(XI) Higher relative abundances of *Escherichia/Shigella*, *Enterococcus*, *Akkermansia*, *Parasutterella*, *Lactobacillus* and unclassified taxa from the families Enterobacteriaceae, Burkholderiales and Desulfovibrionaceae are associated with higher levels of VRE intestinal colonization in mice.

(XII) Oral inoculation of mice with a bacterial mixture containing three isolates belonging to the taxa *Alistipes*, *Barnesiella*, *Oscillibacter* and an isolate from the family Ruminococacceae decreases by 3 orders of magnitude the VRE intestinal colonization levels.

Study of the mechanisms by which the identified protective commensal bacteria decrease VRE intestinal colonization.

(XIII) Oral inoculation of mice with a bacterial mixture containing three isolates belonging to the taxa *Alistipes*, *Barnesiella*, *Oscillibacter* and an isolate from the family Ruminococacceae restores the expression of genes, reduced upon vancomycin treatment, that codify for saccharides transporters (cellobiose, N-acetyl-galactosamine, N-acetyl-glucosamine), for a serine sensitive receptor and for genes involved in the synthesis of SCFAs.

(XIV) Oral inoculation of mice with a bacterial mixture containing three isolates belonging to the taxa *Alistipes*, *Barnesiella*, *Oscillibacter* and one isolate from the family Ruminococacceae decreases the intestinal concentration of the oligosaccharide cellobiose, the SCFA lactate and the aminoacids proline, serine, leucine and threonine, which are increased upon vancomycin treatment. On the other hand, administration of these bacteria increase the concentration of trimethylamine, phenylalanine, phenol, biliary acids, and the SCFAs butyrate, 2-methylbutyrate, valerate and propionate, which are decrease upon vancomycin treatment.

(XV) During colonization of the murine intestinal tract, VRE expresses the different subunits of the transporters responsible for the internalization of cellobiose an N-acetyl-galactosamine and transporters that allow the internalization of branched-chain aminoacids, proline and arginine.

(XVI) Out of 190 carbon sources tested, N-acetyl-glucosamine, N-acetyl-galactosamine and cellobiose are among the 10 carbon sources that promote the highest VRE growth in vitro under anaerobic conditions. Moreover, VRE can grow using uniquely the aminoacid serine as a carbon source in vitro under anaerobic conditions.

(XVII) Although additional studies should be performed for validation, the results presented in this thesis support a model in which the administered bacteria *Alistipes*, *Barnesiella*, *Oscillibacter* and the unclassified isolate from the family Ruminococacceae decrease the intestinal levels of nutrients that can be utilized by VRE for growth, which reduces the VRE capacity of colonizing the intestinal tract.

Resumen de la Tesis

Introducción

El tracto gastrointestinal está colonizado por centenares de especies de bacterias comensales. Se ha visto en los últimos años que dichas bacterias presentan un papel crucial en la prevención y resolución de enfermedadesinfecciosas. La microbiota comensal inhibe a patógenos oportunistas mediante mecanismos (i) de interacción directa, por producción de sustancias inhibitorias o competición por nutrientes (ii) indirectos, por la inducción del sistema inmune. El tratamiento con antibióticos altera la composición de la microbiota intestinal promoviendo la colonización intestinal por patógenos oportunistas [179]. Dicha situación es preocupante especialmente en el ámbito hospitalario debido al conjunto de (I) un uso intensivo de los antibióticos, (II) una alta concentración de patógenos multi-resistentes, (III) la presencia de pacientes especialmente susceptibles por su situación fisiológica (catéteres) y baja inmunidad [23]. Por ello, se registran un gran número de enfermedades nosocomiales, especialmente complicadas de tratar por el carácter multi-resistente de las cepas colonizadoras. Entre ellas, *Enterococcus faecium* Vancomicina Resistente (EVR) es importante porque su prevalencia ha aumentado de 6.2% en 2011 a 7.9% en 2014 en Europa y es resistente a la mayoría de los antibióticos disponibles [30].

Objetivos

En la primera parte del presente trabajo, hemos investigado la relación entre el tratamiento antibiótico y la alteración de la microbiota intestinal. Los objetivos de esta primera parte son: (I) entender las alteraciones en la microbiota asociadas con el tratamiento por vancomicina tanto en pacientes como en el modelo de ratón. Vancomicina es uno de los antibióticos relacionados con la colonización intestinal y con septicemias por EVR, por ello quisimos evaluar la influencia de las alteraciones producidas en la microbiota por este antibiótico con la capacidad de colonización de EVR. (II) Estudiar la influencia de otros antibióticos de diferente espectro anti-bacteriano al de vancomicina sobre la microbiota intestinal así como la capacidad de recuperación de la misma tras el cese de tratamiento antibiótico utilizando un modelo de ratón

Conociendo el efecto de diferentes antibióticos sobre la microbiota, nos centramos en la relación entre alteraciones de la microbiota y la capacidad de colonizar de EVR. En este caso nos planteamos: (III) relacionar las alteraciones de la microbiota provocadas por diferentes antibióticos con la pérdida de resistencia a la colonización por EVR, (IV) identi-

ficar bacterias comensales de la microbiota intestinal que protegen frente a la colonización intestinal por el EVR, (V) restaurar la resistencia frente a la colonización mediante la administración de dichas bacterias, (VI) identificar mecanismos por los cuales estas cepas bacterianas comensales protegen frente a EVR.

Metodología

Estudio de los cambios producidos por vancomicina en la microbiota intestinal de pacientes. Para cumplir el primer objetivo, hemos analizado los datos de la microbiota fecal de un estudio clínico realizado en pacientes con artritis reumatoide (AR) [212]. El propósito del estudio era evaluar la posible acción benéfica de la vancomicina sobre AR mediante la disminución de bacterias pro-inflamatorias, sensibles a dicho antibiótico. Para ello, un grupo de pacientes con AR nuevamente diagnosticada se reclutaron y recibieron vancomicina vía oral durante dos semanas (250 mg cuatro veces al día) seguido de metotrexato (tratamiento para la AR). Como control, un grupo de pacientes solamente recibió metotrexato. Entre los criterios de inclusión, los pacientes no debían haber recibido tratamiento antibiótico en los últimos tres meses. Por tanto, este estudio nos permitió evaluar los cambios de la microbiota asociados al tratamiento por vancomicina sin previo tratamiento antibiótico (lo cuál aun no se había descrito en comparación con otros estudios donde los pacientes evaluados, además de recibir vancomicina habían recibido otros antibióticos).

Se recolectaron muestras fecales antes del tratamiento, al finalizar el tratamiento con vancomicina y a las 2, 6, 14 y 22 semanas tras finalizar el tratamiento. Se extrajo el ADN bacteriano de estas muestras y se determinó su composición microbiana mediante secuención masiva de la región V2-V3 del 16s rRNA, un gen presente en todas las bacterianas que permite clasificarlas taxonómicamente.

Se evaluó la diversidad bacteriana en las muestras según el tratamiento recibido así como su recuperación tras el cese del tratamiento. Para ello se utilizó la distancia de disimilitud de la microbiota (Unweighted Unifrac distance), se calcularon el número de OTUs (unidad taxonómica operacional, similar al nivel de filogenético de especie), el indice de filodiversidad y el indice de Shannon. Por otro lado se identificaron las abundancias relativas a nivel de filo, género y OTUs para cada una de las muestras. Con el fin de analizar cambios en cada uno de los parámetros analizados, se utilizó el test no-paramétrico de wilcoxon y se comparó cada uno de los tiempos post-tratamiento con el tiempo pre-tratamiento. Estos análisis nos indicaron las alteraciones en la microbiota que ocurrieron en la mayoría de los pacientes. Por otro lado, evaluamos también las alteraciones en la microbiota a nivel individual para cada uno de los sujetos, calculando que porcentaje de las OTUs presentes antes del tratamiento que se recuperaban tras el cese del tratamiento antibiótico. Así, hemos demostrado que la la tasa de recuperación de la microbiota después de la retirada del tratamiento era muy variable entre sujetos. Mientras que algunos pacientes llegaban a recuperar la mayor parte de su microbiota, otros no llegaban a recuperar el 89% de las bacterias más abundantes de su microbiota, incluso 22 semanas tras parar el tratamiento antibiótico.

Estudio del efecto de los cambios producidos en la microbiota tras el tratamiento con vancomicina en la capacidad de colonización de EVR. Como a nivel clínico un porcentaje de los pacientes tratados con vancomicina son colonizados por patógenos, a nivel intestinal, después de la finalización del tratamiento con vancomicina, decidimos evaluar si la diferencia observada en la recuperación de la microbiota podría influenciar la capacidad de colonización intestinal por el patógeno EVR. Para ello, hemos desarrollado un modelo de infección por EVR en ratones tratados con vancomicina.

Los experimentos con ratones fueron llevado a cabo en el "Servei Central de Suport a la Investigació Experimental" de la Universidad de Valencia, utilizando hembras de 7 semanas C57BL6J compradas a la compañía Charles River laboratories. Se trataron los ratones con vancomicina (en el agua de bebida, 0.5g/l) durante una semana, se cogió una muestra fecal para analizar la composición de la microbiota, posteriormente se infectaron oralmente con 10E6 UFCs200 ul de EVR y se evaluaron los niveles de colonización intestinal a los dos días post-infección. Por otro lado, para otro grupo de ratones, se dejó un periodo de recuperación de dos semanas entre el final del tratamiento y la toma de muestra y infección. Para evaluar la colonización intestinal por EVR a los dos días de la infección, se plaquearón varias diluciones de una muestra fecal en medio BEA agar complementado con vancomicina y ampicilina.

En nuestro modelo de tratamiento oral con vancomicina en ratones, se inducen cambios drásticos y consistentes en la microbiota intestinal, tál y como se observó en humanos. Utilizando un número de muestras elevado, se observó también una variabilidad inter-individual que nos permitió evaluar la relación entre tasa de recuperación de la microbiota después de finalizar el tratamiento y capacidad de colonización por EVR. Gracias a este modelo, hemos podido demostrar que la diferencia en la tasa de recuperación de la microbiota después de finalizar el tratamiento es clinicamente relevante ya que una recuperación de la microbiota menor en ratón se correlaciona con una capacidad de colonización por EVR significativamente más alta.

Estudio de las alteraciones de la microbiota provocadas por el tratamiento con antibióticos de distinto espectro y de su efecto en la capacidad de colonización de EVR. Posteriormente, hemos utilizado este modelo de infección en ratones para investigar el impacto de antibióticos de varios espectros en la microbiota intestinal y relacionar esas alteraciones con la capacidad de EVR de colonizar el intestino. Además, estos datos nos permitieron identificar bacterias comensales de la microbiota capaces de proteger frente a la colonización por EVR.

Para ello, hemos tratado los ratones con antibióticos de distinto espectro (ciprofloxacina, neomicina, ceftriaxona, ampicilina, clindamicina y vancomicina), lo cual nos permitió conseguir diferentes tipos de disbiosis (alteración de la microbiota). Posteriormente, hemos infectado vía oral con EVR a los ratones tratados. Los niveles de EVR excretados en heces fueron determinados por recuento de colonias en medio específico y se analizó la composición de la microbiota de los ratones gracias a la secuenciación masiva del gen 16s rRNA. En este caso también, se infectaron ratones durante el tratamiento o después de dos semanas de recuperación.

Para cuantificar las alteraciones asociados a diferentes tratamientos antibióticos, hemos evaluado la perdida de biodiversidad (número de OTUs, Shannon) y de biomasa (ng ADN/g heces), una aproximación de la carga bacteriana total. Se observó una dismin-

ución de la biomasa y del indice de Shannon después de todos los tratamientos excepto ciprofloxacina. Las alteraciones más drásticas fueron asociadas al tratamiento con clindamicina y vancomicina.

Por otro lado, también hemos evaluado cuanto diferentes eran las microbiota resultantes tras la administración de los diferentes antibióticos mediante NMDS (Non-metric Multi-Dimensional Scaling) y determinado los taxa significativamente alterados (a nivel de phylum y genus). Además, hemos evaluado la perdida de diversidad intra-filo e intra-género, lo que nos ha permitido determinar que durante el tratamiento con clindamicina, apenas 5 OTUs consituyen el 97% de la microbiota total. Realizando los mismos análisis sobre las muestras obtenidas después de dos semanas de recuperación, se observó una recuperación de la microbiota en la mayoría de los casos y el establecimiento de un estado de microbiota alternativo en el caso de los ratones tratados con ceftriaxona y clindamicina.

Posteriormente, el análisis conjunto de las alteraciones producidas por los distintos antibióticos con los niveles de EVR permitieron identificar aquellas bacterias comensales intestinales que se asocian a la resistencia frente a la infección (su eliminación por determinados antibióticos se asocia a un incremento de los niveles intestinales de EVR). Para ello, se realizó una correlación de spearman y se seleccionaron las bacterias por las cuales obtuvimos una correlación negativa entre su abundancia y los niveles de EVR detectados en heces a los dos días de la infección (p-valor ajustado <0.05). Como segundo criterio, las bacterias tenían que estar presente en todos los ratones no tratados. De este modo, evitamos seleccionar bacterias no detectadas normalmente en la microbiota de los ratones pero que hubieran expandido en los tratamientos que promueven bajos niveles de colonización intestinal por EVR y nos aseguramos la posibilidad de aislar las bacterias seleccionadas para testar su capacidad protectora . Se confirmaron los resultados mediante un LDA ("linear discriminant analysis"), el cual está menos influenciado por valores extremos. De este modo, se determinó que la presencia de los géneros bacterianos *Alistipes*, *Allobaculum*, *Barnesiella*, *Oscillibacter* y de miembros no identificados de las familias Lachospiraceae, Porphyromonadaceae y Ruminococacceae se asocian a la resistencia frente a la colonización por EVR. Por otra parte, se detectó que los géneros bacterianos *Escherichia/Shigella*, *Enterococcus*, *Akkermansia*, *Parasutterella*, *Lactobacillus* y miembros no identificados de las familias Enterobacteriaceae, Burkholderiales y Desulfovibrionaceae se asocian positivamente con la colonización por EVR.

Aislamiento de bacterias comensales de interés. Para testar la capacidad protectora de las bacterias asociadas a la resistencia frente a la colonización por EVR, fue necesario aislarlas desde la flora intestinal de ratones no tratados. Para ello, se han estudiado los medios y las condiciones adecuadas para su crecimiento y aislamiento. Concretamente, se crecieron muestras cecales en varios medios de cultivos, se recogieron las comunidades bacterianas crecidas y se secuenció el gen 16s rRNA por secuenciación masiva mediante Miseq Illumina para determinar que bacterias eran capaces de crecer en cada condición. Se determinó el indice de Shannon para averiguar que medio presentaba la mayor diversidad bacteriana al mismo tiempo que se identificaron cada una de los taxones presentes en cada medio. Todas las bacterias de interés crecían en el medio "Columbia Blood Agar" incubado a 37ºC en condiciones anaérobicas por lo que utilizamos este medio para aislar las bacterias de interés. Posteriormente, una vez seleccionado el mejor medio para aislar las bacterias de interés, crecimos una muestra cecal en el medio "Columbia Blood Agar"

en una dilución adecuada para obtener colonias individuales. Las colonias crecidas se aislaron y se identificaron mediante secuenciación del gen 16s rRNA por la metodología de Sanger. Esta aproximación nos permitió aislar la mayoría de los taxones de interés excepto *Oscillibacter*, que presentaba una abundancia relativa inferior a 0.01% en las condiciones de cultivo utilizadas. En este caso, se diseñaron una par de cebadores específicos de la familia Ruminococcaceae (a la cual pertenece el género *Oscillibacter*) para poder determinar directamente por PCR (sin necesidad de secuenciar el gen 16s rRNA) si la bacteria crecida era este taxón o no. Finalmente, fuimos capaces de conseguir aislados de las diferentes bacterias de interés, incluyendo *Oscillibacter*.

Estudio de la capacidad protectora frente a la colonización por EVR de las bacterias comensales aisladas. Posteriormente, la capacidad protectora de las bacterias aisladas fue testada en un modelo de ratones tratados con vancomicina. Se trataron los ratones una semana con vancomicina, se les administró una mezcla de las bacterias de las cuales queríamos testar la capacidad protectora los tres días siguientes a la finalización del tratamiento. Dos semanas tras parar el tratamiento antibiótico, se recogió una muestra fecal que nos permitió verificar mediante secuenciación masiva del gen 16s rRNA si dichas bacterias habían colonizado los ratones. Tras la recogida de dicha muestra, los ratones se infectaron vía oral con EVR. Los niveles de colonización intestinal por EVR se determinaron mediante plaqueo de muestras fecales a los dos días tras la infección, como en los previos experimentos ya descritos. Como grupo control, se infectó un grupo de ratones tratados con vancomicina que no recibió la mezcla de bacterias protectoras.

En este modelo de ratones tratados con vancomicina se administraron *Alistipes*, *Allobaculum*, *Barnesiella*, *Oscillibacter* y miembros no identificados de las familia Ruminococacceae. No se administraron bacterias de la familia Lachnospiraceae (también asociadas con la resistencia frente a EVR, porque de manera habitual el ratón recupera estás bacterias tras el tratamiento con vancomicina mientras que no recupera las bacterias que si se administraron). Mediante este modelo, se obtuvo una disminución de más de 100 veces en la capacidad de EVR de colonizar el intestino, en el grupo que recibió las bacterias comensales en comparación con el grupo de ratones que no las recibió. Como control del experimento, administramos la bacteria *Klebsiella pneumoniae*, no asociada con la resistencia a la colonización por EVR. Como cabía esperar, la administración de dicha bacteria no confirió protección.

Posteriormente, verificamos que la biomasa (aproximación de la abundancia bacteriana) de los ratones tratados con vancomicina no estaba disminuida con respecto a los ratones no tratados, indicando que probablemente el mecanismo por el cual las bacterias comensales suministradas protegían frente a EVR no se trataba de un mecanismo de inhibición inespecífico debido a la ocupación de un nicho vacío.

