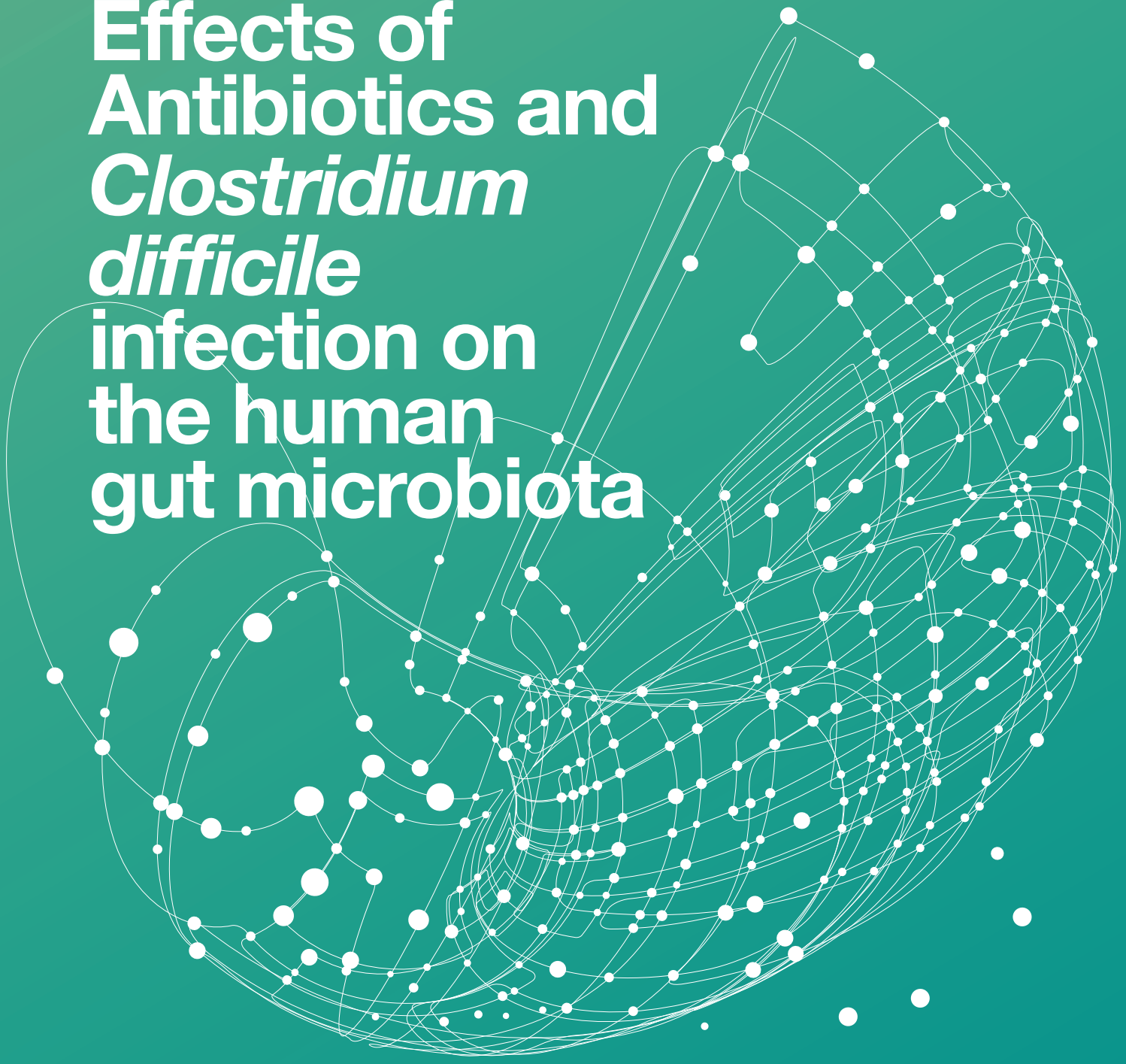
Effects of Antibiotics and *Clostridium difficile* infection on the human gut microbiota



Doctoranda : **ANA ELENA PÉREZ COBAS**

Para optar al grado de Doctor en Biodiversidad por la Universitat de València

FACULTAT DE CIÈNCIES BIOLÒGIQUES

Unidad Mixta de Investigación en Genómica y Salud de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO) y el Instituto Cavanilles de Biodiversidad y Biología Evolutiva de la Universitat de València

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"The emergence of antibiotic resistance is the most eloquent example of Darwin's principle of evolution that there ever was"

DAVID LIVERMORE

Amparo Latorre y Andrés Moya, Catedráticos del Departamento de Genética de la Universitat de València, y María José Gosalbes, Investigadora del Centro de Investigación Biomédica en Red (Epidemiología y Salud Pública) (CIBERESP), certifican que la memoria titulada "Effects of antibiotics and *Clostridium difficile* infection on the human gut microbiota " ha sido realizada bajo su dirección en la unidad mixta de investigación en Genómica y Salud FISABIO-UVEG/Instituto Cavanilles por Ana Elena Pérez Cobas para optar al grado de doctor en Biodiversidad por la Universitat de València.

Y para que así conste, firman el presente certificado.

Amparo Latorre

Andrés Moya

María José Gosalbes

Valencia, a _____

A mi mamut
A mis abuelos
A Mima y Carmen

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Quiero agradecer en primer lugar a mis directores de tesis Pepa, Amparo y Andrés. A Pepa por su apoyo constante y su capacidad para superar cualquier dificultad, a Amparo por sus consejos y por haberme dado la oportunidad de trabajar en diferentes proyectos, y a Andrés por abrirme las puertas del Cavanilles, que ha representado el inicio de esta historia. También quiero hacer un agradecimiento especial a Alejandro Artacho, que me inició en el misterioso mundo de la progRamación y por hacer sencillo lo complicado a la hora de trabajar. Por otro lado quiero agradecer a Eric y a su grupo de París, por haberme tratado maravillosamente durante mi estancia y enseñarme como trabajan los verdaderos bioinformáticos. Finalmente, quiero agradecer en general, a la gente del área de Genómica y Salud por haber hecho funcionar nuestro laboratorio desde cero y por su colaboración durante mis años de tesis.