Antes de averiguar el mecanismo de protección frente a la infección por EVR, intentamos simplificar la mezcla de bacterias administrada. Para ello, se utilizó el modelo descrito para probar la actividad protectora de la mezcla de bacterias, administrando las bacterias individualmente. Ninguna bacteria administrada por si misma consiguió una disminución de la colonización por EVR igual a la obtenida tras la administración de la mezcla completa, aunque varias de las bacterias administradas si que disminuyeron la capacidad de colonización por EVR pero en menor medida. Posteriormente, se administró la mezcla de bacterias, eliminando *Oscillibacter* de la misma (individualmente no producía

ningún tipo de disminución de la colonización por EVR) ó *Allobaculum* (los análisis por LDA no lo habían identificado como una de las bacterias más relevantes dentro de las asociadas con la resistencia frente a EVR). Como resultado, decidimos administrar una nueva mezcla de bacterias que contenía *Alistipes*, *Barnesiella*, *Oscillibacter* y un aislado no identificado de las familia Ruminococacceae.

Estudio del mecanismo de protección por el cual las bacterias comensales identificadas protegen frente a EVR. Posteriormente, para averiguar el mecanismo de protección frente a la infección por EVR, nos hemos centrado en dos enfoques complementarios, (i) la determinación de las alteraciones a nivel intestinal, entre otros el cambio en la disponibilidad de los diferentes nutrientes, asociados con la administración de la mezcla de bacterias y (ii) la caracterización de los requerimientos genéticos y nutricionales de EVR para colonizar el intestino y crecer.

(i) Para determinar como nuestros probióticos influencian la colonización por EVR, nos hemos centrado en mecanismos de interacción directa (competición por nutrientes, producción de sustancias inhibitorias). No hemos estudiado mecanismos de acción indirecta (inducción de la respuesta inmune) ya que en un estudio anterior se describió que la microbiota es capaz de eliminar EVR en ausencia de componentes fundamentales del sistema inmune [211].

Hemos decidido investigar la actividad de los probióticos mediante técnicas ómicas que nos permiten apreciar tanto la producción de substancia inhibitorias como mecanismos de competición nutricional.

Para conseguir las muestras necesarias para dicho estudio, hemos tratados ratones con vancomicina durante una semana y posteriormente los ratones alojados de dos en dos en jaulas, recibieron las bacterias protectoras o PBS (Tampón fosfato salino). Dos semanas después de finalizar el tratamiento, se separaron los ratones y uno fue sacrificado para conseguir el contenido del ciegomientras que el otro ratón fue infectado con EVR para determinar los niveles de colonización intestinal. Ratones que se encuentran en la misma jaula recuperan una microbiota muy similar tras el cese del tratamiento antibiótico por lo que el ratón infectado con EVR nos indicaba si esa microbiota protege o no frente a la infección, mientras que el otro ratón de la misma jaula nos aporta las muestras necesarias para los estudios ómicos.

Posteriormente, se realizó un análisis meta-transcriptómico sobre las muestras de estos ratones tratados que recibieron la mezcla de bacterias protectoras o no, así como de ratones no tratados, con el fin de determinar que funciones bacterianas se recuperaban gracias a la administración de las bacterias protectoras. 28 KOs (Kegg Orthology, ORFs anotados con la base de datos KEGG) no se detectaron después del tratamiento con vancomicina pero estaban expresados en el grupo de ratones que recibió la mezcla de bacterias protectoras y en los ratones no tratados. Estos KO codifican entre otros para transportadores que permiten la internalización de azucares (transportadores lactosa/L-arabinosa; para la celobiosa, el myo-inositol, la N-acetil-galactosamina y el D-glucosaminato), así como su metabolización (restauración del la vía de las pentosas fosfatos). Se detectó también el incremento de un receptor sensitivo a la serina así como KOs correspondientes a pasos de la síntesis de la histidina y de la lisina.

Para averiguar si estas diferencias en expresión génica entre el grupo de ratones que recibió las bacterias intestinales o no se reflejaban en cambios en la concentración de

los diferentes metabolitos, hemos analizado el metaboloma intestinal de las muestras anteriores. Este análisis se hizó en colaboración con el centro de investigación Príncipe Felipe (CIPF) mediante estudio de resonancia magnética nuclear (RMN). Gracias a este estudio, hemos confirmado que (i) la disponibilidad intestinal en azucares es baja en ratones no tratados (ii) dicha disponibilidad aumenta drásticamente después del tratamiento con vancomicina (iii) la administración de nuestras bacterias protectoras disminuye la disponibilidad intestinal de azucares después del tratamiento. Concretamente, hemos podido demostrar estas conclusiones para la celobiosa (un azúcar de la dieta). Además, se ha constatado que las bacterias protectoras modifican el metaboloma intestinal incrementando algunos metabolitos que se han asociado con la interferencia frente a la colonización intestinal por patógenos (i.e. ácidos grasos de cadena corta, fenol). Por otra parte, se detectó una alteración importante de las concentraciones de varios aminoácidos. Entre ellos destacamos un aumento de la concentración de serina durante el tratamiento y su disminución en el grupo de ratones que recibió las bacterias protectoras.

Estos resultados sugieren que las bacterias administradas podrían estar compitiendo con EVR para el consumo de diferentes azucares y posiblemente de algunos amino ácidos. Además, el incremento en ácidos grasos de cadena corta y fenol podría participar en la resistencia a la colonización por EVR inhibiendo su crecimiento.

Por otro lado, averiguamos los requerimientos nutricionales de EVR.Con este fin, hemos identificado los genes expresados por EVR *in vivo*. En primer lugar hemos secuenciado el genoma de EVR. En segundo lugar, utilizando secuenciación masiva, hemos obtenido el transcriptoma de EVR *in vivo* en ratones tratados con antibióticos y colonizados por EVR. Se secuenció el meta-transcriptoma completo de la muestra y posteriormente se mapearon las secuencias obtenidas contra el genoma de EVR. Así, se determinó el transcriptoma de EVR *in vivo* en presencia de la microbiota intestinal.

Los resultados obtenidos indican que unos de los genes altamente expresados *in vivo* por EVR corresponden a transportadores de N-acetil-galactosamina y celobiosa, justo los azúcares que podrían estar siendo utilizados por las bacterias comensales, como indicaron nuestros estudios metabolómicos y metatranscriptómicos. Por otra parte, EVR expresaba transportadores para la internalización de varios amino ácidos (i.e un transportador para la internalización de glicina betaina/prolina, arginina/ornitina y aminoácidos ramificados).

Posteriormente, para determinar que relevancia tiene la disponibilidad de diferentes fuentes de carbono para el crecimiento de EVR, hemos realizado un "array" de nutrientes (metodología Biolog). Básicamente, un "array" consiste en una placa de 96 pocillos con una única fuente de carbono por cada pocillo. Se inocula el "array" con EVR en un medio que contenga los nutrientes necesarios a su crecimiento excepto el carbono. Posteriormente, midiendo el crecimiento de EVR en cada pocillo, se puede determinar que fuentes de carbono la bacteria testada utiliza y con que eficacia.

Hemos puesto a punto la metodología necesaria con el fin de poder realizar dichos "arrays" en las condiciones anaeróbicas que se encontrarían en el intestino (aplicación de un aceite en la superficie del pocillo del "array" con el fin de impedir la entrada de oxígeno). Los resultados obtenidos del "array" de nutrientes indican que el crecimiento de EVR está influenciado por el tipo de fuente de carbono disponible. Cabe destacar que unos de los azúcares que permiten un mayor crecimiento de EVR son la N-acetilgalactosa y la celobiosa, los azúcares que podrían estar siendo secuestrados tras la administración de

las bacterias protectoras identificadas. Curiosamente, EVR es capaz de crecer utilizando a la serina como única fuente de carbono.

El conjunto de nuestros resultados sugiere la hipótesis de una competición nutricional para la celobiosa y la N-acetilgalactosa como mecanismo de protección frente a la colonización intestinal por EVR. Se ha visto que EVR utiliza muy eficientemente estos dos azucares *in vitro* y que expresa los transportadores para su utilización *in vivo*. Por otra parte, la administración de las bacterias protectoras disminuye la disponibilidad de azucares *in vivo*, entre los cuales se pudo identificar la celobiosa. Además, se vio un incremento de transportadores para el consumo tanto de celobiosa como de N-acetilgalactosa en el grupo de ratones que recibió las bacterias protectoras.

Además, se ha visto que EVR puede utilizar la serina como fuente de carbono y que las concentraciones de este aminoácido están incrementadas después del tratamiento con vancomicina y disminuidas en el grupo de ratones que recibió las bacterias protectoras. En concordancia con la disminución de las concentraciones de serina en el grupo de ratones que recibió las bacterias protectoras, se detectó la expresión de un receptor sensor de serina (tsr). Sin embargo, el análisis realizado no nos permitió identificar transportadores asociados a la utilización de serina *in vivo* por EVR.

Finalmente, el incremento de ácidos grasos de cadena corta y de fenol en el grupo de ratones que recibió las bacterias protectoras podría intervenir en el mecanismo de protección frente a la colonización por EVR, inhibiendo su crecimiento.

Para confirmar que nuestras bacterias compiten con EVR mediante la utilización de nutrientes requeridos para su crecimiento, especialmente de los azucares celobiosa y la N-acetilgalactosa, será necesario confirmar (i) confirmar la disminución de N-acetilgalactosa mediante espectrometría de masas, ya que no pudo ser detectada por NMR, (ii) realizar ensayos de competición por el nutriente entre las bacterias comensales administradas y EVR con el fin de demostrar su papel como mecanismo protector frente a EVR.

Conclusiones

Estudio del efecto de la administración oral de vancomicina sobre la composición de la microbiota intestinal humana.

(I) La vancomicina oral induce cambios drásticos y permanentes en la microbiota intestinal humana, incluyendo la eliminación de todas las unidades taxonómicas operativas (OTUs) pertenecientes al filo Bacteroidetes.

(II) Al parar el tratamiento con vancomicina, la tasa de recuperación de la microbiota varía considerablemente entre distintos sujetos, lo que podría influir, tal y como se ha validado en ratones, el nivel de susceptibilidad a la colonización intestinal por el *Enterococo*-vancomicina resistentes (EVR).

Estudio de las alteraciones en la microbiota intestinal causadas por la administración de diferentes antibióticos de distinto espectro sobre la capacidad de colonización intestinal por EVR.

(III) Los tratamientos con antibióticos de diferente espectro (ciprofloxacino, neomicina, ceftriaxona, ampicilina, clindamicina y vancomicina), causan alteraciones más amplias en la microbiota intestinal murina de lo esperado según su espectro de acción.

(IV) Todos los antibióticos estudiados (ciprofloxacino, neomicina, ceftriaxona, ampicilina, clindamicina y vancomicina) provocan una disminución de la riqueza de la microbiota de ratón. Dos semanas después del final del tratamiento, la riqueza de la microbiota sólo recupera sus niveles basales en el caso del tratamiento con neomicina. La mayoría de los antibióticos estudiados (con la excepción de la ciprofloxacina) también causan una disminución en la diversidad y biomasa de la microbiota de ratón. Dos semanas después de la retirada de los tratamientos antibióticos, la diversidad de microbiota no recupera los niveles basales excepto después del tratamiento con neomicina. Por el contrario, la biomasa de la microbiota recupera sus niveles basales dos semanas después del cese de cualquiera de los antibióticos estudiados.

(V) Todos los tratamientos probados en el presente trabajo (ciprofloxacina, neomicina, ceftriaxona, ampicilina, clindamicina, vancomicina) provocan una disminución en la abundancia de los filos Actinobacteria y TM7 y los géneros *Turicibacter*, *Ruminococcus*, *Allobaculum* y *Coprobacillus* en la microbiota fecal de ratón.

(VI) La abundancia del filo Firmicutes en muestras fecales de ratón se ve disminuida por el tratamiento con ampicilina, ceftriaxona y clindamicina, mientras que la vancomicina, cuyo espectro de acción está dirigido frente a este filo, no disminuye la abundancia de este filo en muestras fecales tanto de ratón como humanas. No obstante, a excepción de la ciprofloxacina, todos los antibióticos disminuyen la riqueza bacteriana detectada dentro del filo Firmicutes en muestras fecales de ratones.

(VII) La abundancia de filo Bacteroidetes disminuye con el tratamiento con vancomicina, pero no ciprofloxacina, neomicina, ceftriaxona, ampicilina o clindamicina, que de hecho inducen un aumento del género *Bacteroides*, incluído dentro del filo Bacteroidetes. Por otro lado, la riqueza bacteriana dentro de este filo aumenta después del tratamiento con neomicina y disminuye después de ciprofloxacina, ceftriaxona, clindamicina y vancomicina. Dos semanas después de parar el tratamiento con vancomicina, clindamicina o ceftriaxona, los ratones recuperan una microbiota con menor abundancia del filo Bacteroidetes.

(VIII) Vancomicina y clindamicina aumentan la abundancia del filo Proteboacteria mientras que el tratamiento con ciprofloxacino y ceftriaxona disminuye la abundancia de este filo en muestras fecales de ratón. Las alteraciones observadas dentro de este filo se detectan durante el tratamiento y dos semanas después de su retirada.

(IX) Los tratamientos antibióticos que causan más alteraciones en la microbiota intestinal promueven niveles más altos de colonización intestinal de EVR en el intestino de ratón, siendo en orden decreciente, vancomicina, clindamicina, ampicilina, ceftriaxona, neomicina y ciprofloxacina, los tratamientos que promueven los niveles más altos de colonización intestinal de EVR. Dos semanas después de la retirada del tratamiento, vancomicina, clindamicina y ceftriaxona aún promueven niveles altos de colonización intestinal por EVR, la ampicilina y la ciprofloxacina promueven

niveles intermedios y la neomicina, al igual que los ratones no tratados, no permite la colonización intestinal por EVR.

Estudio de la capacidad protectora de los aislados comensales bacterianos frente a la colonización intestinal por EVR.

(X) Una mayor abundancia relativa de *Alistipes*, *Allobaculum*, *Barnesiella*, *Oscillibacter* y taxones no clasificados de las familias Lachnospiraceae, Porphyromonadaceae y Ruminococacceae se asocia con resistencia frente a la colonización intestinal por EVR en ratones.

(XI) Mayores abundancias relativas de *Escherichia Shigella*, *Enterococcus*, *Akkermansia*, *Parasutterella*, *Lactobacillus* y taxones no clasificados de las familias Enterobacteriaceae, Burkholderiales y Desulfovibrionaceae se asocian con mayores niveles de colonización intestinal por EVR en ratones.

(XII) La inoculación oral de ratones con una mezcla bacteriana que contiene tres aislados bacterianos pertenecientes a los taxones *Alistipes*, *Barnesiella*, *Oscillibacter* y un aislado bacteriano de la familia Ruminococacceae disminuye en más de 3 órdenes de magnitud los niveles de colonización intestinal por EVR.

Estudio de los mecanismos por los cuales las bacterias comensales protectoras identificadas disminuyen la colonización intestinal de EVR.

(XIII) La inoculación oral de ratones con una mezcla bacteriana que contiene tres aislados bacterianos pertenecientes a los taxones *Alistipes*, *Barnesiella*, *Oscillibacter* y un aislado de la familia Ruminococacceae restauran la expresión de genes, reducidos tras el tratamiento con vancomicina, que codifican para transportadores de azúcares (celobiose, N Acetil-galactosamina, N-acetil-glucosamina), para un receptor sensible a serina y para genes implicados en la síntesis de ácidos grasos de cadena corta (AGCC).

(XIV) La inoculación oral de ratones con una mezcla bacteriana que contiene tres aislados bacterianos pertenecientes a los taxones *Alistipes*, *Barnesiella*, *Oscillibacter* y un aislado de la familia Ruminococacceae disminuyen la concentración intestinal del oligosacárido celobiosa, el AGCC lactato y los aminoácidos prolina, serina, leucina y treonina, que incrementan con el tratamiento con vancomicina. Por otra parte, la administración de estas bacterias aumenta la concentración de trimetilamina, fenilalanina, fenol, ácidos biliares y el butirato, 2-metilbutirato, valerato y propionato de AGCC, que disminuyen con el tratamiento con vancomicina.

(XV) Durante la colonización del tracto intestinal de ratón, EVR expresa las diferentes subunidades de los transportadores responsables de la internalización de celobiosa y N-acetil-galactosamina y transportadores que permiten la internalización de aminoácidos ramificados, prolina y arginina.

(XVI) De las 190 fuentes de carbono analizadas, la N-acetil-glucosamina, la N-acetil-galactosamina y la celobiosa se encuentran entre las 10 fuentes de carbono que promueven el mayor crecimiento de EVR *in vitro* en condiciones anaerobias. Además, EVR puede crecer utilizando únicamente serina como fuente de carbono *in vitro* en condiciones anaeróbicas.

(XVII) Si bien deben realizarse estudios adicionales para su validación, los resultados presentados en esta tesis apoyan un modelo en el que las bacterias *Alistipes*, *Barnesiella*, *Oscillibacter* y el aislado no clasificado de la familia Ruminococaceae disminuyen la concentración intestinal de nutrientes que pueden ser utilizados por EVR para su crecimiento, lo que reduciría la capacidad de EVR de colonizar el tracto intestinal.

Bibliography

[1] R T Mayon-White, G Ducel, T Kereselidze, and E Tikomirov. An international survey of the prevalence of hospital-acquired infection. *J. Hosp. Infect.*, 11 Suppl A:43–48, feb 1988.

[2] L. Nicolle G. Ducel, J. Fabry. Prevention of hospital-acquired infections : A practical Guide 2nd edition. Technical report, World Health Organization, 2002.

[3] Adrian G Barnett, Katie Page, Megan Campbell, Elizabeth Martin, Rebecca Rashleigh-Rolls, Kate Halton, David L Paterson, Lisa Hall, Nerina Jimmieson, Katherine White, and Nicholas Graves. The increased risks of death and extra lengths of hospital and ICU stay from hospital-acquired bloodstream infections: a case-control study. *BMJ Open*, 3(10):e003587, oct 2013.