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1

INTRODUCTION

1. INTRODUCTION

1.1. A GLANCE TO THE MICROBIAL ECOLOGY HISTORY

Microbial ecology is a research field in microbiology that focuses on the role of microbes in the environment and their interactions. It is a significant area of study since prokaryotes are the most abundant form of life on the planet, and has a main impact in the entire biosphere (Moya et al. 2008, Whitman et al. 1998).

Anton van Leeuwenhoek, a Dutch tradesman and scientist, was the first observing microorganisms after scraping his own teeth in 1683, initiating one of the widest fields of biology: microbiology. The Golden Era of microbiology began 200 years later, mainly by the contributions of Louis Pasteur, Ferdinand Cohn and Robert Koch. The later scientist, known as the father of modern bacteriology, confirmed through studies in tuberculosis, anthrax and cholera, that microorganisms are causative agents of disease. He also set the standards for medical microbiology, with his famous postulates that link a specific microorganism with a specific disease, being essential the isolation and pure culture of the agent.

A few years later, Martinus Beijerinck, a Dutch microbiologist and botanist, introduced the enriched culture, a medium with specific properties which allows the growth of particular microorganisms isolated from the environment. Beijerinck together with the Russian microbiologist Sergei Winogradsky, were the pioneers in the study of microorganisms in a physiologic and ecological point of view. Winogradsky is well-known for discovering the chemosynthesis, the process by which organisms derive energy through the oxidation of inorganic compounds.

Later, a main discovery came from the microbial ecologist Robert Edward Hungate when developed the first techniques for the culturing of anaerobic microbes during his studies on the bovine rumen. From the mid- to late 20th century, enrichment culture of microorganisms, microscopy and biochemical tests enabled great advances in the study of microbial communities. Despite the great contributions derived from culture techniques, an important limitation of these techniques is the large number of bacteria from several environments that are unable to grow in culture media, a phenomenon known as "the great plate anomaly" (Staley & Konopka 1985). In fact, it has been estimated that more than 99% of bacteria remain uncultured (Amann et al. 1995, Eilers et al. 2000).

A breakthrough came by 1980 when the American microbiologist and biophysicist Carl Woese and colleagues proposed to classify the life in three domains (bacteria, archaea and eukarya) using the small ribosomal subunit for a phylogenetic reconstruction of the tree of life (Woese & Fox 1977). Since then, the 16S rRNA gene (SSU) has been widely used for the taxonomic affiliation of bacteria (Fox et al. 1980). The develop of polymerase chain reaction (PCR) facilitated the cloning and sequencing of the rRNA encoding genes allowing to describe the composition of mixed communities without cultivation (Giovannoni et al. 1990). In parallel, other methods to explore microbial populations based on the SSU rRNA genes such as different types of *in situ* hybridization and fingerprinting techniques were also developed (Amann et al. 1990, Liu et al. 1997, Muyzer 1999).

In 1998, Handelsman and collaborators defined, for the first time, metagenomics as the analysis of the collective genomes (total DNA) that are present in a specific environment (Handelsman et al. 1998). This culture-independent method opened the doors to describe not only the composition but also the genetic potential and ecological interactions of whole microbial ecosystems.

The usage of these culture-independent techniques (rRNA-based approaches and metagenomics) has been improved since the development of new sequencing technologies, which have increased the sequencing depth by orders of magnitude. To gain more insights, not only in the potential functions but also in activity biomarkers, other meta-"omics" approaches such as metatranscriptomic (messenger RNA), metaproteomic (proteins) or metametabolomic (metabolites) were later developed (Zoetendal et al. 2008).

In 2006, the first microbial ecology studies based on pyrosequencing (454 platform) were reported and since then, a variety of microbial communities have been explored such as those living in marine water, soils, the bovine rumen or the human gut (Brulc et al. 2009, Qin et al. 2010, Roesch et al. 2007, Sogin et al. 2006). Nowadays, several projects to study the ecology of microbial communities are ongoing. As a consequence, a lot of information is being generated and the scientific

community is currently investing much effort in the development of bioinformatics tools for the ecological analyses. Several programs, databases and websites are now involved in dealing with the huge amount of data that microbial ecologists produce from their research (Robinson et al. 2010).

1.2. OVERVIEW OF THE HUMAN MICROBIOTA

Bacteria have inhabited Earth from at least 2.5 billion years and are the most abundant organisms on the biosphere (Brocks et al. 1999, Whitman et al. 1998). Thus, our predecessors adapted to live in a microbial world. Indeed, it has been described that animals have carried resident microorganisms since at least the emergence of sponges (Hoffmeister & Martin 2003). The vertebrate-associated bacterial communities have a diversity pattern very different of the free-living communities from marine, terrestrial or freshwater environments. This suggests that co-evolution over hundred millions years has selected a specific community of microbes adapted to the stable environment which is the human body (Ley et al. 2008b).

The human body harbours complex microbial communities (defined as microbiota) with ~ 10^{14} microbial symbionts, outnumbering the human cells by at least 10-fold (Savage 1977). The collective genomes of our indigenous microbes is defined as the microbiome and their total number of genes also exceeds those of the human genome. The majority of microorganisms and the largest diversity is found in the intestinal tract, which contains in average 10^{12} cells per gram of faeces and around 100-fold more genes than the human genome (Hooper & Gordon 2001, Savage 1977, Yang et al. 2009). Moreover, the microbiome variability between individuals is huge compared to the human genome variation. The human genome is about 99.9% similar among individuals, but can be 80-90% different in terms of the microbiome (values obtained from gut and skin studies) (Ursell et al. 2012).

The traditional idea that all microorganisms are parasitic, causing disease, has changed to a non-pathogenic vision of microbe-host interactions, being most of the relationships described as commensals (one partner is benefited and the other remains unharmed) or mutualists (both partners benefit) (Dethlefsen et al. 2007). The human-associated microbial communities benefit the host in different ways, being the best known those performed by the gut microbiota. Our intestinal symbionts participate in food digestion, energy metabolism, development and maintenance of the immune system, synthesis of vitamins, renewal of gut epithelial cells or pathogen resistance, among others (Backhed et al. 2004, Hattori & Taylor 2009, Hooper 2004, Leser & Molbak 2009, Montalto et al. 2009). On the other hand, the intestinal microbial residents benefit from stable growth conditions and constant source of nutrients (Savage 1977).

Microbes associated with the human body include mainly bacteria which comprise the bulk of biomass, but archaea, viruses, and eukaryotes are also present (Breitbart et al. 2008, Eckburg et al. 2005, Marchesi 2010). The bacterial diversity of the human body shows a limited number of phyla but a high richness at strain and species levels. In free-living microbial communities of environments such as soil or water, it has been found more than 50 bacterial phyla, but only 4 are dominant in the human microbiota: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Aas et al. 2005, Eckburg et al. 2005, Gao et al. 2007, Ley et al. 2008b, Pei et al. 2004). Thus, the diversity pattern of the human microbiota is the result of strong selective forces along the co-evolution time of the microbial communities and the host and each individual presents at species and strain levels a unique microbiota (Blaser & Falkow 2009, Dethlefsen et al. 2007, Eckburg et al. 2005, Ley et al. 2008b) (Figure 1.1).

Microbial habitats are found mainly in the skin surfaces, the eyes, the respiratory, the urinary and the reproductive systems, the oral cavity, and the gastrointestinal tract (GIT), being the most diverse the mouth and the gut (Wilson 2008). Each site has its own physicochemical and biologic features and thus, it presents a specialized set of microorganisms (Dethlefsen et al. 2007). In this regard, it has been described that the differences along time within a specific habitat are smaller than between habitats and that also stable inter-individual differences are found between body sites (Caporaso et al. 2011, Costello et al. 2009).

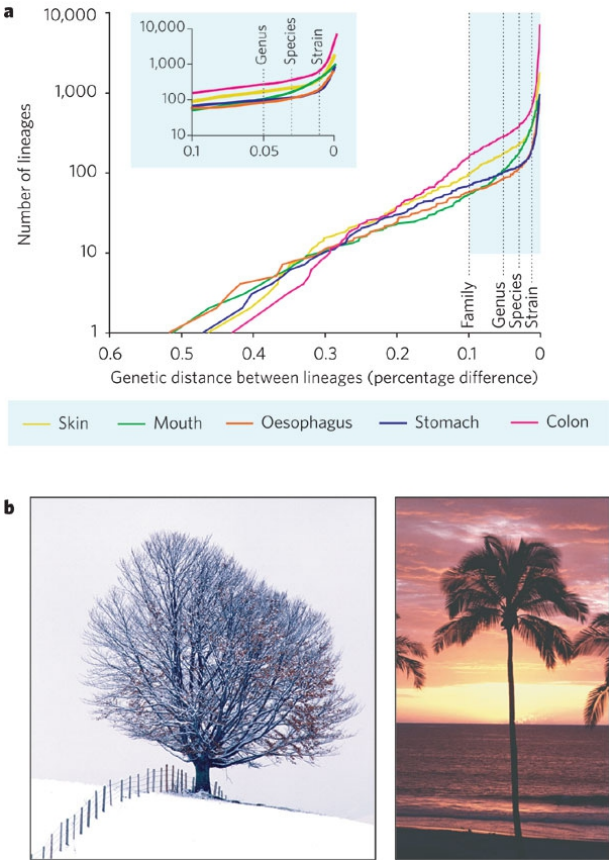


Figure 1.1. Patterns of diversity of human-associated microbial communities. a. Lineage-by-distance analysis of 16S rRNA gene sequences from human microbial communities in specific habitats. b. 16S rRNA gene-based patterns of microbial diversity displayed as dendograms. In soil and aquatic environments, they generally resemble the tree shape on the left, with new branches arising at all distances from the root. In invertebrate-associated communities, they resemble the tree shape on the right, with few branches arising close to the root and many branches arising close to the branch tips (Adapted from Dethlefsen et al. 2007).

1.2.1. THE SKIN

The human skin harbours a complex microbial ecosystem, which is affected by environmental factors such as humidity, temperature or light exposure and host factors as immune status, gender, genotype, hygiene habits, or cosmetic use (Fredricks 2001, Roth & James 1988). The microbial communities of the skin live in a wide range of physiologically and topographically niches and protect against the invasion of harmful organisms (Grice & Segre 2011). The first molecular study based on 16S rRNA gene analysis was performed by Gao and colleagues, being Actinobacteria, Firmicutes and Proteobacteria the major observed phyla (Gao et al. 2007). They found no differences between regions of the body with the same properties, while large variations over time and between individuals were observed. A more recent study of 20 skin sites also showed that

physiologically comparable sites harbour similar bacterial communities and that the diversity and stability are dependent on specific characteristics of the site (sebaceous, moist or dry). It was also described that the stability of the skin microbiota over time is site-dependent. In general, the most abundant species of the skin belonged to *Corynebacterium* and *Propionibacterium* genera (Actinobacteria phylum), followed by *Staphylococcus* (Firmicutes phylum) (Findley et al. 2013, Grice et al. 2009).

Little is known about the functional potential of our skin microbiome. Until now, metagenomic studies of human skin have not been reported, mainly by the critical amount of starting material, host DNA contamination, or lack of reference genomes for skin isolates (Grice & Segre 2011). The development of new methods to solve some of these issues will allow a functional characterization of our skin microbiota and its role in health and disease.