[4] Marta Riu, Pietro Chiarello, Roser Terradas, Maria Sala, Enric Garcia-Alzorriz, Xavier Castells, Santiago Grau, and Francesc Cots. Cost Attributable to Nosocomial Bacteremia. Analysis According to Microorganism and Antimicrobial Sensitivity in a University Hospital in Barcelona. *PLoS One*, 11(4):e0153076, 2016.

[5] David K Warren, Wasim W Quadir, Christopher S Hollenbeak, Alexis M Elward, Michael J Cox, and Victoria J Fraser. Attributable cost of catheter-associated bloodstream infections among intensive care patients in a nonteaching hospital. *Crit. Care Med.*, 34(8):2084–2089, aug 2006.

[6] Richard P Wenzel. Health care-associated infections: major issues in the early years of the 21st century. *Clin. Infect. Dis.*, 45:S85–8, jul 2007.

[7] P. Zarb, B. Coignard, J. Griskeviciene, A. Muller, V. Vankerckhoven, K. Weist, M. M. Goossens, S. Vaerenberg, S. Hopkins, B. Catry, D. L. Monnet, H. Goossens, and C. Suetens. The european centre for disease prevention and control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. Technical report, ECDC, 2012.

[8] Victor Daniel Rosenthal, Dennis George Maki, Yatin Mehta, Hakan Leblebicioglu, Ziad Ahmed Memish, Haifaa Hassan Al-Mousa, Hanan Balkhy, Bijie Hu, Carlos Alvarez-Moreno, Eduardo Alexandrino Medeiros, Anucha Apisarnthanarak, Lul Raka, Luis E. Cuellar, Altaf Ahmed, Josephine Anne Navoa-Ng, Amani Ali El-Kholy, Souha Sami Kanj, Ider Bat-Erdene, Wieslawa Duszynska, Nguyen Van Truong, Leonardo N. Pazmino, Lucy Chai See-Lum, Rosalia Fernandez-Hidalgo,

Gabriela Di-Silvestre, Farid Zand, Sona Hlinkova, Vladislav Belskiy, Hussain Al-Rahma, Marco Tulio Luque-Torres, Nesil Bayraktar, Zan Mitrev, Vaidotas Gurskis, Dale Fisher, Ilham Bulos Abu-Khader, Kamal Berechid, Arnaldo Rodriguez-Sanchez, Florin George Horhat, Osiel Requejo-Pino, Nassya Hadjieva, Nejla Ben-Jaballah, Elias Garcia-Mayorca, Luis Kushner-Davalos, Srdjan Pasic, Luis E. Pedrozo-Ortiz, Eleni Apostolopoulou, Nepomuceno Mejia, May Osman Gamar-Elanbya, Kushlani Jayatilleke, Miriam De Lourdes-Duenas, and Guadalupe Aguirre-Avalos. International Nosocomial Infection Control Consortiu (INICC) report, data summary of 43 countries for 2007-2012. Device-associated module. *Am. J. Infect. Control*, 42:942–956, 2014.

[9] E J Bassett, M S Keith, G J Armelagos, D L Martin, and A R Villanueva. Tetracycline-labeled human bone from ancient Sudanese Nubia (A.D. 350). *Science*, 209(4464):1532–4, sep 1980.

[10] Mark L. Nelson, Andrew Dinardo, Jeffery Hochberg, and George J. Armelagos. Brief communication: Mass spectroscopic characterization of tetracycline in the skeletal remains of an ancient population from Sudanese Nubia 350-550 CE. *Am. J. Phys. Anthropol.*, 143(1):151–154, sep 2010.

[11] Rustam I. Aminov. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Front. Microbiol.*, 1(December):1–7, 2010.

[12] R Khosla, D D Verma, A Kapur, R V Aruna, and N Khanna. Streptogramins: a new class of antibiotics. *Indian J. Med. Sci.*, 53(3):111–119, mar 1999.

[13] J Preudhomme, A Belloc, Y Charpentie, and P Tarridec. An antibiotic formed from 2 groups of compounds with synergistic action: pristinamycin. *C. R. Hebd. Seances Acad. Sci.*, 260:1309–1312, jan 1965.

[14] Nickie D Greer. Tigecycline (Tygacil): the first in the glycylcycline class of antibiotics. *Proc. (Bayl. Univ. Med. Cent).*, 19(2):155–161, apr 2006.

[15] Michael A Kohanski, Daniel J Dwyer, and James J Collins. How antibiotics kill bacteria : from targets to networks. *Nat Rev Microbiol. 2010*, 8(6):423–435, 2010.

[16] C D Freeman, N E Klutman, and K C Lamp. Metronidazole. A therapeutic review and update. *Drugs*, 54(5):679–708, nov 1997.

[17] Eric G Pamer. Resurrecting the intestinal microbiota to combat antibiotic- resistant pathogens. *Science*, 352(6285):535–538, 2016.

[18] Rustam I. Aminov and Roderick I. Mackie. Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol. Lett.*, 271(1993):147–161, 2007.

[19] Andrew C Singer, Helen Shaw, Vicky Rhodes, and Alwyn Hart. Review of Antimicrobial Resistance in the Environment and its Relevance to Environmental Regulators. *Front. Microbiol.*, 7(November):1–22, 2016.

[20] Carla Novais, Ana R Freitas, Eduarda Silveira, Patrícia Antunes, Ricardo Silva, Teresa M Coque, and Luísa Peixe. Spread of multidrug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment. *J. Antimicrob. Chemother.*, 68(12):2746–54, dec 2013.

[21] European Commission. Communication from the Commission to the European Parliament and the Council - Action plan against the rising threats from Antimicrobial Resistance. Technical report, 2011.

[22] Shelley S. Magill, Jonathan R. Edwards, Wendy Bamberg, Zintars G. Beldavs, Ghinwa Dumyati, Marion a. Kainer, Ruth Lynfield, Meghan Maloney, Laura McAllister-Hollod, Joelle Nadle, Susan M. Ray, Deborah L. Thompson, Lucy E. Wilson, and Scott K. Fridkin. Multistate Point-Prevalence Survey of Health Care–Associated Infections. *N. Engl. J. Med.*, 370:1198–1208, 2014.

[23] Cesar a Arias and Barbara E Murray. The rise of the Enterococcus: beyond vancomycin resistance. *Nat. Rev. Microbiol.*, 10(4):266–78, apr 2012.

[24] R R Facklam. Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Appl. Microbiol.*, 26(2):138–145, aug 1973.

[25] Gunter Klein. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int. J. Food Microbiol.*, 88(2-3):123–131, dec 2003.

[26] B E Murray. The life and times of the Enterococcus. *Clin. Microbiol. Rev.*, 3(1):46–65, jan 1990.

[27] H Graudal. The classification of motile Streptococci within the Enterococcus group. *Acta Pathol. Microbiol. Scand.*, 41(5):403–410, 1957.

[28] A J Martinez-Murcia and M D Collins. Enterococcus sulfureus, a new yellow-pigmented Enterococcus species. *FEMS Microbiol. Lett.*, 64(1):69–74, may 1991.

[29] Francois Lebreton, Rob J L Willems, and Michael S Gilmore. Enterococcus Diversity, Origins in Nature, and Gut Colonization. In Editors. Gilmore MS, Clewell DB, Ike Y, et al., editor, *Enterococci From Commensals to Lead. Causes Drug Resist. Infect.* Boston: Massachusetts Eye and Ear Infirmary;, 2014.

[30] Ecdc. *European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2015.* 2015.

[31] J D Martin and J O Mundt. Enterococci in insects. *Appl. Microbiol.*, 24(4):575–580, oct 1972.

[32] J O Mundt. Occurrence of enterococci in animals in a wild environment. *Appl. Microbiol.*, 11:136–140, mar 1963.

[33] L A Devriese, K Ceyssens, U M Rodrigues, and M D Collins. Enterococcus columbae, a species from pigeon intestines. *FEMS Microbiol. Lett.*, 59(3):247–251, sep 1990.

[34] A de Vaux, G Laguerre, C Divies, and H Prevost. Enterococcus asini sp. nov. isolated from the caecum of donkeys (Equus asinus). *Int. J. Syst. Bacteriol.*, 48 Pt 2:383–387, apr 1998.

[35] M Ostrolenk and A C Hunter. The Distribution of Enteric Streptococci. *J. Bacteriol.*, 51(6):735–741, jun 1946.

[36] L A Devriese and K De Pelsmaecker. The anal region as a main carrier site of Staphylococcus intermedius and Streptococcus canis in dogs. *Vet. Rec.*, 121(13):302–303, sep 1987.

[37] J O Mundt. Occurrence of enterococci on plants in a wild environment. *Appl. Microbiol.*, 11:141–144, mar 1963.

[38] J O Mundt. Occurrence of Enterococci: Bud, Blossom, and Soil Studies. *Appl. Microbiol.*, 9(6):541–544, nov 1961.

[39] Richard L Whitman, Dawn A Shively, Heather Pawlik, Meredith B Nevers, and Muruleedhara N Byappanahalli. Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol.*, 69(8):4714–4719, aug 2003.

[40] S B Grant, B F Sanders, A B Boehm, J A Redman, J H Kim, R D Mrse, A K Chu, M Gouldin, C D McGee, N A Gardiner, B H Jones, J Svejkovsky, G V Leipzig, and A Brown. Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality. *Environ. Sci. Technol.*, 35(12):2407–2416, jun 2001.

[41] Gregory J Imamura, Rachelle S Thompson, Alexandria B Boehm, and Jennifer A Jay. Wrack promotes the persistence of fecal indicator bacteria in marine sands and seawater. *FEMS Microbiol. Ecol.*, 77(1):40–49, jul 2011.

[42] Brian D Badgley, Bina S Nayak, and Valerie J Harwood. The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. *Water Res.*, 44(20):5857–5866, dec 2010.

[43] Brian D Badgley, Florence I M Thomas, and Valerie J Harwood. The effects of submerged aquatic vegetation on the persistence of environmental populations of Enterococcus spp. *Environ. Microbiol.*, 12(5):1271–1281, may 2010.

[44] T Muller, A Ulrich, E M Ott, and M Muller. Identification of plant-associated enterococci. *J. Appl. Microbiol.*, 91(2):268–278, aug 2001.

[45] Muruleedhara N Byappanahalli, Meredith B Nevers, Asja Korajkic, Zachery R Staley, and Valerie J Harwood. Enterococci in the environment. *Microbiol. Mol. Biol. Rev.*, 76(4):685–706, dec 2012.

[46] R Maarit Niemi, Tuula Ollinkangas, Lars Paulin, Pavel Svec, Peter Vandamme, Antti Karkman, Marcel Kosina, and Kristina Lindstrom. Enterococcus rivorum sp. nov., from water of pristine brooks. *Int. J. Syst. Evol. Microbiol.*, 62(Pt 9):2169–2173, sep 2012.

[47] A Korajkic, M J Brownell, and V J Harwood. Investigation of human sewage pollution and pathogen analysis at Florida Gulf coast beaches. *J. Appl. Microbiol.*, 110(1):174–183, jan 2011.

[48] D M Ferguson, D F Moore, M A Getrich, and M H Zhowandai. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *J. Appl. Microbiol.*, 99(3):598–608, 2005.

[49] K Obiri-Danso and K Jones. Distribution and seasonality of microbial indicators and thermophilic campylobacters in two freshwater bathing sites on the River Lune in northwest England. *J. Appl. Microbiol.*, 87(6):822–832, dec 1999.

[50] Elizabeth Halliday and Rebecca J Gast. Bacteria in beach sands: an emerging challenge in protecting coastal water quality and bather health. *Environ. Sci. Technol.*, 45(2):370–379, jan 2011.

[51] Kevan M Yamahara, Blythe A Layton, Alyson E Santoro, and Alexandria B Boehm. Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. *Environ. Sci. Technol.*, 41(13):4515–4521, jul 2007.

[52] Michael S. Gilmore, Francois Lebreton, and Willem van Schaik. Genomic Transition of Enterococci from Gut Commensals to Leading Causes of Multidrug-resistant Hospital Infection in the Antibiotic Era. *Curr. Opin. Microbiol.*, 16(1):10–16, 2013.

[53] Yoriko Deguchi and Takashi Morishita. Nutritional Requirements in Multiple Auxotrophic Lactic Acid Bacteria: Genetic Lesions Affecting Amino Acid Biosynthetic Pathways in Lactococcus lactis, Enterococcus faecium , and Pediococcus acidilactici. *Biosci. Biotechnol. Biochem.*, 56(6):913–918, jan 1992.

[54] Nadine Veith, Margrete Solheim, Koen W. A. van Grinsven, Brett G. Olivier, Jennifer Levering, Ruth Grosseholz, Jeroen Hugenholtz, Helge Holo, Ingolf Nes, Bas Teusink, and Ursula Kummer. Using a Genome-Scale Metabolic Model of Enterococcus faecalis V583 To Assess Amino Acid Uptake and Its Impact on Central Metabolism. *Appl. Environ. Microbiol.*, 81(5):1622–1633, mar 2015.

[55] S S Nowlan, R H Deibel, Sandra Simpson-Nowlan, and R H Deibel. Nutritional Characteristics and Growth Relationship to Thymine, Folate, and Folinate. *J. Bacteriol.*, 94(2):297–299, aug 1967.

[56] L A Devriese, B. Pot, and M. D. Collins. Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups, 1993.

[57] Melanie Zischka, Carsten T Künne, Jochen Blom, Dominique Wobser, Türkân Sakınç, Kerstin Schmidt-Hohagen, P Wojtek Dabrowski, Andreas Nitsche, Johannes Hübner, Torsten Hain, Trinad Chakraborty, Burkhard Linke, Alexander Goesmann, Sonja Voget, Rolf Daniel, Dietmar Schomburg, Rüdiger Hauck, Hafez M Hafez, Petra Tielen, Dieter Jahn, Margrete Solheim, Ewa Sadowy, Jesper Larsen, Lars B Jensen, Patricia Ruiz-Garbajosa, Dianelys Quiñones Pérez, Theresa Mikalsen, Jennifer Bender, Matthias Steglich, Ulrich Nübel, Wolfgang Witte, and Guido Werner.

Comprehensive molecular, genomic and phenotypic analysis of a major clone of Enterococcus faecalis MLST ST40. *BMC Genomics*, 16(1):175, mar 2015.

[58] Joana Barros, Margarida Andrade, Hajer Radhouani, Maria Lopez, Gilberto Igrejas, Patricia Poeta, and Carmen Torres. Detection of vanA-containing Enterococcus species in faecal microbiota of gilthead seabream (Sparus aurata). *Microbes Environ.*, 27(4):509–511, 2012.

[59] Alexandre Goncalves, Gilberto Igrejas, Hajer Radhouani, Tiago Santos, Ricardo Monteiro, Rui Pacheco, Eva Alcaide, Irene Zorrilla, Rodrigo Serra, Carmen Torres, and Patricia Poeta. Detection of antibiotic resistant enterococci and Escherichia coli in free range Iberian Lynx (Lynx pardinus). *Sci. Total Environ.*, 456-457:115–119, jul 2013.

[60] A Goncalves, G Igrejas, H Radhouani, S Correia, R Pacheco, T Santos, R Monteiro, A Guerra, F Petrucci-Fonseca, F Brito, C Torres, and P Poeta. Antimicrobial resistance in faecal enterococci and Escherichia coli isolates recovered from Iberian wolf. *Lett. Appl. Microbiol.*, 56(4):268–274, apr 2013.

[61] R Leclercq, E Derlot, J Duval, and P Courvalin. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *N. Engl. J. Med.*, 319(3):157–161, jul 1988.

[62] A H Uttley, R C George, J Naidoo, N Woodford, A P Johnson, C H Collins, D Morrison, A J Gilfillan, L E Fitch, and J Heptonstall. High-level vancomycin-resistant enterococci causing hospital infections. *Epidemiol. Infect.*, 103(1):173–181, aug 1989.

[63] Louis B Rice, Lenore Carias, Susan Rudin, Carl Vael, Herman Goossens, Carola Konstabel, Ingo Klare, Sreedhar R Nallapareddy, Wenxiang Huang, and Barbara E Murray. A potential virulence gene, hylEfm, predominates in Enterococcus faecium of clinical origin. *J. Infect. Dis.*, 187(3):508–512, feb 2003.

[64] Helen L Leavis, Rob J L Willems, Willem J B van Wamel, Frank H Schuren, Martien P M Caspers, and Marc J M Bonten. Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium. *PLoS Pathog.*, 3(1):e7, jan 2007.

[65] Willem van Schaik, Janetta Top, David R Riley, Jos Boekhorst, Joyce E P Vrijenhoek, Claudia M E Schapendonk, Antoni Pa A Hendrickx, Isaac J Isaäc J Nijman, Marc J M Bonten, Hervé Herve Tettelin, and Rob J L Willems. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen Enterococcus faecium and identification of a large transferable pathogenicity island. *BMC Genomics*, 11:239, apr 2010.

[66] Jessica Galloway-Peña, Jung Hyeob Roh, Mauricio Latorre, Xiang Qin, and Barbara E. Murray. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of enterococcus faecium. *PLoS One*, 7(1), 2012.

[67] F. Lebreton, W. van Schaik, A. Manson McGuire, P. Godfrey, A. Griggs, V. Mazumdar, J. Corander, L. Cheng, S. Saif, S. Young, Q. Zeng, J. Wortman, B. Birren, R. J. L. Willems, A. M. Earl, and M. S. Gilmore. Emergence of Epidemic Multidrug-Resistant Enterococcus faecium from Animal and Commensal Strains. *MBio*, 4(4):1–10, aug 2013.

[68] Ana M. Guzman Prieto, Willem van Schaik, Malbert R C Rogers, Teresa M. Coque, Fernando Baquero, Jukka Corander, and Rob J L Willems. Global emergence and dissemination of enterococci as nosocomial pathogens: Attack of the clones? *Front. Microbiol.*, 7(May):1–15, 2016.