1.2.2. THE EYE

The ocular surface is continually exposed to the environment and, as a consequence, to several types of microbes throughout life. Several studies based on cultivation techniques have shown that the most common bacteria isolated from the conjunctiva, lids or tears belong to the genera *Staphylococcus* (Firmicutes), *Propionibacterium* and *Corynebacterium* (Actinobacteria) (Willcox 2013).

The first non-cultivable molecular studies of the ocular surface have identified other taxa in addition to those obtained by cultivation, mainly *Bacillus, Streptococcus* (both Firmicutes), *Rhodococcus* (Actinobacteria), *Bradyrhizobium*, *Acinetobacter*, *Brevundimonas*, *Aquabacterium*, *Sphingomonas*, *Pseudomonas* and *Klebsiella* (Proteobacteria) (Dong et al. 2011a, Graham et al. 2007).

Moreover, the composition of the eye microbiota seems to be extremely variable between individuals, with some individuals colonized by diverse types of microbes and others by a narrow spectrum of them (Lee et al. 2012).

Since the eye-associated microbiota has been poorly studied, little is known about their possible role in human health. The human tears contain a diverse range of antimicrobial components and molecules of the innate and adaptive immune system (McDermott 2013). Thus, it is possible that the role of eye microbial communities could be related to protection against pathogens by stimulating human defense systems (Willcox 2013).

In addition, the ocular microbiota composition is affected by external factors as the use of contact lenses and its effect may vary depending on the length of lens wear, the type of polymer of which they are made or the frequency of use (Fleiszig & Efron 1992, Iskeleli et al. 2005, Larkin & Leeming 1991, Willcox 2013).

Further research would clarify the existence of a normal ocular surface microbiota, its acquisition mode or its changes associated to pathologies as pathogen invasions associated to the use of contact lenses (Stapleton et al. 1995).

1.2.3. THE RESPIRATORY SYSTEM

The respiratory system supplies oxygen and removes carbon dioxide from the blood. It consists of the respiratory tract for air conduction, a respiratory membrane for gaseous exchange and a ventilator mechanism for delivering gases to and from the respiratory membrane. The respiratory tract is divided into the upper (nose and pharynx) and the lower tract (larynx, trachea, bronchi, bronchioles, alveolar ducts, alveolar sacs and alveoli), being the upper tract the main area populated by microorganisms (Wilson 2008). Given that bacteria present in the respiratory tract cannot be cultivated, it was supossed that airways were sterile. Recently, several works have shown not only the presence of bacteria in the airways, but also an association of specific bacterial load and composition with health and disease status (Marsland et al. 2013).

Charlson and colleagues, by means of molecular analyses (16S rRNA quantitative PCR and pyrosequencing), characterized multiple sites along the respiratory tract, showing a microbiota that decreases in biomass from the upper to the lower tract, but with a similar phylogenetic composition (Charlson et al. 2011). The predominant families of the

nasopharyngeal niche are Staphylococcaceae (Firmicutes), Propionibacteriaceae and Corynebacteriaceae (both Actinobacteria), being similar to the skin composition, while the oropharynx showed a composition closer to that of the gastrointestinal microbiota (Firmicutes, Proteobacteria and Bacteroidetes phyla) (Charlson et al. 2011, Lemon et al. 2010).

Cigarette smoke is the main factor for chronic obstructive pulmonary disease, which is one of the major chronic airway diseases. Smokers present greater variation in the type and relative abundance of bacteria for both sites, oropharynx and nasopharynx, than non-smokers. The altered microbiota associated to cigarette smoking may facilitate pathogen colonization of the upper respiratory tract (Charlson et al. 2011). Generally, a greater diversity has been associated with health conditions of the respiratory system (Blainey et al. 2012, Erb-Downward et al. 2011, Hilty et al. 2010).

Sampling the upper respiratory tract is by far easier compared to the lower respiratory tract. It remains to be proven if the microbes detected on the lower respiratory tract are resident or transient that were inhaled from the upper tract, a possible contamination from the oropharynx in the collection of samples, or individuals incorrectly categorized as healthy (Huang & Lynch 2011).

Recent studies suggest that shifts in airway microbiome could be associated with chronic airway diseases such as allergy, asthma, chronic obstructive pulmonary disease or cystic fibrosis. It is still unknown if the changes are the main cause of disease or a consequence of inflammation, but the association suggests a microbe-host cross talk whose functional consequences need to be addressed (Marsland et al. 2013).

Pioneer studies in mice suggest a positive role of the airway microbiota in differentiation, maturation and regulation of immune system cells, antiviral immunity, and inflammatory responses (Abt et al. 2012, Herbst et al. 2011, Larsen et al. 2012, Olszak et al. 2012). Since this field is quite recent, future research will help to address the establishment of the airway microbiota, as well as its role in health and disease.

1.2.4. THE URINARY SYSTEM

The urinary system is responsible for the removal of waste products of metabolism and the regulation of the chemical composition, volume and pressure of body fluids. The system consists of two kidneys, two ureters, a bladder and a urethra. Historically, urine has been considered sterile, nevertheless urine (representing the bladder microbiota) contains several bacteria which are not cultivated but that have been described by 16S rRNA gene sequencing (Nelson et al. 2010, Siddiqui et al. 2011).

The microbiota of urinary system is mainly dominated by Firmicutes, but there are also large differences between sexes regarding the presence and abundance of many of the genera. For instance, male show a lack of members from Actinobacteria and Bacteroidetes that are present in females (Lewis et al. 2013, Nelson et al. 2010, Siddiqui et al. 2011).

The female microbiota of the urinary system shares some bacterial members with the vaginal microbiota and with the male urogenital tract (Dong et al. 2011b, Ling et al. 2010). The most abundant genera are *Lactobacillus* (Firmicutes), *Prevotella* (Bacteroidetes) and *Gardnerella* (Actinobacteria), but a high inter-individual variation in the community structure is also present (Siddiqui et al. 2011). In general, the urine microbiota from males and females overlaps in composition to other microbial communities found in the superficial skin, colon or vagina, being the predominant genera: *Lactobacillus* (Firmicutes), *Corynebacterium* (Actinobacteria), *Streptococcus* (Firmicutes) and *Sneathia* spp. (Fusobacteria) (Eckburg et al. 2005, Gao et al. 2007, Ling et al. 2010). There is a substantial inter-individual variation in the urine microbiome of males even at the phylum level and greater that in female (Nelson et al. 2010).

1.2.5. THE REPRODUCTIVE SYSTEM

The female reproductive system consists of the ovaries, fallopian tubes, uterus, cervix vagina and the vulva. The cervix, vagina

and vulva are those organs colonized by microbial communities, being the vaginal ecosystem the most extensively studied.

The indigenous microbiota of the vagina plays a role in protecting against colonization by pathogenic organisms, including those related to symptomatic bacterial vaginosis, sexually transmitted infections and urinary tract infections (Gupta et al. 1998, Hillier et al. 1992, Wijgert et al. 2000).

The genus *Lactobacillus* has been considered as a keystone of the vaginal microbial community in reproductive-age women and prevents colonization by potentially harmful microorganisms, through the lactic acid production which results in a low protective pH (Boskey et al. 1999). Also, some *Lactobacillus* species has an antimicrobial action including the production of target-specific bacteriocins and broad-spectrum peroxidases, which also contribute to fight pathogen invasion (Eschenbach et al. 1989). Its importance is emphasized by the fact that disruption of the equilibrium of the normal vaginal microbiota can trigger a bacterial vaginosis which is a highly prevalent disorder in reproductive age women (Ravel et al. 2013, Srinivasan et al. 2012). Species of the *Lactobacillus* genus are dominant in the majority of healthy women, but a diverse group of strictly anaerobic microorganisms has also been found in high abundance in some women (Ma et al. 2012, Ravel et al. 2011).

The microbial communities of the vagina show different behaviour among women by changing markedly on some of them over short periods of time, while on others are relatively stable along time (Ma et al. 2012). Moreover, the vaginal microbiota changes continuously along woman life, from childhood to puberty, during the reproductive years and menopause with several factors influencing the composition and stability of the ecosystem, mainly the menstrual cycle and sexual activity. Thus, this ecosystem is a result of adaptive co-evolutionary processes integrating host physiology and sexual hormone levels with the composition and functions of the microbial community.

The genital microbiota of male is present in the lower male genital tract, mostly in the urethra and coronal sulcus. The microbial composition of the urogenital tract has been characterized in the previous epigraph (1.2.4. The urinary system). The upper genital tract (including prostate tissue and vas deferens) is generally germ-free except in case of infections, as prostatitis (Mändar 2013).

The male genital microbiota has an impact on the microbiota of the reproductive system of females, promoting an increase of vaginosis probability, although contradictory results have been found (Mändar 2013). Future studies will be required to study the interaction between the male and female genital microbiota and its relationship to diseases such as vaginosis that represents a high cost for public health.

1.2.6. THE ORAL CAVITY

The oral cavity is the entrance to the GIT and consists of a complex system of tissues and organs mainly involved in selecting and processing the food into a suitable form for passage into the rest of the GIT (Wilson 2008).

After the gut, the oral cavity constitutes the second most complex habitat of the human body with around 1000 species of bacteria present (Dewhirst et al. 2010, Human Microbiome Project Consortium 2012). Based on the taxonomic composition, three distinct bacterial communities have been identified in different areas of the oral cavity: the buccal mucosa, gingivae and hard palate (first group), saliva, tongue, tonsils and throat (second group) and supra- and sub-gingival plaque (third group) (Segata et al. 2012).

The bacterial community of the mouth is dominated by Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes and Fusobacteria (Dewhirst et al. 2010). At genus level, *Actinomyces* (Actinobacteria), *Streptococcus* (Firmicutes), *Neisseria* (Proteobacteria), *Veillonella* (Firmicutes), *Porphyromonas* (Bacteroidetes), *Granulicatella* (Firmicutes), *Corynebacterium* (Actinobacteria), *Rothia* (Actinobacteria) and *Fusobacterium* (Fusobacteria) are predominant in a healthy oral microbiota (Zarco et al. 2012). It has been proposed that one of the most important roles of the oral microbiota is to avoid infections by opportunistic pathogens such as *Staphylococcus aureus* or *Candida* species, mainly by competing for binding to host cell receptors (Jenkinson & Douglas 2002).

Poor oral hygiene is greatly responsible for accumulating plaque will lead to overgrowth of some bacteria that may become pathogenic, reducing the biodiversity of the oral cavity, and ultimately causing two of the most common human

pathologies: the dental caries and the periodontal diseases (Wade 2013, Zaura et al. 2009). The microbiota of dental caries shows a taxonomic and functional composition that differs to the normal microbiota with a lower microbial diversity and a lower presence of some protective functions as antimicrobial peptide production or quorum sensing, among other differences (Belda-Ferre et al. 2012).

1.3. THE GUT MICROBIAL ECOSYSTEM

The digestive system consists in the GIT and the accessory digestive organs (teeth, tongue, salivary glands, liver, gallbladder and pancreas). Its main function is to process the food by breaking down the dietary compounds in small molecules that are absorbed and distributed throughout the body (Wilson 1998). The human gut is anatomically divided in different sections: the oral cavity, esophagus, stomach, small intestine (divided in duodenum, jejunum, and ileum), cecum, colon or distal gut (divided in ascending, transverse and descending colon) and rectum.

1.3.1. COMPOSITION, DIVERSITY AND SOURCES OF VARIATION OF THE GUT MICROBIAL COMMUNITIES

The GIT is colonized by a wide variety of microorganisms and each section harbours a specific microbial community according to the properties of the compartment. The number of microorganisms and the complexity increase from the stomach to the rectum, being the colon the most densely populated region with up to 10^{12} bacteria per gram of luminal material (Leser & Molbak 2009, Marchesi 2011, Whitman et al. 1998) (Figure 1.2).
The digestion process starts in the mouth with the mechanical and enzymatic digestion. The microbial communities of the oral cavity share high levels of diversity as well as phyla composition (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria) with other compartments of the GIT (Marchesi 2010) (detailed description of the oral cavity in the epigraph 1.2.6).