[69] Gregory W. Goldberg and Luciano A. Marraffini. Resistance and tolerance of foreign genetic elements by prokaryotic immune systems — curating the genome. *Nat. Rev. Immunol.*, 15(11):717–724, 2015.

[70] Kelli L Palmer and Michael S Gilmore. Multidrug-resistant enterococci lack CRISPR-cas. *MBio*, 1(4), jan 2010.

[71] Valerie J Price, Wenwen Huo, Ardalan Sharifi, and Kelli L Palmer. CRISPR-Cas and Restriction-Modificatio Act Additively against Conjugative Antibiotic Resistance Plasmid Transfer in Enterococcus faecalis. *Mol. Biol. Physiol.*, 1(3):1–13, 2016.

[72] J Parkhill, M Sebaihia, a Preston, L Murphy, N Thomson, D Harris, M Holden, C Churcher, S Bentley, K Mungall, a Cerdeno-Tarraga, L Temple, K James, B Harris, M Quail, M Achtman, R Atkin, S Baker, D Basham, N Bason, I Cherevach, T Chillingworth, M Collins, a Cronin, P Davis, J Doggett, T Feltwell, a Goble, N Hamlin, H Hauser, S Holroyd, K Jagels, S Leather, S Moule, H Norberczak, S O'Neil, D Ormond, C Price, E Rabbinowitsch, S Rutter, M Sanders, D Saunders, K Seeger, S Sharp, M Simmonds, J Skelton, R Squares, S Squares, K Stevens, L Unwin, S Whitehead, B Barrell, and D Maskell. Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. *Nat Genet*, 35(1):32–40, 2003.

[73] Brian L. Hollenbeck and Louis B. Rice. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*, 3:421–433, 2012.

[74] F Sifaoui, M Arthur, L Rice, and L Gutmann. Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in Enterococcus faecium. *Antimicrob. Agents Chemother.*, 45(9):2594–2597, sep 2001.

[75] Maria Camila Montealegre, Jung Hyeob Roh, Meredith Rae, Milya G Davlieva, Kavindra V Singh, Yousif Shamoo, and Barbara E Murray. Differential Penicillin-Binding Protein 5 (PBP5) Levels in the Enterococcus faecium Clades with Different Levels of Ampicillin Resistance. *Antimicrob. Agents Chemother.*, 61(1), jan 2017.

[76] Y Costa, M Galimand, R Leclercq, J Duval, and P Courvalin. Characterization of the chromosomal aac(6')-Ii gene specific for Enterococcus faecium. *Antimicrob. Agents Chemother.*, 37(9):1896–1903, sep 1993.

[77] Kavindra V Singh, George M Weinstock, and Barbara E Murray. An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. *Antimicrob. Agents Chemother.*, 46(6):1845–1850, jun 2002.

[78] Lea M Hurlimann, Valentina Corradi, Michael Hohl, Guido V Bloemberg, D Peter Tieleman, and Markus A Seeger. The Heterodimeric ABC Transporter EfrCD Mediates Multidrug Efflux in Enterococcus faecalis. *Antimicrob. Agents Chemother.*, 60(9):5400–5411, sep 2016.

[79] A Portillo, F Ruiz-Larrea, M Zarazaga, A Alonso, J L Martinez, and C Torres. Macrolide resistance genes in Enterococcus spp. *Antimicrob. Agents Chemother.*, 44(4):967–971, apr 2000.

[80] Sabina Leanti La Rosa, Lars-Gustav Snipen, Barbara E Murray, Rob J L Willems, Michael S Gilmore, Dzung B Diep, Ingolf F Nes, and Dag Anders Brede. A genomic virulence reference map of Enterococcus faecalis reveals an important contribution of phage03-like elements in nosocomial genetic lineages to pathogenicity in a Caenorhabditis elegans infection model. *Infect. Immun.*, 83(5):2156–2167, may 2015.

[81] Agot Aakra, Heidi Vebo, Lars Snipen, Helmut Hirt, Are Aastveit, Vivek Kapur, Gary Dunny, Barbara E Murray, and Ingolf F Nes. Transcriptional response of Enterococcus faecalis V583 to erythromycin. *Antimicrob. Agents Chemother.*, 49(6):2246–2259, jun 2005.

[82] Louis B Rice, Samuel Bellais, Lenore L Carias, Rebecca Hutton-Thomas, Robert A Bonomo, Patrick Caspers, Malcolm G P Page, and Laurent Gutmann. Impact of specific pbp5 mutations on expression of beta-lactam resistance in Enterococcus faecium. *Antimicrob. Agents Chemother.*, 48(8):3028–3032, aug 2004.

[83] Tristan O'Driscoll and Christopher W Crank. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infect. Drug Resist.*, 8:217–30, jan 2015.

[84] Patrice Courvalin. Vancomycin resistance in gram-positive cocci. *Clin. Infect. Dis.*, 42 Suppl 1:S25–34, jan 2006.

[85] Theresa Mikalsen, Torunn Pedersen, Rob Willems, Teresa M Coque, Guido Werner, Ewa Sadowy, Willem van Schaik, Lars Bogø Jensen, Arnfinn Sundsfjord, and Kristin Hegstad. Investigating the mobilome in clinically important lineages of Enterococcus faecium and Enterococcus faecalis. *BMC Genomics*, 16:282, jan 2015.

[86] Esther Heikens, Marc J M Bonten, and Rob J L Willems. Enterococcal surface protein Esp is important for biofilm formation of Enterococcus faecium E1162. *J. Bacteriol.*, 189(22):8233–8240, nov 2007.

[87] Esther Heikens, Masja Leendertse, Lucas M Wijnands, Miranda van Luit-Asbroek, Marc J M Bonten, Tom van der Poll, and Rob J L Willems. Enterococcal surface

protein Esp is not essential for cell adhesion and intestinal colonization of Enterococcus faecium in mice. *BMC Microbiol.*, 9:19, jan 2009.

[88] Masja Leendertse, Esther Heikens, Lucas M Wijnands, Miranda van Luit-Asbroek, Gwendoline J D Teske, Joris J T H Roelofs, Marc J M Bonten, Tom van der Poll, and Rob J L Willems. Enterococcal surface protein transiently aggravates Enterococcus faecium-induced urinary tract infection in mice. *J. Infect. Dis.*, 200(7):1162–1165, oct 2009.

[89] Esther Heikens, Kavindra V Singh, Karen D Jacques-Palaz, Miranda van Luit-Asbroek, Evelien A N Oostdijk, Marc J M Bonten, Barbara E Murray, and Rob J L Willems. Contribution of the enterococcal surface protein Esp to pathogenesis of Enterococcus faecium endocarditis. *Microbes Infect.*, 13(14-15):1185–1190, dec 2011.

[90] Sreedhar R Nallapareddy, George M Weinstock, and Barbara E Murray. Clinical isolates of Enterococcus faecium exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. *Mol. Microbiol.*, 47(6):1733–1747, mar 2003.

[91] Jouko Sillanpaa, Sreedhar R Nallapareddy, Vittal P Prakash, Xiang Qin, Magnus Hook, George M Weinstock, and Barbara E Murray. Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in Enterococcus faecium. *Microbiology*, 154(Pt 10):3199–3211, oct 2008.

[92] Antoni P A Hendrickx, Miranda van Luit-Asbroek, Claudia M E Schapendonk, Willem J B van Wamel, Johanna C Braat, Lucas M Wijnands, Marc J M Bonten, and Rob J L Willems. SgrA, a nidogen-binding LPXTG surface adhesin implicated in biofilm formation, and EcbA, a collagen binding MSCRAMM, are two novel adhesins of hospital-acquired Enterococcus faecium. *Infect. Immun.*, 77(11):5097–5106, nov 2009.

[93] Sreedhar R Nallapareddy, Kavindra V Singh, and Barbara E Murray. Contribution of the collagen adhesin Acm to pathogenesis of Enterococcus faecium in experimental endocarditis. *Infect. Immun.*, 76(9):4120–4128, sep 2008.

[94] Ana M. Guzmán Prieto, Rolf T. Urbanus, Xinglin Zhang, Damien Bierschenk, C. Arnold Koekman, Miranda van Luit-Asbroek, Janneke P. Ouwerkerk, Marieke Pape, Fernanda L. Paganelli, Dominique Wobser, Johannes Huebner, Antoni P. a. Hendrickx, Marc J. M. Bonten, Rob J. L. Willems, and Willem van Schaik. The N-terminal domain of the thermo-regulated surface protein PrpA of Enterococcus faecium binds to fibrinogen, fibronectin and platelets. *Sci. Rep.*, 5(November):18255, 2015.

[95] Jouko Sillanpää, Sreedhar R Nallapareddy, Kavindra V Singh, Vittal P Prakash, Timothy Fothergill, Hung Ton-That, and Barbara E Murray. Characterization of the ebp(fm) pilus-encoding operon of Enterococcus faecium and its role in biofilm

formation and virulence in a murine model of urinary tract infection. *Virulence*, 1(4):236–246, 2010.

[96] Eun Bae Kim and Maria L. Marco. Nonclinical and clinical enterococcus faecium strains, but not enterococcus faecalis strains, have distinct structural and functional genomic features. *Appl. Environ. Microbiol.*, 80(1):154–165, 2014.

[97] Esther Heikens, Willem van Schaik, Helen L Leavis, Marc J M Bonten, and Rob J L Willems. Identification of a novel genomic island specific to hospital-acquired clonal complex 17 Enterococcus faecium isolates. *Appl. Environ. Microbiol.*, 74(22):7094–7097, nov 2008.

[98] Xinglin Zhang, Janetta Top, Mark De Been, Damien Bierschenk, Malbert Rogers, Masja Leendertse, Marc J M Bonten, Tom Van Der Poll, Rob J L Willems, and Willem van Schaik. Identification of a genetic determinant in clinical enterococcus faecium strains that contributes to intestinal colonization during antibiotic treatment. *J. Infect. Dis.*, 207(11):1780–1786, jun 2013.

[99] Jenny a. Laverde Gomez, Willem van Schaik, Ana R. Freitas, Teresa M. Coque, Keith E. Weaver, Maria Victoria Francia, Wolfgang Witte, and Guido Werner. A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates. *Int. J. Med. Microbiol.*, 301(2):165–175, feb 2011.

[100] Louis B Rice, Viera Lakticova, Lenore L Carias, Susan Rudin, Rebecca Hutton, Steven H Marshall, Viera Lakticová, Lenore L Carias, Susan Rudin, Rebecca Hutton, and Steven H Marshall. Transferable capacity for gastrointestinal colonization in Enterococcus faecium in a mouse model. *J. Infect. Dis.*, 199(3):342–9, feb 2009.

[101] Cesar A Arias, Diana Panesso, Kavindra V Singh, Louis B Rice, and Barbara E Murray. Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium. *Antimicrob. Agents Chemother.*, 53(10):4240–6, oct 2009.

[102] David S. Kim, Kavindra V. Singh, Sreedhar R. Nallapareddy, Xiang Qin, Diana Panesso, Cesar a. Arias, and Barbara E. Murray. The fms21 (pilA)-fms20 locus encoding one of four distinct pili of Enterococcus faecium is harboured on a large transferable plasmid associated with gut colonization and virulence. *J. Med. Microbiol.*, 59:505–507, 2010.

[103] Ana R Freitas, Ana P Tedim, Carla Novais, Patricia Ruiz-Garbajosa, Guido Werner, Jenny A Laverde-Gomez, Rafael Canton, Luisa Peixe, Fernando Baquero, and Teresa M Coque. Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the Enterococcus faecium CC17 polyclonal subcluster. *Antimicrob. Agents Chemother.*, 54(6):2660–2665, jun 2010.

[104] Diana Panesso, Maria C Montealegre, Sandra Rincón, Maria F Mojica, Louis B Rice, Kavindra V Singh, Barbara E Murray, and Cesar a Arias. The hylEfm gene in pHylEfm of Enterococcus faecium is not required in pathogenesis of murine peritonitis. *BMC Microbiol.*, 11:20, 2011.

[105] Curtis J Donskey, Jennifer A Hanrahan, Rebecca A Hutton, Louis B Rice, and In Cleveland. Effect of Parenteral Antibiotic Administration on Persistence of Vancomycin- Resistant. *J. Infect. Dis.*, 180:384–390, 1999.

[106] L B Rice. Antibiotics and gastrointestinal colonization by vancomycin-resistant enterococci. *Eur. J. Clin. Microbiol. Infect. Dis.*, 24(12):804–14, dec 2005.

[107] Nelson I. Agudelo Higuita and Mark M Huycke. Enterococcal Disease, Epidemiology, and Implications for Treatment. *Enterococci From Commensals to Lead. Causes Drug Resist. Infect.*, pages 1–35, 2014.

[108] Mini Kamboj, Rachel Blair, Natalie Bell, Janet Sun, Janet Eagan, and Kent Sepkowitz. What is the source of bloodstream infection due to vancomycin-resistant enterococci in persons with mucosal barrier injury? *Infect. Control Hosp. Epidemiol.*, 35(January 2013):99–101, 2014.

[109] Mitsugu Owari, Masafumi Wasa, Takaharu Oue, Satoko Nose, and Masahiro Fukuzawa. Glutamine prevents intestinal mucosal injury induced by cyclophosphamide in rats. *Pediatr. Surg. Int.*, 28(3):299–303, mar 2012.

[110] Jason Ramsingh, Carsten Bolln, Robert Hodnett, and Ahmed Al-Ani. Neutropenic enterocolitis affecting the transverse colon: an unusual complication of chemotherapy. *BMJ Case Rep.*, 2014, may 2014.

[111] S. Miyazaki, T. Fujikawa, I. Kobayashi, T. Matsumoto, K. Tateda, and K. Yamaguchi. Development of systemic bacteraemia after oral inoculation of vancomycin-resistant enterococci in mice. *J. Med. Microbiol.*, 50(2001):695–701, 2001.

[112] Carles Ubeda, Ying Taur, Robert R Jenq, Michele J Equinda, Tammy Son, Miriam Samstein, Agnes Viale, Nicholas D Socci, Marcel R M Van Den Brink, Mini Kamboj, and Eric G Pamer. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J. Clin. Invest.*, 120(12):4332–4342, 2010.

[113] Ying Taur, Joao B Xavier, Lauren Lipuma, Carles Ubeda, Jenna Goldberg, Asia Gobourne, Yeon Joo Lee, Krista A Dubin, Nicholas D Socci, Agnes Viale, Miguel-Angel Perales, Robert R Jenq, Marcel R M van den Brink, and Eric G Pamer. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin. Infect. Dis.*, 55(7):905–914, oct 2012.

[114] Ana L Flores-Mireles, Jennifer N Walker, Aaron Potretzke, Henry L 4th Schreiber, Jerome S Pinkner, Tyler M Bauman, Alyssa M Park, Alana Desai, Scott J Hultgren, and Michael G Caparon. Antibody-Based Therapy for Enterococcal Catheter-Associated Urinary Tract Infections. *MBio*, 7(5), oct 2016.

[115] Abhishek Deshpande, Kelly Hurless, Jennifer L. Cadnum, Laurent Chesnel, Lihong Gao, Luisa Chan, Sirisha Kundrapu, Alexander Polinkovsky, and Curtis J. Donskey. Effect of Surotomycin, a Novel Cyclic Lipopeptide Antibiotic, on Intestinal Colonization with Vancomycin-Resistant Enterococci and Klebsiella pneumoniae in Mice. *Antimicrob. Agents Chemother.*, 60(6):3333–3339, jun 2016.

[116] Abhishek Deshpande, Kelly Hurless, Jennifer L. Cadnum, Laurent Chesnel, Lihong Gao, Luisa Chan, Sirisha Kundrapu, Alexander Polinkovsky, and Curtis J. Donskey. Effect of Fidaxomicin versus Vancomycin on Susceptibility to Intestinal Colonization with Vancomycin-Resistant Enterococci and Klebsiella pneumoniae in Mice. *Antimicrob. Agents Chemother.*, 60(7):3988–3993, jul 2016.

[117] Laureen Crouzet, Lionel Rigottier-Gois, and Pascale Serror. Potential use of probiotic and commensal bacteria as non-antibiotic strategies against vancomycin-resistant enterococci. *FEMS Microbiol. Lett.*, 362(8):1–17, apr 2015.

[118] Eamonn P Culligan and Roy D Sleator. Advances in the Microbiome: Applications to Clostridium difficile Infection. *J. Clin. Med.*, 5(9), sep 2016.

[119] Barbara a Methé, Karen E Nelson, Mihai Pop, Heather H Creasy, Michelle G Giglio, Curtis Huttenhower, Dirk Gevers, Joseph F Petrosino, Sahar Abubucker, H Jonathan, Asif T Chinwalla, Ashlee M Earl, Michael G Fitzgerald, Robert S Fulton, Kymberlie Hallsworth-pepin, Elizabeth a Lobos, Ramana Madupu, Vincent Magrini, John C Martin, Makedonka Mitreva, Donna M Muzny, Erica J Sodergren, Aye M Wollam, Kim C Worley, Jennifer R Wortman, Sarah K Young, Qiandong Zeng, Kjersti M Aagaard, Olukemi O Abolude, Emma Allen-vercoe, J Eric, Lucia Alvarado, Gary L Andersen, Scott Anderson, Elizabeth Appelbaum, Harindra M Arachchi, Gary Armitage, Cesar a Arze, Tulin Ayvaz, Carl C Baker, Lisa Begg, Tsegahiwot Belachew, Veena Bhonagiri, Monika Bihan, Martin J Blaser, Toby Bloom, J Vivien Bonazzi, Paul Brooks, Gregory a Buck, J Christian, Dana a Busam, Joseph L Campbell, Shane R Canon, Brandi L Cantarel, Patrick S Chain, I-min a Chen, Lei Chen, Shaila Chhibba, Ken Chu, M Dawn, Jose C Clemente, Sandra W Clifton, Sean Conlan, Jonathan Crabtree, A Cutting, Noam J Davidovics, Catherine C Davis, Todd Z Desantis, Kimberley D Delehaunty, Floyd E Dewhirst, Elena Deych, and Yan Ding. A framework for human microbiome research. *Nature*, 486(7402):215–221, jun 2012.

[120] Gregory P. Donaldson, S. Melanie Lee, and Sarkis K. Mazmanian. Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.*, 14(1):20–32, 2016.

[121] Junjie Qin, Ruiqiang Li, Jeroen Raes, Manimozhiyan Arumugam, Solvsten Burgdorf, Chaysavanh Manichanh, Trine Nielsen, Nicolas Pons, Takuji Yamada, Daniel R Mende, Junhua Li, Junming Xu, Shaochuan Li, Dongfang Li, Jianjun Cao, Bo Wang, Huiqing Liang, Huisong Zheng, Yinlong Xie, Julien Tap, Patricia Lepage, Marcelo Bertalan, Jean-michel Batto, Torben Hansen, Denis Le Paslier, Allan Linneberg, H Bjørn Nielsen, Eric Pelletier, Pierre Renault, Yan Zhou, Yingrui Li, Xiuqing Zhang, Songgang Li, Nan Qin, and Huanming Yang. A human gut microbial gene catalog established by metagenomic sequencing. *Nature*, 464(7285):59–65, 2010.

[122] Julia Oh, Allyson L. Byrd, Clay Deming, Sean Conlan, Heidi H. Kong, Julia A. Segre, Betty Barnabas, Robert Blakesley, Gerry Bouffard, Shelise Brooks, Holly Coleman, Mila Dekhtyar, Michael Gregory, Xiaobin Guan, Jyoti Gupta, Joel Han, Shi-ling Ho, Richelle Legaspi, Quino Maduro, Cathy Masiello, Baishali Maskeri,

Jenny McDowell, Casandra Montemayor, James Mullikin, Morgan Park, Nancy Riebow, Karen Schandler, Brian Schmidt, Christina Sison, Mal Stantripop, James Thomas, Pamela Thomas, Meg Vemulapalli, Alice Young, Heidi H. Kong, and Julia A. Segre. Biogeography and individuality shape function in the human skin metagenome. *Nature*, 514(7520):59–64, oct 2014.

[123] Peter J Turnbaugh, Ruth E Ley, Michael a Mahowald, Vincent Magrini, Elaine R Mardis, and Jeffrey I Gordon. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(December):1027–1031, 2006.

[124] Peter J Turnbaugh, Vanessa K Ridaura, Jeremiah J Faith, Federico E Rey, Rob Knight, and Jeffrey I Gordon. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.*, 1(6):6ra14, nov 2009.

[125] Manon D. Schulz, Çiğdem Atay, Jessica Heringer, Franziska K. Romrig, Sarah Schwitalla, Begüm Aydin, Paul K. Ziegler, Julia Varga, Wolfgang Reindl, Claudia Pommerenke, Gabriela Salinas-Riester, Andreas Böck, Carl Alpert, Michael Blaut, Shawn W. Sara C. Polson, Lydia Brandl, Thomas Kirchner, Florian R. Greten, Shawn W. Sara C. Polson, and Melek C. Arkan. High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. *Nature*, 514(7523):508–12, aug 2014.

[126] Jose U. Scher, Carles Ubeda, Alejandro Artacho, Mukundan Attur, Sandrine Isaac, Soumya M. Reddy, Shoshana Marmon, Andrea Neimann, Samuel Brusca, Tejas Patel, Julia Manasson, Eric G. Pamer, Dan R. Littman, and Steven B. Abramson. Decreased Bacterial Diversity Characterizes the Altered Gut Microbiota in Patients With Psoriatic Arthritis, Resembling Dysbiosis in Inflammatory Bowel Disease. *Arthritis Rheumatol.*, 67(1):128–139, jan 2015.

[127] Lea-Maxie Haag and Britta Siegmund. Intestinal Microbiota and the Innate Immune System – A Crosstalk in Crohn's Disease Pathogenesis. *Front. Immunol.*, 6(September):489, sep 2015.

[128] Hiutung Chu, Arya Khosravi, Indah P Kusumawardhani, Alice H K Kwon, Anilton C Vasconcelos, Larissa D Cunha, Anne E Mayer, Yue Shen, Wei-Li Wu, Amal Kambal, Stephan R Targan, Ramnik J Xavier, Peter B Ernst, Douglas R Green, Dermot P B McGovern, Herbert W Virgin, and Sarkis K Mazmanian. Gene-microbiota interactions contribute to the pathogenesis of inflammatory bowel disease. *Science*, 9948(May):1–10, 2016.

[129] Jacques Amar, Chantal Chabo, Aurélie Waget, Pascale Klopp, Christelle Vachoux, Luis G Bermúdez-Humarán, Natalia Smirnova, Mathieu Bergé, Thierry Sulpice, Sampo Lahtinen, Arthur Ouwehand, Philippe Langella, Nina Rautonen, Philippe J Sansonetti, and Rémy Burcelin. Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment. *EMBO Mol. Med.*, 3(9):559–572, sep 2011.

[130] Alison C Bested, Alan C Logan, and Eva M Selhub. Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part II - contemporary contextual research. *Gut Pathog.*, 5(1):5, jan 2013.

[131] Aiden Haghikia, Stefanie Jörg, Alexander Duscha, Johannes Berg, Arndt Manzel, Anne Waschbisch, Anna Hammer, De Hyung Lee, Caroline May, Nicola Wilck, Andras Balogh, Annika I Ostermann, Nils Helge Schebb, Denis A Akkad, Diana A Grohme, Markus Kleinewietfeld, Stefan Kempa, Jan Thöne, Seray Demir, Dominik N Müller, Ralf Gold, and Ralf A Linker. Dietary Fatty Acids Directly Impact Central Nervous System Autoimmunity via the Small Intestine. *Immunity*, 43(4):817–829, oct 2015.

[132] Ana E Duran-Pinedo, Tsute Chen, Ricardo Teles, Jacqueline R Starr, Xiaoshan Wang, Keerthana Krishnan, and Jorge Frias-Lopez. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J.*, 8(8):1659–1672, aug 2014.

[133] RE Ley, Micah Hamady, and Catherine Lozupone. Evolution of mammals and their gut microbes. *Science*, 320(5883):1647–1651, 2008.

[134] Aurélien Trompette, Eva S Gollwitzer, Koshika Yadava, Anke K Sichelstiel, Norbert Sprenger, Catherine Ngom-Bru, Carine Blanchard, Tobias Junt, Laurent P Nicod, Nicola L Harris, and Benjamin J Marsland. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat. Med.*, 20(2):159–166, feb 2014.

[135] Benjamin J. Marsland, Aurélien Trompette, and Eva S. Gollwitzer. The gut-lung axis in respiratory disease. *Ann. Am. Thorac. Soc.*, 12(November):S150–S156, nov 2015.

[136] The Human Microbiome Project Consortium. Structure, Function and Diversity of the Healthy Human Microbiome. *Nature*, 486(7402):207–214, jun 2012.

[137] Catherine a Lozupone, Jesse I Stombaugh, Jeffrey I Gordon, Janet K Jansson, and Rob Knight. Diversity, stability and resilience of the human gut microbiota. *Nature*, 489(7415):220–230, sep 2012.

[138] Julien Tap, Stanislas Mondot, Florence Levenez, Eric Pelletier, Christophe Caron, Jean Pierre Furet, Edgardo Ugarte, Rafael Muñoz-Tamayo, Denis L E Paslier, Renaud Nalin, Joel Dore, and Marion Leclerc. Towards the human intestinal microbiota phylogenetic core. *Environ. Microbiol.*, 11:2574–2584, 2009.

[139] Frederik Bäckhed, Claire M Fraser, Yehuda Ringel, Mary Ellen Sanders, R Balfour Sartor, Philip M Sherman, James Versalovic, Vincent Young, and B Brett Finlay. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe*, 12:611–622, 2012.

[140] Jason Lloyd-Price, Galeb Abu-Ali, and Curtis Huttenhower. The healthy human microbiome. *Genome Med.*, 8(1):51, 2016.

[141] Manimozhiyan Arumugam, Jeroen Raes, Eric Pelletier, Denis Le Paslier, Takuji Yamada, Daniel R Mende, Gabriel R Fernandes, Julien Tap, Thomas Bruls, Jean-Michel Batto, Marcelo Bertalan, Natalia Borruel, Francesc Casellas, Leyden Fernandez, Laurent Gautier, Torben Hansen, Masahira Hattori, Tetsuya Hayashi, Michiel Kleerebezem, Ken Kurokawa, Marion Leclerc, Florence Levenez, Chaysavanh Manichanh, H Bjørn Nielsen, Trine Nielsen, Nicolas Pons, Julie Poulain, Junjie Qin, Thomas Sicheritz-Ponten, Sebastian Tims, David Torrents, Edgardo Ugarte, Erwin G Zoetendal, Jun Wang, Francisco Guarner, Oluf Pedersen, Willem M de Vos, Søren Brunak, Joel Doré, María Antolín, François Artiguenave, Hervé M Blottiere, Mathieu Almeida, Christian Brechot, Carlos Cara, Christian Chervaux, Antonella Cultrone, Christine Delorme, Gérard Denariaz, Rozenn Dervyn, Konrad U Foerstner, Carsten Friss, Maarten van de Guchte, Eric Guedon, Florence Haimet, Wolfgang Huber, Johan van Hylckama-Vlieg, Alexandre Jamet, Catherine Juste, Ghalia Kaci, Jan Knol, Omar Lakhdari, Severine Layec, Karine Le Roux, Emmanuelle Maguin, Alexandre Mérieux, Raquel Melo Minardi, Christine M'rini, Jean Muller, Raish Oozeer, Julian Parkhill, Pierre Renault, Maria Rescigno, Nicolas Sanchez, Shinichi Sunagawa, Antonio Torrejon, Keith Turner, Gaetana Vandemeulebrouck, Encarna Varela, Yohanan Winogradsky, Georg Zeller, Jean Weissenbach, S Dusko Ehrlich, Peer Bork, and Arumugam. Enterotypes of the human gut microbiome. *Nature*, 473(7346):174–80, may 2011.

[142] Margaret L. Zupancic, Brandi L. Cantarel, Zhenqiu Liu, Elliott F. Drabek, Kathleen A. Ryan, Shana Cirimotich, Cheron Jones, Rob Knight, William A. Walters, Daniel Knights, Emmanuel F. Mongodin, Richard B. Horenstein, Braxton D. Mitchell, Nanette Steinle, Soren Snitker, Alan R. Shuldiner, and Claire M. Fraser. Analysis of the Gut Microbiota in the Old Order Amish and Its Relation to the Metabolic Syndrome. *PLoS One*, 7(8):e43052, aug 2012.

[143] Joseph J Gillespie, Alice R Wattam, Stephen A Cammer, Joseph L Gabbard, Maulik P Shukla, Oral Dalay, Timothy Driscoll, Deborah Hix, Shrinivasrao P Mane, Chunhong Mao, Eric K Nordberg, Mark Scott, Julie R Schulman, Eric E Snyder, Daniel E Sullivan, Chunxia Wang, Andrew Warren, Kelly P Williams, Tian Xue, Hyun Seung Yoo, Chengdong Zhang, Yan Zhang, Rebecca Will, Ronald W Kenyon, and Bruno W Sobral. PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species. *Infect. Immun.*, 79(11):4286–98, nov 2011.

[144] Alice R. Wattam, David Abraham, Oral Dalay, Terry L. Disz, Timothy Driscoll, Joseph L. Gabbard, Joseph J. Gillespie, Roger Gough, Deborah Hix, Ronald Kenyon, Dustin Machi, Chunhong Mao, Eric K. Nordberg, Robert Olson, Ross Overbeek, Gordon D. Pusch, Maulik Shukla, Julie Schulman, Rick L. Stevens, Daniel E. Sullivan, Veronika Vonstein, Andrew Warren, Rebecca Will, Meredith J.C. Wilson, Hyun Seung Yoo, Chengdong Zhang, Yan Zhang, and Bruno W. Sobral. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Res.*, 42(D1):D581–D591, jan 2014.

[145] Peter J Turnbaugh, Micah Hamady, Tanya Yatsunenko, Brandi L Cantarel, Alexis Duncan, Ruth E Ley, Mitchell L Sogin, William J Jones, Bruce A Roe, Jason P

Affourtit, Michael Egholm, Bernard Henrissat, Andrew C Heath, Rob Knight, and Jeffrey I Gordon. A core gut microbiome in obese and lean twins. *Nature*, 457(7228):480–484, jan 2009.

[146] Sahar Abubucker, Nicola Segata, Johannes Goll, Alyxandria M. Schubert, Jacques Izard, Brandi L. Cantarel, Beltran Rodriguez-Mueller, Jeremy Zucker, Mathangi Thiagarajan, Bernard Henrissat, Owen White, Scott T. Kelley, Barbara Methe, Patrick D. Schloss, Dirk Gevers, Makedonka Mitreva, and Curtis Huttenhower. Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput. Biol.*, 8(6), 2012.

[147] Ling Chun Kong, Bridget A Holmes, Aurelie Cotillard, Fatiha Habi-Rachedi, Remi Brazeilles, Sophie Gougis, Nicolas Gausseres, Patrice D Cani, Soraya Fellahi, Jean Philippe Bastard, Sean P Kennedy, Joel Dore, Stanislav Dusko Ehrlich, Jean Daniel Zucker, Salwa W Rizkalla, and Karine Clement. Dietary patterns differently associate with inflammation and gut microbiota in overweight and obese subjects. *PLoS One*, 9(10), 2014.

[148] Ludovica F. Buttó and Dirk Haller. Dysbiosis in intestinal inflammation: Cause or consequence. *Int. J. Med. Microbiol.*, 306(5):302–309, 2016.

[149] Bärbel Stecher, David Berry, and Alexander Loy. Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiol. Rev.*, 37(5):793–829, sep 2013.

[150] Ellen L Zechner. Inflammatory disease caused by intestinal pathobionts. *Curr. Opin. Microbiol.*, 35:64–69, feb 2017.

[151] Paul B Eckburg, Elisabeth M Bik, Charles N Bernstein, Elizabeth Purdom, Les Dethlefsen, Michael Sargent, Steven R Gill, Karen E Nelson, and David A Relman. Diversity of the human intestinal microbial flora. *Science*, 308(5728):1635–1638, 2005.

[152] Peter a Bron, Peter van Baarlen, and Michiel Kleerebezem. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat. Rev. Microbiol.*, 10(1):66–78, jan 2012.

[153] Bärbel Stecher and Wolf-Dietrich Hardt. Mechanisms controlling pathogen colonization of the gut. *Curr. Opin. Microbiol.*, 14(1):82–91, feb 2011.

[154] Seth Rakoff-Nahoum, Justin Paglino, Fatima Eslami-Varzaneh, Stephen Edberg, and Ruslan Medzhitov. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. *Cell*, 118(2):229–241, jul 2004.

[155] Simone Becattini, Ying Taur, and Eric G Pamer. Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends Mol. Med.*, 22(6):458–78, jun 2016.

[156] Filipe De Vadder, Petia Kovatcheva-Datchary, Daisy Goncalves, Jennifer Vinera, Carine Zitoun, Adeline Duchampt, Fredrik Bäckhed, and Gilles Mithieux.

Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell*, 156(1-2):84–96, jan 2014.

[157] Justin L Sonnenburg and Fredrik Backhed. Diet-microbiota interactions as moderators of human metabolism. *Nature*, 535(7610):56–64, jul 2016.

[158] Mercedes Gomez de Agüero, Stephanie C Ganal-Vonarburg, Tobias Fuhrer, Sandra Rupp, Yasuhiro Uchimura, Hai Li, Anna Steinert, Mathias Heikenwalder, Siegfried Hapfelmeier, Uwe Sauer, Kathy D McCoy, and Andrew J Macpherson. The maternal microbiota drives early postnatal innate immune development. *Science*, 351:1296–302, 2016.

[159] Yasmine Belkaid and Timothy W Hand. Role of the microbiota in immunity and inflammation. *Cell*, 157(1):121–41, mar 2014.

[160] Torsten Olszak, Dingding An, Sebastian Zeissig, Mp Miguel Pinilla Vera, Julia Richter, Andre Franke, Jonathan N Glickman, Reiner Siebert, Rebecca M Baron, Dennis L Kasper, and Richard S Blumberg. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*, 336(6080):489–493, 2012.

[161] Duane R. Wesemann, Andrew J. Portuguese, Robin M. Meyers, Michael P. Gallaghera, Kendra Cluff-Jonesa, Jennifer M. Mageea, Rohit A. Panchakshari, Scott J. Rodig, Thomas B. Keplerf, and Frederick W. Alt. Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature*, 501(7465):112–115, 2013.

[162] Katharina Brandl, George Plitas, Coralia N. Mihu, Carles Ubeda, Ting Jia, Martin Fleisher, Bernd Schnabl, Ronald P. DeMatteo, and Eric G. Pamer. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature*, 455(7214):804–7, oct 2008.

[163] Maayan Levy, Christoph A. Thaiss, David Zeevi, Lenka Dohnalová, Gili Zilberman-Schapira, Jemal Ali Mahdi, Eyal David, Alon Savidor, Tal Korem, Yonatan Herzig, Meirav Pevsner-Fischer, Hagit Shapiro, Anette Christ, Alon Harmelin, Zamir Halpern, Eicke Latz, Richard A. Flavell, Ido Amit, Eran Segal, and Eran Elinav. Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling. *Cell*, 163:1428–1443, 2015.

[164] Maria G Dominguez-Bello, Elizabeth K Costello, Monica Contreras, Magda Magris, Glida Hidalgo, Noah Fierer, and Rob Knight. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U. S. A.*, 107(26):11971–11975, 2010.

[165] Derrick M Chu, Jun Ma, Amanda L Prince, Kathleen M Antony, Maxim D Seferovic, and Kjersti M Aagaard. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat. Med.*, 2017.