Moving down the gut we found the throat and esophagus, whose microbial communities have been less explored than those from other areas of the human body. The few conducted studies have shown Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and candidate division TM7 as the most abundant phyla. The throat harbours a stable microbial ecosystem with a high similarity between individuals and a low diversity (at phylotype level) compared to other regions of the GIT. The dominant genera for both, esophagus and throat, are *Streptococcus* (Firmicutes) and *Prevotella* (Bacteroidetes), but *Actinomyces* (Actinobacteria), *Gemella* (Firmicutes), *Rothia* (Actinobacteria), *Granulicatella* (Firmicutes), *Haemophilus* (Proteobacteria) and *Veillonella* (Firmicutes) are also present (Andersson et al. 2008, Pei et al. 2004).
The food passes from the esophagus to the stomach where it is performed the next part of digestion after chewing: the release of gastric acid and proteases. This organ is a markedly different habitat to the oral cavity or the intestine, with a strong selective pressure due its low pH (1-2 in adult humans) and other antimicrobial factors. Used to be thought that due these extreme conditions, a very low (or any) significant microbiota inhabits the stomach, except the opportunistic pathogen *Helicobacter pylori*. However, Bik and collaborators (2006) reported the presence of a microbiota mainly distributed over the most abundant phyla of the GIT: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (in decreasing order of abundance), although the biomass and microbial diversity is lower than in highly populated regions of the GIT as the oral cavity or the lower intestine. The most common genera described on the human stomach are *Caulobacter* (Proteobacteria), *Actinobacillus* (Proteobacteria), *Corynebacterium* (Actinobacteria), *Rothia* (Actinobacteria) and *Gemella* (Firmicutes).

The composition of the stomach microbiota is highly variable between individuals, even for the most abundant taxa. This variability could be due to the acid in the stomach that kills many microbes derived from the esophagus and from the ingested food, frequently reshaping the resident and transient microbial populations there present.

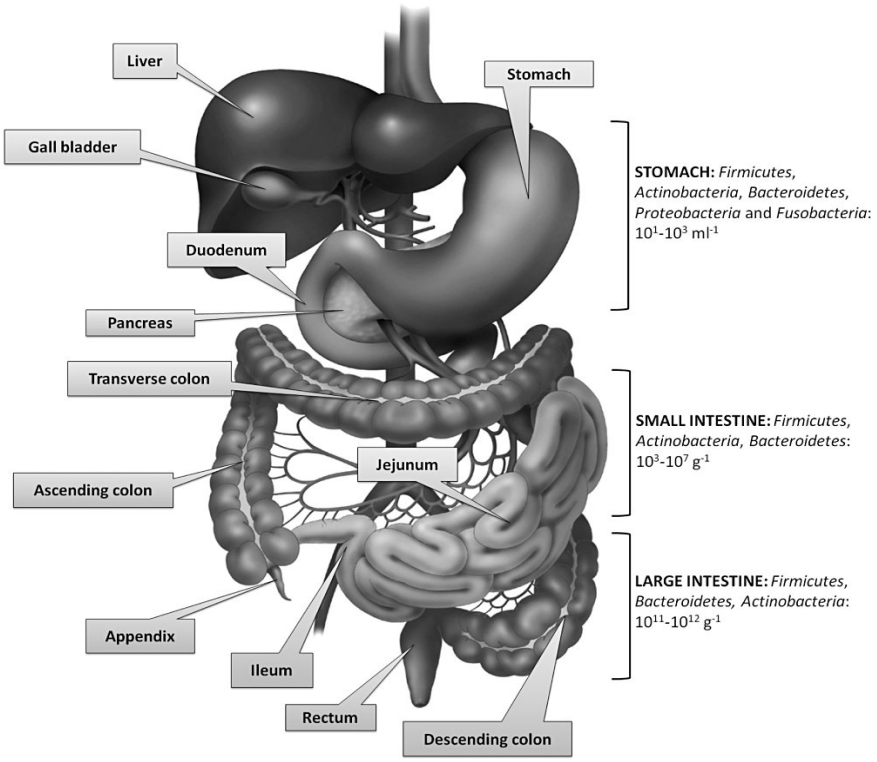


Figure 1.2. Overview of the gastrointestinal tract, main bacterial phyla and their abundance in each region (Adapted from Marchesi, 2011).

Moreover, the infection by *H. pylori*, which is well known as the agent causing of gastritis, has been associated to an extremely low diversity of the stomach microbial ecosystem, since during infection the pathogen can constitute the 93%-97% of the total community (Andersson et al. 2008, Bik et al. 2006).

Most of the digestion and absorption of food take place in the small intestine which comprises duodenum, jejunum and ileum. The small intestine presents a large absorptive surface area of epithelium due to the villi (finger-like projections from the epithelial lining) and the microvilli (microscopic cellular membrane protrusions). The epithelium is covered by mucus layers which act as a mechanical barrier that separates the luminal bacteria from the epithelium.

The food passes from the stomach to the duodenum where is blended with bile, bicarbonate and digestive enzymes. In healthy humans the intestinal content (chyme) passes from the duodenum to the ileum in 1-4 hours and the bacterial populations increase from 10^{4} to 10^{8} bacteria per ml of intestinal content (Laux et al., 2005). In the small intestine the bacteria deconjugate bile and produce vitamins and amino acids for the host (Conly & Stein, Tannock et al. 1994, Torrallardona et al. 2003).