[166] Julie Mirpuri, Megan Raetz, Carolyn R Sturge, Cara L Wilhelm, Alicia Benson, Rashmin C Savani, Lora V Hooper, and Felix Yarovinsky. Proteobacteria-specific IgA regulates maturation of the intestinal microbiota. *Gut Microbes*, 5(1):28–39, jan 2014.

[167] Daniel Garrido, David C. Dallas, and David a. Mills. Consumption of human milk glycoconjugates by infant-associated bifidobacteria: Mechanisms and implications. *Microbiol. (United Kingdom)*, 159:649–664, 2013.

[168] H Zhang, J B Sparks, S V Karyala, R Settlage, and X M Luo. Host adaptive immunity alters gut microbiota. *Isme J*, 9(3):770–781, 2015.

[169] Xinglin Zhang, Fernanda L Paganelli, Damien Bierschenk, Annemarie Kuipers, Marc J M Bonten, Rob J L Willems, and Willem van Schaik. Genome-Wide Identification of Ampicillin Resistance Determinants inEnterococcus faecium. *PLoS Genet.*, 8(6):e1002804, jun 2012.

[170] Margarita Martinez-Medina, Jérémy Denizot, Nicolas Dreux, Frédéric Robin, Elisabeth Billard, Richard Bonnet, Arlette Darfeuille-Michaud, and Nicolas Barnich. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut*, 63(1):116–24, jan 2014.

[171] Benjamin P Willing, Shannon L Russell, and B Brett Finlay. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat. Rev. Microbiol.*, 9(4):233–43, apr 2011.

[172] Christopher A. Lopez, Dawn D. Kingsbury, Eric M. Velazquez, and Andreas J. Bäumler. Collateral Damage: Microbiota-derived Metabolites and Immune Function in the Antibiotic Era. *Cell Host Microbe*, 16(2):156–163, 2014.

[173] Les Dethlefsen, Sue Susan M. Huse, Mitchell L. Sogin, and David a. Relman. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLoS Biol.*, 6(11):e280, nov 2008.

[174] Mary C Rea, Alleson Dobson, Orla O'Sullivan, Fiona Crispie, Fiona Fouhy, Paul D Cotter, Fergus Shanahan, Barry Kiely, Colin Hill, and R Paul Ross. Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc. Natl. Acad. Sci. U. S. A.*, 108 Suppl:4639–44, mar 2011.

[175] Brittany B. Lewis, Charlie G. Buffie, Rebecca A. Carter, Ingrid Leiner, Nora C. Toussaint, Liza C. Miller, Asia Gobourne, Lilan Ling, and Eric G. Pamer. Loss of Microbiota-Mediated Colonization Resistance to Clostridium difficile Infection with Oral Vancomycin Compared with Metronidazole. *J. Infect. Dis.*, 212(10):1656–1665, nov 2015.

[176] David A Hill, Mark C Siracusa, Michael C Abt, Brian S Kim, Dmytro Kobuley, Masato Kubo, Taku Kambayashi, David F LaRosa, Ellen D Renner, Jordan S Orange, Frederic D Bushman, and David Artis. Commensal bacterial–derived signals

regulate basophil hematopoiesis and allergic inflammation. *Nat Med*, 18(4):538–546, 2012.

[177] L Caetano M Antunes, Jun Han, Rosana B R Ferreira, Petra Lolić, Christoph H Borchers, and B Brett Finlay. Effect of antibiotic treatment on the intestinal metabolome. *Antimicrob. Agents Chemother.*, 55(4):14941–503, apr 2011.

[178] Cecilia Jernberg, Sonja Löfmark, Charlotta Edlund, and Janet K Jansson. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J.*, 1(1):56–66, may 2007.

[179] Charlie G Buffie, Irene Jarchum, Michele Equinda, Lauren Lipuma, Asia Gobourne, Agnes Viale, Carles Ubeda, Joao Xavier, and Eric G Pamer. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. *Infect. Immun.*, 80(1):62–73, jan 2012.

[180] Rosalie Maltby, Mary P Leatham-Jensen, Terri Gibson, Paul S Cohen, and Tyrrell Conway. Nutritional Basis for Colonization Resistance by Human Commensal Escherichia coli Strains HS and Nissle 1917 against E. coli O157:H7 in the Mouse Intestine. *PLoS One*, 8(1):e53957, jan 2013.

[181] Katharine M Ng, Jessica A Ferreyra, Steven K Higginbottom, Jonathan B Lynch, Purna C Kashyap, Smita Gopinath, Natasha Naidu, Biswa Choudhury, Bart C Weimer, Denise M Monack, and Justin L Sonnenburg. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature*, 502(7469):96–99, oct 2013.

[182] Maria Camila Montealegre, Kavindra V Singh, and Barbara E Murray. Gastrointestinal Tract Colonization Dynamics by Different Enterococcus faecium Clades. *J. Infect. Dis.*, pages 1–9, 2015.

[183] Nicole J Pultz, Lansing C Hoskins, and Curtis J Donskey. Vancomycin-resistant Enterococci may obtain nutritional support by scavenging carbohydrate fragments generated during mucin degradation by the anaerobic microbiota of the colon. *Microb. Drug Resist.*, 12(1):63–67, 2006.

[184] Sushma Kommineni, Daniel J. Bretl, Vy Lam, Rajrupa Chakraborty, Michael Hayward, Pippa Simpson, Yumei Cao, Pavlos Bousounis, Christopher J. Kristich, and Nita H. Salzman. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature*, 526(7575):719–722, 2015.

[185] Martina Sassone-Corsi, Sean-Paul Nuccio, Henry Liu, Dulcemaria Hernandez, Christine T. Vu, Amy A. Takahashi, Robert A. Edwards, and Manuela Raffatellu. Microcins mediate competition among Enterobacteriaceae in the inflamed gut. *Nature*, 540(7632):280–283, oct 2016.

[186] R Shin, M Suzuki, and Y Morishita. Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic Escherichia coli O157:H7. *J. Med. Microbiol.*, 51(3):201–206, mar 2002.

[187] Ansel Hsiao, a. M. Shamsir Ahmed, Sathish Subramanian, Nicholas W. Griffin, Lisa L. Drewry, William a. Petri, Rashidul Haque, Tahmeed Ahmed, and Jeffrey I. Gordon. Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. *Nature*, 515(7527):423–426, sep 2014.

[188] Sara D Lawhon, Russell Maurer, Mitsu Suyemoto, and Craig Altier. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. *Mol. Microbiol.*, 46(5):1451–1464, dec 2002.

[189] Charlie G. Buffie, Vanni Bucci, Richard R. Stein, Peter T. McKenney, Lilan Ling, Asia Gobourne, Daniel No, Hui Liu, Melissa Kinnebrew, Agnes Viale, Eric Littmann, Marcel R. M. van den Brink, Robert R. Jenq, Ying Taur, Chris Sander, Justin Cross, Nora C. Toussaint, Joao B. Xavier, and Eric G. Pamer. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature*, 517(7533):205–8, oct 2014.

[190] Casey M Theriot, Mark J Koenigsknecht, Paul E Carlson, Gabrielle E Hatton, Adam M Nelson, Bo Li, Gary B Huffnagle, Jun Z Li, and Vincent B Young. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nat. Commun.*, 5:3114, 2014.

[191] Meital Gury-BenAri, Christoph A. Thaiss, Nicolas Serafini, Deborah R. Winter, Amir Giladi, David Lara-Astiaso, Maayan Levy, Tomer Meir Salame, Assaf Weiner, Eyal David, Hagit Shapiro, Mally Dori-Bachash, Meirav Pevsner-Fischer, Erika Lorenzo-Vivas, Hadas Keren-Shaul, Franziska Paul, Alon Harmelin, Gérard Eberl, Shalev Itzkovitz, Amos Tanay, James P. Di Santo, Eran Elinav, and Ido Amit. The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell*, 166(5):1231–1246.e13, aug 2016.

[192] Yan Zheng, Patricia A Valdez, Dimitry M Danilenko, Yan Hu, Susan M Sa, Qian Gong, Alexander R Abbas, Zora Modrusan, Nico Ghilardi, Frederic J de Sauvage, and Wenjun Ouyang. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.*, 14(3):282–289, mar 2008.

[193] Lucille C Rankin, Mathilde J H Girard-Madoux, Cyril Seillet, Lisa A Mielke, Yann Kerdiles, Aurore Fenis, Elisabeth Wieduwild, Tracy Putoczki, Stanislas Mondot, Olivier Lantz, Dieter Demon, Anthony T Papenfuss, Gordon K Smyth, Mohamed Lamkanfi, Sebastian Carotta, Jean-Christophe Renauld, Wei Shi, Sabrina Carpentier, Tim Soos, Christopher Arendt, Sophie Ugolini, Nicholas D Huntington, Gabrielle T Belz, and Eric Vivier. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat. Immunol.*, 17(2):179–186, feb 2016.

[194] Arya Khosravi, Alberto Yáñez, Jeremy G Price, Andrew Chow, Miriam Merad, Helen S Goodridge, and Sarkis K Mazmanian. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe*, 15(3):374–381, mar 2014.

[195] Carles Ubeda, Ana Djukovic, and Sandrine Isaac. Roles of the intestinal microbiota in pathogen protection. *Clin. Transl. Immunol.*, 6(2):e128, 2017.

[196] Kenya Honda and Dan R Littman. The microbiota in adaptive immune homeostasis and disease. *Nature*, 535(7610):75–84, jul 2016.

[197] P Michetti, M J Mahan, J M Slauch, J J Mekalanos, and M R Neutra. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. *Infect. Immun.*, 60(5):1786–1792, may 1992.

[198] P Michetti, N Porta, M J Mahan, J M Slauch, J J Mekalanos, A L Blum, J P Kraehenbuhl, and M R Neutra. Monoclonal immunoglobulin A prevents adherence and invasion of polarized epithelial cell monolayers by Salmonella typhimurium. *Gastroenterology*, 107(4):915–923, oct 1994.

[199] F M Apter, P Michetti, L S Winner, J A Mack, J J Mekalanos, and M R Neutra. Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against Vibrio cholerae and cholera toxin by use of monoclonal IgA antibodies in vivo. *Infect. Immun.*, 61(12):5279–5285, dec 1993.

[200] Richard A. Strugnell and Odilia L.C. Wijburg. The role of secretory antibodies in infection immunity. *Nat. Rev. Microbiol.*, 8(1):656–667, 2010.

[201] Emma Slack, Maria L. Balmer, and Andrew J. Macpherson. B cells as a critical node in the microbiota-host immune system network. *Immunol. Rev.*, 260(1):50–66, jul 2014.

[202] K. Atarashi, T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, T. Taniguchi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science*, 331(6015):337–341, jan 2011.

[203] Koji Atarashi, Takeshi Tanoue, Kenshiro Oshima, Wataru Suda, Yuji Nagano, Hiroyoshi Nishikawa, Shinji Fukuda, Takuro Saito, Seiko Narushima, Koji Hase, Sangwan Kim, Joëlle V Fritz, Paul Wilmes, Satoshi Ueha, Kouji Matsushima, Hiroshi Ohno, Bernat Olle, Shimon Sakaguchi, Tadatsugu Taniguchi, Hidetoshi Morita, Masahira Hattori, and Kenya Honda. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*, 500(7461):232–236, aug 2013.

[204] Wafa N Al-Nassir, Ajay K Sethi, Yuejin Li, Michael J Pultz, Michelle M Riggs, and Curtis J Donskey. Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of Clostridium difficile-associated disease. *Antimicrob. Agents Chemother.*, 52(7):2403–2406, jul 2008.

[205] R D Poduval, R P Kamath, M Corpuz, E P Norkus, and C S Pitchumoni. Clostridium difficile and vancomycin-resistant enterococcus: the new nosocomial alliance. *Am. J. Gastroenterol.*, 95(12):3513–3515, dec 2000.

[206] Csaba Fehér and Josep Mensa. A Comparison of Current Guidelines of Five International Societies on Clostridium difficile Infection Management. *Infect. Dis. Ther.*, pages 207–230, 2016.

[207] Melissa A Kinnebrew, Carles Ubeda, Lauren A Zenewicz, Nichole Smith, Richard A Flavell, and Eric G Pamer. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. *J. Infect. Dis.*, 201(4):534–543, feb 2010.

[208] Michael C Abt, Charlie G Buffie, Bože Sušac, Simone Becattini, Rebecca A Carter, Ingrid Leiner, James W Keith, David Artis, Lisa C Osborne, and Eric G Pamer. TLR-7 activation enhances IL-22-mediated colonization resistance against vancomycin-resistant enterococcus. *Sci. Transl. Med.*, 8(327):327ra25, 2016.

[209] M Dodin and D E Katz. Faecal microbiota transplantation for Clostridium difficile infection. *Int. J. Clin. Pract.*, 68(3):363–368, mar 2014.

[210] Giovanni Cammarota, Gianluca Ianiro, Herbert Tilg, Mirjana Rajilić-Stojanović, Patrizia Kump, Reetta Satokari, Harry Sokol, Perttu Arkkila, Cristina Pintus, Ailsa Hart, Jonathan Segal, Marina Aloi, Luca Masucci, Antonio Molinaro, Franco Scaldaferri, Giovanni Gasbarrini, Antonio Lopez-Sanroman, Alexander Link, Pieter De Groot, Willem M De Vos, Christoph Högenauer, Peter Malfertheiner, Eero Mattila, Tomica Milosavljević, Max Nieuwdorp, Maurizio Sanguinetti, Magnus Simren, and Antonio Gasbarrini. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut*, 66(4):569–580, 2017.

[211] Carles Ubeda, Vanni Bucci, Silvia Caballero, Ana Djukovic, Nora C Toussaint, Michele Equinda, Lauren Lipuma, Lilan Ling, Asia Gobourne, Daniel No, Ying Taur, Robert R Jenq, Marcel R M van den Brink, Joao B Xavier, Eric G. Pamera, and Eric G Pamer. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. *Infect. Immun.*, 81(3):965–973, mar 2013.

[212] Jose U Scher, Andrew Sczesnak, Randy S Longman, Nicola Segata, Carles Ubeda, Craig Bielski, Tim Rostron, Vincenzo Cerundolo, Eric G Pamer, Steven B Abramson, Curtis Huttenhower, and Dan R Littman. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife*, 2:e01202, nov 2013.

[213] Daniel Aletaha, Tuhina Neogi, Alan J. Silman, Julia Funovits, David T. Felson, Clifton O. Bingham, Neal S. Birnbaum, Gerd R. Burmester, Vivian P. Bykerk, Marc D. Cohen, Bernard Combe, Karen H. Costenbader, Maxime Dougados, Paul Emery, Gianfranco Ferraccioli, Johanna M W Hazes, Kathryn Hobbs, Tom W J Huizinga, Arthur Kavanaugh, Jonathan Kay, Tore K. Kvien, Timothy Laing, Philip Mease, Henri a. Ménard, Larry W. Moreland, Raymond L. Naden, Theodore Pincus, Josef S. Smolen, Ewa Stanislawska-Biernat, Deborah Symmons, Paul P. Tak, Katherine S. Upchurch, Jiří Vencovský, Frederick Wolfe, and Gillian Hawker. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.*, 62(9):2569–2581, 2010.

[214] Silvia Caballero, Rebecca Carter, Xu Ke, Bože Sušac, Ingrid M. Leiner, Grace J. Kim, Liza Miller, Lilan Ling, Katia Manova, and Eric G. Pamer. Distinct but Spatially Overlapping Intestinal Niches for Vancomycin-Resistant Enterococcus faecium and Carbapenem-Resistant Klebsiella pneumoniae. *PLOS Pathog.*, 11:e1005132, 2015.

[215] Patrick D Schloss, Sarah L Westcott, Thomas Ryabin, Justine R Hall, Martin Hartmann, Emily B Hollister, Ryan A Lesniewski, Brian B Oakley, Donovan H Parks, Courtney J Robinson, Jason W Sahl, Blaz Stres, Gerhard G Thallinger, David J Van Horn, and Carolyn F Weber. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, 75(23):7537–7541, dec 2009.

[216] Robert Schmieder and Robert Edwards. Quality control and preprocessing of metagenomic datasets. *Bioinformatics*, 27(6):863–864, mar 2011.

[217] Erik Aronesty. ea-utils : "Command-line tools for processing biological sequencing data", 2011.

[218] Elmar Pruesse, Christian Quast, Katrin Knittel, Bernhard M Fuchs, Wolfgang Ludwig, Jörg Peplies, and Frank Oliver Glöckner. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.*, 35(21):7188–7196, 2007.

[219] Brian J Haas, Dirk Gevers, Ashlee M Earl, Mike Feldgarden, Doyle V Ward, Georgia Giannoukos, Dawn Ciulla, Diana Tabbaa, Sarah K Highlander, Erica Sodergren, Barbara Methé, Todd Z DeSantis, The Human Microbiome Human Microbiome Consortium, Joseph F Petrosino, Rob Knight, and Bruce W Birren. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.*, 21(3):494–504, mar 2011.

[220] R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 27(16):2194–2200, aug 2011.

[221] Susan M Huse, David Mark Welch, Hilary G Morrison, and Mitchell L Sogin. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ. Microbiol.*, 12(7):1889–1898, jul 2010.

[222] Torbjørn Rognes, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé. VSEARCH: a versatile open source tool for metagenomics. *PeerJ Prepr.*, 4:e2409v1, 2016.

[223] Melanie Schirmer, Umer Z Ijaz, Rosalinda D'Amore, Neil Hall, William T Sloan, and Christopher Quince. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nucleic Acids Res.*, 43(6):e37, mar 2015.

[224] CE Shannon. A mathematical theory of communication. *Bell Syst. Tech. J.*, 27:379–656, 1948.

[225] Qiong Wang, George M Garrity, James M Tiedje, and James R Cole. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.*, 73(16):5261–5267, aug 2007.

[226] Catherine Lozupone and Rob Knight. UniFrac : a New Phylogenetic Method for Comparing Microbial Communities UniFrac : a New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.*, 71(12):8228–8235, 2005.

[227] Luke Sheneman, Jason Evans, and James a. Foster. Clearcut: A fast implementation of relaxed neighbor joining. *Bioinformatics*, 22(22):2823–2824, 2006.

[228] Henning Seedorf, Nicholas W Griffin, Vanessa K Ridaura, Alejandro Reyes, Jiye Cheng, Federico E Rey, Michelle I Smith, Gabriel M Simon, Rudolf H Scheffrahn, Dagmar Woebken, Alfred M Spormann, William Van Treuren, Luke K Ursell, Megan Pirrung, Adam Robbins-Pianka, Brandi L Cantarel, Vincent Lombard, Bernard Henrissat, Rob Knight, and Jeffrey I Gordon. Bacteria from Diverse Habitats Colonize and Compete in the Mouse Gut. *Cell*, 159:1–14, oct 2014.

[229] Jari Oksanen, F Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R Minchin, R B O'Hara, Gavin L Simpson, Peter Solymos, M Henry H Stevens, Eduard Szoecs, and Helene Wagner. *vegan: Community Ecology Package*, 2017.

[230] K R Clarke and M Ainsworth. A method of linking multivariate community structure to environmental variables. *Mar. Ecol. Prog. Ser.*, 92:205–219, 1993.

[231] Yoav Benjamini and Yosef Hochberg. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B*, 57:289–300, 1995.

[232] R R Development Core Team. R: A language and environment for statistical computing., 2008.

[233] Nicola Segata, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S Garrett, and Curtis Huttenhower. Metagenomic biomarker discovery and explanation. *Genome Biol.*, 12(6):R60, jan 2011.

[234] Kevin E Ashelford, Andrew J Weightman, and John C Fry. PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Res.*, 30(15):3481–9, aug 2002.

[235] Marcel Martin. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17(1):10, may 2011.

[236] Laurent Modolo and Emmanuelle Lerat. UrQt: an efficient software for the Unsupervised Quality trimming of NGS data. *BMC Bioinformatics*, 16(1):137, apr 2015.

[237] Anton Bankevich, Sergey Nurk, Dmitry Antipov, Alexey A. Gurevich, Mikhail Dvorkin, Alexander S. Kulikov, Valery M. Lesin, Sergey I. Nikolenko, Son Pham, Andrey D. Prjibelski, Alexey V. Pyshkin, Alexander V. Sirotkin, Nikolay Vyahhi, Glenn Tesler, Max A. Alekseyev, and Pavel A. Pevzner. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.*, 19(5):455–477, may 2012.

[238] T. Seemann. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, 30(14):2068–2069, jul 2014.

[239] R. D. Finn, J. Tate, J. Mistry, P. C. Coggill, S. J. Sammut, H.-R. Hotz, G. Ceric, K. Forslund, S. R. Eddy, E. L. L. Sonnhammer, and A. Bateman. The Pfam protein families database. *Nucleic Acids Res.*, 36(Database):D281–D288, dec 2007.

[240] Jaime Huerta-Cepas, Damian Szklarczyk, Kristoffer Forslund, Helen Cook, Davide Heller, Mathias C. Walter, Thomas Rattei, Daniel R. Mende, Shinichi Sunagawa, Michael Kuhn, Lars Juhl Jensen, Christian von Mering, and Peer Bork. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.*, 44(D1):D286–D293, jan 2016.

[241] M Kanehisa and S Goto. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.*, 28(1):27–30, jan 2000.

[242] Sean R Eddy. A new generation of homology search tools based on probabilistic inference. *Genome Inform.*, 23(1):205–211, oct 2009.

[243] Michael Reck, Jürgen Tomasch, Zhiluo Deng, Michael Jarek, Peter Husemann, and Irene Wagner-Döbler. Stool metatranscriptomics: A technical guideline for mRNA stabilisation and isolation. *BMC Genomics*, 16:494, jan 2015.

[244] Christian Quast, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo Yarza, Jörg Peplies, and Frank Oliver Glöckner. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.*, 41(Database issue):D590–D596, jan 2013.

[245] Ben Langmead and Steven L Salzberg. Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, 9(4):357–359, mar 2012.

[246] Mouse Genome Sequencing Consortium, Robert H Waterston, Kerstin Lindblad-Toh, Ewan Birney, Jane Rogers, Josep F Abril, Pankaj Agarwal, Richa Agarwala, Rachel Ainscough, Marina Alexandersson, Peter An, Stylianos E Antonarakis, John Attwood, Robert Baertsch, Jonathon Bailey, Karen Barlow, Stephan Beck, Eric Berry, Bruce Birren, Toby Bloom, Peer Bork, Marc Botcherby, Nicolas Bray, Michael R Brent, Daniel G Brown, Stephen D Brown, Carol Bult, John Burton, Jonathan Butler, Robert D Campbell, Piero Carninci, Simon Cawley, Francesca Chiaromonte, Asif T Chinwalla, Deanna M Church, Michele Clamp, Christopher Clee, Francis S Collins, Lisa L Cook, Richard R Copley, Alan Coulson, Olivier Couronne, James Cuff, Val Curwen, Tim Cutts, Mark Daly, Robert David, Joy

Davies, Kimberly D Delehaunty, Justin Deri, Emmanouil T Dermitzakis, Colin Dewey, Nicholas J Dickens, Mark Diekhans, Sheila Dodge, Inna Dubchak, Diane M Dunn, Sean R Eddy, Laura Elnitski, Richard D Emes, Pallavi Eswara, Eduardo Eyras, Adam Felsenfeld, Ginger A Fewell, Paul Flicek, Karen Foley, Wayne N Frankel, Lucinda A Fulton, Robert S Fulton, Terrence S Furey, Diane Gage, Richard A Gibbs, Gustavo Glusman, Sante Gnerre, Nick Goldman, Leo Goodstadt, Darren Grafham, Tina A Graves, Eric D Green, Simon Gregory, Roderic Guigó, Mark Guyer, Ross C Hardison, David Haussler, Yoshihide Hayashizaki, LaDeana W Hillier, Angela Hinrichs, Wratko Hlavina, Timothy Holzer, Fan Hsu, Axin Hua, Tim Hubbard, Adrienne Hunt, Ian Jackson, David B Jaffe, L Steven Johnson, Matthew Jones, Thomas A Jones, Ann Joy, Michael Kamal, Elinor K Karlsson, Donna Karolchik, Arkadiusz Kasprzyk, Jun Kawai, Evan Keibler, Cristyn Kells, W James Kent, Andrew Kirby, Diana L Kolbe, Ian Korf, Raju S Kucherlapati, Edward J Kulbokas, David Kulp, Tom Landers, J P Leger, Steven Leonard, Ivica Letunic, Rosie Levine, Jia Li, Ming Li, Christine Lloyd, Susan Lucas, Bin Ma, Donna R Maglott, Elaine R Mardis, Lucy Matthews, Evan Mauceli, John H Mayer, Megan McCarthy, W Richard McCombie, Stuart McLaren, Kirsten McLay, John D McPherson, Jim Meldrim, Beverley Meredith, Jill P Mesirov, Webb Miller, Tracie L Miner, Emmanuel Mongin, Kate T Montgomery, Michael Morgan, Richard Mott, James C Mullikin, Donna M Muzny, William E Nash, Joanne O Nelson, Michael N Nhan, Robert Nicol, Zemin Ning, Chad Nusbaum, Michael J O'Connor, Yasushi Okazaki, Karen Oliver, Emma Overton-Larty, Lior Pachter, Genís Parra, Kymberlie H Pepin, Jane Peterson, Pavel Pevzner, Robert Plumb, Craig S Pohl, Alex Poliakov, Tracy C Ponce, Chris P Ponting, Simon Potter, Michael Quail, Alexandre Reymond, Bruce A Roe, Krishna M Roskin, Edward M Rubin, Alistair G Rust, Ralph Santos, Victor Sapojnikov, Brian Schultz, Jörg Schultz, Matthias S Schwartz, Scott Schwartz, Carol Scott, Steven Seaman, Steve Searle, Ted Sharpe, Andrew Sheridan, Ratna Shownkeen, Sarah Sims, Jonathan B Singer, Guy Slater, Arian Smit, Douglas R Smith, Brian Spencer, Arne Stabenau, Nicole Stange-Thomann, Charles Sugnet, Mikita Suyama, Glenn Tesler, Johanna Thompson, David Torrents, Evanne Trevaskis, John Tromp, Catherine Ucla, Abel Ureta-Vidal, Jade P Vinson, Andrew C Von Niederhausern, Claire M Wade, Melanie Wall, Ryan J Weber, Robert B Weiss, Michael C Wendl, Anthony P West, Kris Wetterstrand, Raymond Wheeler, Simon Whelan, Jamey Wierzbowski, David Willey, Sophie Williams, Richard K Wilson, Eitan Winter, Kim C Worley, Dudley Wyman, Shan Yang, Shiaw-Pyng Yang, Evgeny M Zdobnov, Michael C Zody, and Eric S Lander. Initial sequencing and comparative analysis of the mouse genome. *Nature*, 420(6915):520–562, dec 2002.

[247] Wenhan Zhu, Alexandre Lomsadze, and Mark Borodovsky. Ab initio gene identification in metagenomic sequences. *Nucleic Acids Res.*, 38(12):e132, jul 2010.

[248] Milot Mirdita, Lars Von Den Driesch, Clovis Galiez, Maria J Martin, Johannes Oding, and Martin Steinegger. Uniclust databases of clustered and deeply annotated protein sequences and alignments. *Nucleic Acids Res.*, 45, 2017.

[249] Heng Li and Richard Durbin. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25(14):1754–1760, jul 2009.

[250] Turki Abujamel, Jennifer L. Cadnum, Lucy a. Jury, Venkata C K Sunkesula, Sirisha Kundrapu, Robin L. Jump, Alain C. Stintzi, and Curtis J. Donskey. Defining the Vulnerable Period for Re-Establishment of Clostridium difficile Colonization after Treatment of C. difficile Infection with Oral Vancomycin or Metronidazole. *PLoS One*, 8(10):1–12, jan 2013.

[251] Curtis J. Donskey, Tanvir K. Chowdhry, Michelle T. Hecker, Claudia K. Hoyen, Jennifer A. Hanrahan, Andrea M. Hujer, Rebecca A. Hutton-Thomas, Christopher C. Whalen, Robert A. Bonomo, and Louis B. Rice. Effect of Antibiotic Therapy on the Density of Vancomycin-Resistant Enterococci in the Stool of Colonized Patients. *N. Engl. J. Med.*, 343(26):1925–1932, dec 2000.

[252] Sandrine Isaac, Jose U. Scher, Ana Djukovic, Nuria Jiménez, Dan R. Littman, Steven B. Abramson, Eric G. Pamer, and Carles Ubeda. Short- and long-term effects of oral vancomycin on the human intestinal microbiota. *J. Antimicrob. Chemother.*, 72(1):128–136, oct 2017.

[253] K. R. Clarke. Non-parametric multivariate analyses of changes in community structure. *Austral Ecol.*, 18(1):117–143, mar 1993.

[254] S S Epstein. The phenomenon of microbial uncultivability. *Curr. Opin. Microbiol.*, 16(5):636–642, oct 2013.

[255] Jean Christophe Lagier, Perrine Hugon, Saber Khelaifia, Pierre Edouard Fournier, Bernard La Scola, and Didier Raoult. The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin. Microbiol. Rev.*, 28(1):237–264, 2015.

[256] K. A. Miller, R. S. Phillips, J. Mrazek, and T. R. Hoover. Salmonella Utilizes D-Glucosaminate via a Mannose Family Phosphotransferase System Permease and Associated Enzymes. *J. Bacteriol.*, 195(18):4057–4066, sep 2013.

[257] Ying Zhao, Junfang Wu, Jia V Li, Ning Yi Zhou, Huiru Tang, and Yulan Wang. Gut microbiota composition modifies fecal metabolic profiles in mice. *J. Proteome Res.*, 12(6):2987–2999, 2013.

[258] J M Swenson, R R Facklam, and C Thornsberry. Antimicrobial susceptibility of vancomycin-resistant Leuconostoc, Pediococcus, and Lactobacillus species. *Antimicrob. Agents Chemother.*, 34(4):543–549, apr 1990.

[259] S. M. Finegold, D. Molitoris, and M. L. Vaisanen. Study of the in vitro activities of rifaximin and comparator agents against 536 anaerobic intestinal bacteria from the perspective of potential utility in pathology involving bowel flora. *Antimicrob. Agents Chemother.*, 53(1):281–286, 2009.

[260] Sydney M Finegold, Denise Molitoris, Marja-liisa Vaisanen, Yuli Song, Chengxu Liu, Mauricio Bolaños, and Mauricio Bolan. In Vitro Activities of OPT-80 and Comparator Drugs against Intestinal Bacteria In Vitro Activities of OPT-80 and Comparator Drugs against Intestinal Bacteria. *Antimicrob. Agents Chemother.*, 48(12):4898–4902, 2004.

[261] Kim L. Credito and Peter C. Appelbaum. Activity of OPT-80, a novel macrocycle, compared with those of eight other agents against selected anaerobic species. *Antimicrob. Agents Chemother.*, 48(11):4430–4434, 2004.

[262] Diane M. Citron, Kerin L. Tyrrell, C. Vreni Merriam, and Ellie J C Goldstein. In vitro activities of CB-183,315, vancomycin, and metronidazole against 556 strains of Clostridium difficile, 445 other intestinal anaerobes, and 56 Enterobacteriaceae species. *Antimicrob. Agents Chemother.*, 56(3):1613–1615, 2012.

[263] Ellie J C Goldstein, Diane M. Citron, Kerin L. Tyrrell, and C. Vreni Merriam. Comparative in vitro activities of SMT19969, a new antimicrobial agent, against Clostridium difficile and 350 gram-positive and gram-negative aerobic and anaerobic intestinal flora isolates. *Antimicrob. Agents Chemother.*, 57(10):4872–4876, 2013.

[264] C Edlund, L Barkholt, B Olsson-Liljequist, and C E Nord. Effect of vancomycin on intestinal flora of patients who previously received antimicrobial therapy. *Clin. Infect. Dis.*, 25(3):729–732, sep 1997.

[265] Sarkis K. Mazmanian, Cui Hua Liu, Arthur O. Tzianabos, and Dennis L. Kasper. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell*, 122(1):107–118, jul 2005.

[266] Thomas J Louie, Kris Cannon, Brendan Byrne, Judy Emery, Linda Ward, Melissa Eyben, and Walter Krulicki. Fidaxomicin preserves the intestinal microbiome during and after treatment of clostridium difficile infection (CDI) and reduces both toxin reexpression and recurrence of CDI. *Clin. Infect. Dis.*, 55(SUPPL.2):S132–S142, aug 2012.

[267] T. J. Louie, B. Byrne, J. Emery, L. Ward, W. Krulicki, D. Nguyen, K. Wu, and K. Cannon. Differences of the Fecal Microflora With Clostridium difficile Therapies. *Clin. Infect. Dis.*, 60(suppl 2):S91–S97, may 2015.

[268] Anne Vrieze, Carolien Out, Susana Fuentes, Lisanne Jonker, Isaie Reuling, Ruud S. Kootte, Els Van Nood, Frits Holleman, Max Knaapen, Johannes a. Romijn, Maarten R. Soeters, Ellen E. Blaak, Geesje M. Dallinga-Thie, Dorien Reijnders, Mariëtte T. Ackermans, Mireille J. Serlie, Filip K. Knop, Jenst J. Holst, Claude Van Der Ley, Ido P. Kema, Erwin G. Zoetendal, Willem M. De Vos, Joost B L Hoekstra, Erik S. Stroes, Albert K. Groen, and Max Nieuwdorp. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *J. Hepatol.*, 60(4):824–831, 2014.

[269] Xuan Zhang, Dongya Zhang, Huijue Jia, Qiang Feng, Donghui Wang, Di Liang, Xiangni Wu, Junhua Li, Longqing Tang, Yin Li, Zhou Lan, Bing Chen, Yanli Li,

Huanzi Zhong, Hailiang Xie, Zhuye Jie, Weineng Chen, Shanmei Tang, Xiaoqiang Xu, Xiaokai Wang, Xianghang Cai, Sheng Liu, Yan Xia, Jiyang Li, Xingye Qiao, Jumana Yousuf Al-Aama, Hua Chen, Li Wang, Qing-jun Wu, Fengchun Zhang, Wenjie Zheng, Yongzhe Li, Mingrong Zhang, Guangwen Luo, Wenbin Xue, Liang Xiao, Jun Li, Wanting Chen, Xun Xu, Ye Yin, Huanming Yang, Jian Wang, Karsten Kristiansen, Liang Liu, Ting Li, Qingchun Huang, Yingrui Li, and Jun Wang. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat. Med.*, 21(8):895–905, jul 2015.

[270] M.-U. Rashid, E. Zaura, M. J. Buijs, B. J. F. Keijser, W. Crielaard, C. E. Nord, and A. Weintraub. Determining the Long-term Effect of Antibiotic Administration on the Human Normal Intestinal Microbiota Using Culture and Pyrosequencing Methods. *Clin. Infect. Dis.*, 60(Suppl 2):S77–S84, 2015.

[271] T. J. Louie, J. Peppe, C. K. Watt, D. Johnson, R. Mohammed, G. Dow, K. Weiss, S. Simon, J. F. John, G. Garber, S.-C. Taber, D. M. Davidson, and Tolevamer Study Investigator Group. Tolevamer, a Novel Nonantibiotic Polymer, Compared with Vancomycin in the Treatment of Mild to Moderately Severe Clostridium difficile-Associated Diarrhea. *Clin. Infect. Dis.*, 43(4):411–420, aug 2006.

[272] Jacques Pepin, Louis Valiquette, Sandra Gagnon, Sophie Routhier, and Isabel Brazeau. Outcomes of Clostridium difficile-associated disease treated with metronidazole or vancomycin before and after the emergence of NAP1/027. *Am. J. Gastroenterol.*, 102(12):2781–2788, dec 2007.