The bacterial community in duodenum and jejunum are strikingly different from that in distal ileum, with a lower diversity and a dominance of *Streptococcus* species which are acid-tolerant bacteria. For its part, the ileum is dominated by Bacteroidetes and *Clostridium* clusters XIVa and IV (Firmicutes) and the composition is more similar to that of the large intestine (Wang et al. 2005). Moreover, biopsies from different regions have revealed that the mucosal microbiota of the small intestine is dominated by the phyla Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia and the most common genera are *Streptococcus* (Firmicutes), *Veillonella* (Firmicutes) and *Clostridium* (Firmicutes) (Marchesi 2010, Wang et al. 2003, 2005).

The transit time in the large intestine (cecum, colon and rectum) is the largest of the digestion process, ranging from 10

hours to various days. The dietary compounds that are not degraded in the upper GIT reach the large intestine supporting the microbiota with nutrients and energy for longer time, which explains why it is the most heavily colonized area of the GIT. Even the great biomass of bacteria in the large intestine, only seven phyla (common to other regions of the GIT) have been found (Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria), being Bacteroidetes and Firmicutes the great majority (more than 95% of the total bacteria) (Eckburg et al. 2005). However, a large diversity has been found at lower phylogenetic levels, with more than 1000 species (Claesson et al. 2009, Rajilić-Stojanović et al. 2009). The bacterial composition in the mucosal community is very similar along the large intestine. On the other hand, there is a high inter-individual and a low intra-individual variation in the bacterial community structure of the gut (Ahmed et al. 2007, Eckburg et al. 2005, Green et al. 2006).

Some of the most prevalent and abundant genera in human gut are *Faecalibacterium*, *Roseburia*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Blautia*, *Coprococcus* (Firmicutes), *Bacteroides*, *Alistipes*, *Parabacteroides* (Bacteroidetes) and *Bifidobacterium* (Actinobacteria) (Arumugam et al. 2011, Tap et al. 2009). It has been attempted to identify a species core in the adult microbiota, being important *Faecalibacterium prausnitzii*, *Roseburia intestinalis* or *Bacteroides uniformis*, but even these, can be in relative abundances lower than 0.5% in some individuals (Lozupone et al. 2012, Qin et al. 2010, Turnbaugh et al. 2009). Thus, the inter-subject variation has not allowed to properly defining a core microbiota, except the fact that Bacteroidetes and Firmicutes are the main phyla (Lozupone et al. 2012, Marchesi 2011). However, a large metagenomic study (33 faecal samples) determined three clusters of the human gut composition, designated as enterotypes, based on phylogenetic profile similarities. The enterotypes are mainly driven by groups of co-occurring species with a high representation of *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) genera (Arumugam et al. 2011). Similar clusters have been found in other two data sets (16S rRNA data from 154 individuals from the United States and metagenomes from 85 individuals from Denmark), but other three data sets failed to show the same pattern (98 adults from the United States, 531 individuals from Malawi, Venezuela and Unites States and 250 adults from the United States) (Qin et al. 2010, The Human Microbiome Project Consortium, 2012 Turnbaugh et al. 2009, Wu et al. 2011, Yatsunenko et al. 2012). Nevertheless, the variation of these last populations (in the adults) was associated to a trade-off between *Bacteroides* and *Prevotella* that seems important for the microbiota equilibrium (Lozupone et al. 2012). Therefore, the enormous variation of the microbiota between individuals, introduced by genotype, age, diet, health status and geographic locations, makes difficult the classification into a limited number of types and it is something to be considered for human gut studies.

Along the GIT two mucus layers have been identified: one firmly attached to the epithelium (known as the inner layer) and other less adherent facing the intestinal lumen (known as the outer layer). However, the bacteria are not uniformly distributed in the layers, being the outer densely populated, while bacteria are absent in the inner layer (Atuma et al. 2001, Johansson et al. 2010). The major component for both layers is the highly glycosylated Muc2 mucin, which is secreted by goblet cells of the epithelium and forms a net-like polymer. The inner mucus is dense, thus protecting epithelial cells from bacterial invasion. For its part, the *O*-glycans of the outer layer serve as nutrients and attachment sites for commensal bacteria (Johansson et al. 2011). Bacteria use the energy obtained from mucin degradation for their own use, but as a result produce short-chain fatty acids (SCFA) that are used by colonocytes as a source of energy, maintaining a balance between microorganisms and host (Louis et al. 2007).

Faeces are the waste product of the digestion process and are expelled through the anus. Most of the studies of the gut microbiota are based on faecal material since its collection is a simple and non-invasive method, but bacteria from the stool (human faeces) are not entirely representative of the mucosal microbiota. In fact, some studies have shown that the microbiota attached to the mucosa is significantly different to that of the faeces, since the mucosal-associated bacteria are between 4-6 orders of magnitude more abundant than the luminal bacteria, which constitute the major fraction of bacteria carried on the faeces. Thus, a lower diversity and the absence of some bacterial groups are typical of faeces respect to biopsies (Durbán et al. 2011, Eckburg et al. 2005). Therefore, even though the stool is a good approximation to study the gut microbiota, it is important to be cautious when extrapolating what is observed in faeces to what is occurring in the gut.

Most of the studies about microbial communities of the human gut are limited to bacteria due to they constitute the great majority. The domain Archaea has been identified in the human microbiota, being *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* the most common species in the distal gut, both from the Methanobacteriaceae family

(Euryarchaeota phylum) (Dridi et al. 2011, Eckburg et al. 2005, Scanlan & Marchesi 2008). These microorganisms through methane production play a role in removing the H_2 produced from the polysaccharides fermentation. Otherwise, H_2 accumulation would reduce the efficiency of processing the dietary carbohydrates (Gill et al. 2006).

Much of the work undertaken on the eukaryotic communities has been based on culture-based approaches and found as the most abundant the fungi *Candida* and *Saccharomyces* spp. (both Ascomycota). On the other hand, culture-independent approaches based on 18S rRNA genes show that *Blastocystis* spp. is one of the main eukaryotes of the human gut. Like in bacteria where a reduced diversity is found at deep phylogenetic levels (phylum), a few dominant lineages of eukaryotes (fungi and *Blastocystis*) are adapted to the gut environment. The eukaryotic communities are also stable across time and unique to individuals (Parfrey et al. 2011, Scanlan & Marchesi 2008).