[273] Marco Falcone, Alessandro Russo, Federica Iraci, Paolo Carfagna, Paola Goldoni, Vincenzo Vullo, and Mario Venditti. Risk Factors and Outcomes for Bloodstream Infections Secondary to Clostridium difficile Infection. *Antimicrob. Agents Chemother.*, 60(1):252–257, jan 2016.

[274] Seth Rakoff-Nahoum, Michael J Coyne, and Laurie E Comstock. An ecological network of polysaccharide utilization among human intestinal symbionts. *Curr. Biol.*, 24(1):40–49, jan 2014.

[275] K. a. Knoop, K. G. McDonald, D. H. Kulkarni, and R. D. Newberry. Antibiotics promote inflammation through the translocation of native commensal colonic bacteria. *Gut*, 65(7):1100–1109, 2016.

[276] Sebastian E Winter, Maria G Winter, Mariana N Xavier, Parameth Thiennimitr, Victor Poon, A Marijke Keestra, Richard C Laughlin, Gabriel Gomez, Jing Wu, Sara D Lawhon, Ina E Popova, Sanjai J Parikh, L Garry Adams, Renée M Tsolis, Valley J Stewart, and Andreas J Bäumler. Host-derived nitrate boosts growth of E. coli in the inflamed gut. *Science*, 339(6120):708–711, feb 2013.

[277] Usha Stiefel, Michelle M Nerandzic, Michael J Pultz, and Curtis J Donskey. Gastrointestinal Colonization with a Cephalosporinase-Producing Bacteroides Species Preserves Colonization Resistance against Vancomycin-Resistant Enterococcus and Clostridium difficile in Cephalosporin-Treated Mice. *Antimicrob. Agents Chemother.*, 58(8):4535–42, aug 2014.

[278] Nathan P McNulty, Meng Wu, Alison R Erickson, Chongle Pan, Brian K Erickson, Eric C Martens, Nicholas a Pudlo, Brian D Muegge, Bernard Henrissat, Robert L Hettich, and Jeffrey I Gordon. Effects of diet on resource utilization by a model human gut microbiota containing Bacteroides cellulosilyticus WH2, a symbiont with an extensive glycobiome. *PLoS Biol.*, 11(8):e1001637, jan 2013.

[279] Gary D Wu, Jun Chen, Christian Hoffmann, Kyle Bittinger, Ying-yu Chen, A Sue, Meenakshi Bewtra, Dan Knights, William A Walters, Rob Knight, Erin Gilroy, Kernika Gupta, Robert Baldassano, Lisa Nessel, and Hongzhe Li. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science (80-.).*, 334(6052):105–108, 2011.

[280] V Shankar, M Gouda, J Moncivaiz, A Gordon, N V Reo, L Hussein, and O Paliy. Differences in Gut Metabolites and Microbial Composition and Functions between Egyptian and U.S. Children Are Consistent with Their Diets. *mSystems*, 2(1):e00169–16, feb 2017.

[281] Falk Hildebrand, Thi Loan Anh Nguyen, Brigitta Brinkman, Roberto Garcia Yunta, Benedicte Cauwe, Peter Vandenabeele, Adrian Liston, Jeroen Raes, Thi Loan, Anh Nguyen, Brigitta Brinkman, Roberto Garcia Yunta, Benedicte Cauwe, Peter Vandenabeele, Adrian Liston, and Jeroen Raes. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol.*, 14(1):R4, jan 2013.

[282] Jun Wang, Miriam Linnenbrink, Sven Künzel, Ricardo Fernandes, Marie-Josée Nadeau, Philip Rosenstiel, and John F Baines. Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice. *Proc. Natl. Acad. Sci. U. S. A.*, 111(26):2703–2710, jul 2014.

[283] Theo S Plantinga, Wendy W C van Maren, Jeroen van Bergenhenegouwen, Marjolijn Hameetman, Stefan Nierkens, Cor Jacobs, Dirk J de Jong, Leo A B Joosten, Belinda van't Land, Johan Garssen, Gosse J Adema, and Mihai G Netea. Differential Toll-like receptor recognition and induction of cytokine profile by Bifidobacterium breve and Lactobacillus strains of probiotics. *Clin. Vaccine Immunol.*, 18(4):621–628, apr 2011.

[284] Muriel Derrien, M Carmen Collado, Kaouther Ben-Amor, Seppo Salminen, and Willem M de Vos. The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. *Appl. Environ. Microbiol.*, 74(5):1646–1648, mar 2008.

[285] Celia Dingemanse, Clara Belzer, Sacha A.F.T. van Hijum, Marie Günthel, Daniela Salvatori, Johan T. den Dunnen, Ed J. Kuijper, Peter Devilee, Willem M. de Vos, GertJan B. van Ommen, and Els C. Robanus-Maandag. Akkermansia muciniphila and Helicobacter typhlonius modulate intestinal tumor development in mice. *Carcinogenesis*, 36(11):1388–1396, nov 2015.

[286] Clara Belzer and Willem M de Vos. Microbes inside–from diversity to function: the case of Akkermansia. *ISME J.*, 6(8):1449–1458, aug 2012.

[287] Noora Ottman, Justus Reunanen, Marjolein Meijerink, Taija E Pietilä, Veera Kainulainen, Judith Klievink, Laura Huuskonen, Steven Aalvink, Mikael Skurnik, Sjef Boeren, Reetta Satokari, Annick Mercenier, Airi Palva, Hauke Smidt, Willem M de Vos, and Clara Belzer. Pili-like proteins of Akkermansia muciniphila modulate host immune responses and gut barrier function. *PLoS One*, 12(3):e0173004, 2017.

[288] C H F Hansen, L Krych, D S Nielsen, F K Vogensen, L H Hansen, S J Sørensen, K Buschard, and A K Hansen. Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. *Diabetologia*, 55(8):2285–2294, aug 2012.

[289] Amandine Everard, Clara Belzer, Lucie Geurts, Janneke P Ouwerkerk, Céline Druart, Laure B Bindels, Yves Guiot, Muriel Derrien, Giulio G Muccioli, Nathalie M Delzenne, Willem M de Vos, and Patrice D Cani. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci.*, 110(22):9066–9071, may 2013.

[290] M Wlodarska, B Willing, K M Keeney, A Menendez, K S Bergstrom, N Gill, S L Russell, B A Vallance, and B B Finlay. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. *Infect. Immun.*, 79(4):1536–1545, apr 2011.

[291] Bhanu Priya Ganesh, Robert Klopfleisch, Gunnar Loh, Michael Blaut, and Bernhard Ryffel. Commensal Akkermansia muciniphila Exacerbates Gut Inflammation in Salmonella Typhimurium-Infected Gnotobiotic Mice. *PLoS One*, 8(9), 2013.

[292] S El Aidy, M Derrien, R Aardema, G Hooiveld, S E Richards, A Dane, J Dekker, R Vreeken, F Levenez, J Doré, E G Zoetendal, P van Baarlen, and M Kleerebezem. Transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonisation of germ-free mice. *Benef. Microbes*, 5(1):67–77, mar 2014.

[293] Hedvig E Jakobsson, Ana M Rodríguez-Piñeiro, André Schütte, Anna Ermund, Preben Boysen, Mats Bemark, Felix Sommer, Fredrik Bäckhed, Gunnar C Hansson, and Malin Ev V Johansson. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Rep.*, 16(2):164–177, feb 2015.

[294] Frédéric Raymond, Amin a Ouameur, Maxime Déraspe, Naeem Iqbal, Hélène Gingras, Bédis Dridi, Philippe Leprohon, Pier-Luc Plante, Richard Giroux, Ève Bérubé, Johanne Frenette, Dominique K Boudreau, Jean-Luc Simard, Isabelle Chabot, Marc-Christian Domingo, Sylvie Trottier, Maurice Boissinot, Ann Huletsky, Paul H Roy, Marc Ouellette, Michel G Bergeron, and Jacques Corbeil. The initial state of the human gut microbiome determines its reshaping by antibiotics. *ISME J.*, 10(3):1–14, mar 2015.

[295] David L Paterson and Robert A Bonomo. Extended-spectrum beta-lactamases: a clinical update. *Clin. Microbiol. Rev.*, 18(4):657–686, oct 2005.

[296] Carles Ubeda, Lauren Lipuma, Asia Gobourne, Agnes Viale, Ingrid Leiner, Michele Equinda, Raya Khanin, and Eric G Pamer. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J. Exp. Med.*, 209(8):1445–1456, jul 2012.

[297] Richard R Stein, Vanni Bucci, Nora C Toussaint, Charlie G Buffie, Gunnar Rätsch, Eric G Pamer, Chris Sander, and João B Xavier. Ecological modeling from time-series inference: insight into dynamics and stability of intestinal microbiota. *PLoS Comput. Biol.*, 9(12):e1003388, jan 2013.

[298] Louis B. Rice, Rebecca Hutton[U+2010]Thomas, Viera Lakticova, Marion S. Helfand, and Curtis J. Donskey. Beta-Lactam Antibiotics and Gastrointestinal Colonization with Vancomycin Resistant Enterococci. *J. Infect. Dis.*, 189(6):1113–1118, mar 2004.

[299] James A. McKinnell, Danielle F. Kunz, Eric Chamot, Mukesh Patel, Rhett M. Shirley, Stephen A. Moser, John W. Baddley, Peter G. Pappas, and Loren G. Miller. Association between Vancomycin-Resistant Enterococci Bacteremia and Ceftriaxone Usage. *Infect. Control Hosp. Epidemiol.*, 33(07):718–724, jul 2012.

[300] Corinne Ferrier Maurice, Henry Joseph Haiser, and Peter James Turnbaugh. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. *Cell*, 152(1-2):39–50, jan 2013.

[301] Alyxandria M Schubert, Hamide Sinani, Patrick D Schloss, Alyxandria M Schubert, Hamide Sinani, and Patrick D Schloss. Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile. *MBio*, 6(4):e00974, jul 2015.

[302] T. Iino, K. Mori, K. Tanaka, K.-i. Suzuki, and S. Harayama. Oscillibacter valericigenes gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. *Int. J. Syst. Evol. Microbiol.*, 57(8):1840–1845, aug 2007.

[303] Yoko Katano, Shun Fujinami, Akatsuki Kawakoshi, Hidekazu Nakazawa, Syoko Oji, Takao Iino, Akio Oguchi, Akiho Ankai, Shigehiro Fukui, Yasuyuki Terui, Sachi Kamata, Takeshi Harada, Satoshi Tanikawa, Ken-Ichiro Suzuki, and Nobuyuki Fujita. Complete genome sequence of Oscillibacter valericigenes Sjm18-20(T) (=NBRC 101213(T)). *Stand. Genomic Sci.*, 6(3):406–14, jul 2012.

[304] A. Naseribafrouei, K. Hestad, E. Avershina, M. Sekelja, A. Linløkken, R. Wilson, and K. Rudi. Correlation between the human fecal microbiota and depression. *Neurogastroenterol. Motil.*, 26(8):1155–1162, aug 2014.

[305] Trevor D Lawley, Simon Clare, Alan W Walker, Mark D Stares, Thomas R Connor, Claire Raisen, David Goulding, Roland Rad, Fernanda Schreiber, Cordelia Brandt,

Laura J Deakin, Derek J Pickard, Sylvia H Duncan, Harry J Flint, Taane G Clark, Julian Parkhill, and Gordon Dougan. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing Clostridium difficile disease in mice. *PLoS Pathog.*, 8(10):e1002995, jan 2012.

[306] Michael S. Gilmore, Marcus Rauch, Matthew M. Ramsey, Paul R. Himes, Sriram Varahan, Janet M. Manson, Francois Lebreton, and Lynn Ernest Hancock. Pheromone killing of multidrug-resistant Enterococcus faecalis V583 by native commensal strains. *Proc. Natl. Acad. Sci.*, 112(23):201500553, 2015.

[307] María S Juárez Tomás, Carolina I Saralegui Duhart, Priscilla R De Gregorio, Esteban Vera Pingitore, and María E Nader-Macías. Urogenital pathogen inhibition and compatibility between vaginal Lactobacillus strains to be considered as probiotic candidates. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 159(2):399–406, dec 2011.

[308] Silvia Caballero, Sohn Kim, Rebecca A. Carter, Ingrid M. Leiner, Boze Susac, Liza Miller, Grace J. Kim, Lilan Ling, Eric G. Pamer. Cooperating Commensals Restore Colonization Resistance to Vancomycin-Resistant Enterococcus faecium *Cell Host Microbe*, 21(5):592–602, 2017.

[309] Mirjana Rajilić-Stojanović and Willem M de Vos. The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol. Rev.*, may 2014.

[310] Hilary P Browne, Samuel C Forster, Blessing O Anonye, Nitin Kumar, B Anne Neville, Mark D Stares, David Goulding, and Trevor D Lawley. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*, 533(7604):543–6, 2016.

[311] Ilias Lagkouvardos, Rüdiger Pukall, Birte Abt, Bärbel U. Foesel, Jan P. Meier-Kolthoff, Neeraj Kumar, Anne Bresciani, Inés Martínez, Sarah Just, Caroline Ziegler, Sandrine Brugiroux, Debora Garzetti, Mareike Wenning, Thi P. N. Bui, Jun Wang, Floor Hugenholtz, Caroline M. Plugge, Daniel A. Peterson, Mathias W. Hornef, John F. Baines, Hauke Smidt, Jens Walter, Karsten Kristiansen, Henrik B. Nielsen, Dirk Haller, Jörg Overmann, Bärbel Stecher, and Thomas Clavel. The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota. *Nat. Microbiol.*, 1(10):16131, aug 2016.

[312] Kumaran Sivagnanam, Vijaya Gs Raghavan, Manesh Shah, Robert L Hettich, Nathan C Verberkmoes, and Mark G Lefsrud. Comparative shotgun proteomic analysis of Clostridium acetobutylicum from butanol fermentation using glucose and xylose. *Proteome Sci.*, 9:66, 2011.

[313] Thorsten W Grebe and Jeff Stock. Bacterial chemotaxis: The five sensors of a bacterium. *Curr. Biol.*, 8(5):154–157, feb 1998.

[314] Alline R Pacheco and Vanessa Sperandio. Enteric Pathogens Exploit the Microbiota-generated Nutritional Environment of the Gut. *Microbiol Spectr.*, 3(3), 2015.

[315] Anna Stincone, Alessandro Prigione, Thorsten Cramer, Mirjam M C Wamelink, Kate Campbell, Eric Cheung, Viridiana Olin-Sandoval, Nana-Maria Grüning, Antje Krüger, Mohammad Tauqeer Alam, Markus A Keller, Michael Breitenbach, Kevin M Brindle, Joshua D Rabinowitz, and Markus Ralser. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biol. Rev. Camb. Philos. Soc.*, 90(3):927–63, aug 2015.

[316] Andreas Brinkkotter, Holger Kloss, C.-A. A. Alpert, Joseph W. Lengeler, Andreas Brinkkötter, Holger Klöß, C.-A. A. Alpert, and Joseph W. Lengeler. Pathways for the utilization of N-acetyl-galactosamine and galactosamine in Escherichia coli. *Mol. Microbiol.*, 37(1):125–135, jul 2000.

[317] Gabriella C van Zanten, Anne Knudsen, Henna Röytiö, Sofia Forssten, Mark Lawther, Andreas Blennow, Sampo J Lahtinen, Mogens Jakobsen, Birte Svensson, and Lene Jespersen. The effect of selected synbiotics on microbial composition and short-chain fatty acid production in a model system of the human colon. *PLoS One*, 7(10):e47212, jan 2012.

[318] Meredith M. Curtis, Zeping Hu, Claire Klimko, Sanjeev Narayanan, Ralph Deberardinis, and Vanessa Sperandio. The Gut Commensal Bacteroides thetaiotaomicron Exacerbates Enteric Infection through Modification of the Metabolic Landscape. *Cell Host Microbe*, 16(6):759–769, dec 2014.

[319] E A Smith and G T Macfarlane. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J. Appl. Bacteriol.*, 81(3):288–302, sep 1996.

[320] Juana Mosele, Alba Macià, and Maria-José Motilva. Metabolic and Microbial Modulation of the Large Intestine Ecosystem by Non-Absorbed Diet Phenolic Compounds: A Review. *Molecules*, 20(9):17429–17468, sep 2015.

[321] Fernando Cardona, Cristina Andrés-Lacueva, Sara Tulipani, Francisco J. Tinahones, and María Isabel Queipo-Ortuño. Benefits of polyphenols on gut microbiota and implications in human health. *J. Nutr. Biochem.*, 24(8):1415–1422, 2013.

[322] Xuetong Fan, Karen Wagner, KimberlyJ. B. Sokorai, and Helen Ngo. Inactivation of Gram-Positive Bacteria by Novel Phenolic Branched-Chain Fatty Acids. *J. Food Prot.*, 80(1):6–14, jan 2017.

[323] Eerdunbayaer, Mohamed Orabi, Hiroe Aoyama, Teruo Kuroda, and Tsutomu Hatano. Structures of New Phenolics Isolated from Licorice, and the Effectiveness of Licorice Phenolics on Vancomycin-Resistant Enterococci. *Molecules*, 19(9):13027–13041, aug 2014.

[324] Sumitkumar Jagani, Rahul Chelikani, and Dong-Shik Kim. Effects of phenol and natural phenolic compounds on biofilm formation by Pseudomonas aeruginosa. *Biofouling*, 25(4):321–324, mar 2009.

[325] Alba Chavez-Dozal, David Hogan, Clayton Gorman, Alvaro Quintanal-Villalonga, and Michele K Nishiguchi. Multiple Vibrio fischeri genes are involved in biofilm formation and host colonization. *FEMS Microbiol. Ecol.*, 81(3):562–73, sep 2012.

[326] Diede Fennema, Ian R Phillips, and Elizabeth A Shephard. Trimethylamine and Trimethylamine N-Oxide, a Flavin-Containing Monooxygenase 3 (FMO3)-Mediated Host-Microbiome Metabolic Axis Implicated in Health and Disease. *Drug Metab. Dispos.*, 44(11):1839–1850, nov 2016.