The viral component of the gut habitat has received little attention, however its number is at least an order of magnitude higher than bacterial number in the distal gut. The first culture-independent studies showed the Siphophage group of viruses as the majority (Breitbart et al. 2003, 2008). Latest, Reyes and colleagues characterized the total virus-like particles isolated from human faecal samples and they found that around 25% of viral genomes were phages and prophages, most of them of double-strand-DNA type and members of Caudovirales order. The intra-individual variation along the time of the viromes was minimal, in contrast to the inter-individual that was extremely high (Minot et al. 2011, Reyes et al. 2010). This high interpersonal variability of human gut viruses may be derived of two sources: the persistence of small portion of the virome over time and the rapid evolution of specific long-term viral residents within the gut (Minot et al. 2013). A substantial portion of the phages in human gut seem to be temperate, so that the genes within phage may alter phenotype of the bacterial host by lysogenic conversion, which may be significant in the functioning of the whole ecosystem (Minot et al. 2011). Recently, the most abundant bacteriophage of human faeces, named crAssphage, has been described, and a *Bacteroides* host was predicted for it (Dutilh et al. 2014). Viruses are critical components of the gut ecosystems, helping in controlling the growth of bacterial populations and protecting mucosal surfaces (Barr et al. 2013).

Further investigation will be necessary to clarify the role of this less known fraction (eukaryotes, archaea, viruses) in the gut ecosystem, as well as, their interactions with other members of the microbiota and with the host.

Then, the gut microbiota of adult human individuals is unique and stable along the time (in the absence of disturbances), and the composition is influenced by several deterministic factors such as host genetics, diet or health status and also by stochastic events (Durbán et al. 2012a, Zoetendal et al. 1998).

1.3.1.1. HOST GENOTYPE

The host genotype contributes to explain the inter-individual variation observed on the human gut microbial composition. Research based on dependent and independent cultivation techniques have shown that in humans there is a positive correlation between the genetic grade of individual's relatedness and the similarity of the microbial composition, being maximum in the case of monozygotic twins (Merwe et al. 1983, Stewart et al. 2005, Turnbaugh et al. 2010). The issue is that in human microbiota investigations, several environmental factors as diet, age, or health status make difficult to evaluate the exact contribution of the host genotype to the microbial variation among individuals. The closest approximation so far is based on mouse studies under controlled environmental conditions, where it has been shown that host genotype effects are distributed across all the gut dominant taxa, and that genomic traits can influence the abundance of specific microbial groups and have pleiotropic effects on different taxa (Benson et al. 2010).

Regarding specific genetic effects on the gut microbiota, studies on mouse model have revealed that the faecal composition is mainly affected by polymorphisms of immune system genes (Toivanen et al. 2001). Besides the impact of immune system, differences on genes involved in the epithelial barrier function (availability of attachment sites) also influence the community structure (Dethlefsen et al. 2006, McKnite et al. 2012).

1.3.1.2. HOST AGE

After birth, a bacterial succession starts that lasts until the establishment of an adult microbiota within the first two years of life (Koenig et al. 2011, Palmer et al. 2007, Vallès et al. 2014). Newborns acquire their microbiota initially from the mother and from the environment they are exposed. The composition of meconium samples suggests that the mother is the first important microbial source of the fetus gut during the intrauterine life (Gosalbes et al. 2013). The colonization of the gut microbiota in early years is influenced by several factors, as maternal microbiota, diet (breast or formula fed), mode of delivery (normal vs. cesarean), gestation (full or preterm) or antibiotic intake (Biasucci et al. 2010, Dominguez-Bello et al. 2010, Favier et al. 2003, Morowitz et al. 2011, Palmer et al. 2007). All these factors contribute to explain the uniqueness of the gut microbiota of early years and also to determine the adult microbiota. The first days of life are characterized by a microbiota dominated by one or two taxonomic groups, but the diversity is increasing as time progresses and the ecosystem structure converges to an assembly characteristic of adults (Favier et al. 2002, Palmer et al. 2007, Vallès et al. 2014). The adult gut community shows limited variations over extended periods in the absence of disturbances, except by fluctuations of minor bacterial groups (Durbán et al. 2012a, Robinson et al. 2010). In old age, the microbiota undergoes extreme changes and the structure is substantially different from that of younger people, with a higher variability among individuals and unusual proportions of the main phyla (Claesson et al. 2011).

1.3.1.3. DIET

Diet is one of the main determinants of the gut microbiota composition since birth. In fact, the faecal microbiota of infants is strongly affected by the transitions between breast milk, formula and solid food (Favier et al. 2002, Harmsen et al. 2000, Vallès et al. 2014). The gut microbial composition depends on different dietary habits. For instance, children from a rural African population with a diet enriched in fiber presented a gut microbiota that had co-evolved with the host to maximize energy intake from plant polysaccharides producing SCFAs that also protect them from inflammation. On the other hand, a less diverse microbiota with a lower presence of beneficial members was found in European children as a consequence of a diet enriched in fat, protein and sugars. The consumption of these type of food are rapidly limiting the adaptive potential of our gut microbiota, thus contributing to explain the great increase in the incidence of intestinal diseases in developed countries (Filippo et al. 2010).

A study on adult humans under different controlled diets has shown that diet-driven changes occurred rapidly, being detectable within 3–4 days (Walker et al. 2011). Also, the short-term consumption of diets with different nutritional composition alters microbial community structure in dependence on the components of the diet. Animal-based diets increase the abundance of bile-tolerant microorganisms (as *Alistipes*, *Bilophila* and *Bacteroides*), while plant-based diets increase the levels of Firmicutes that metabolize plant polysaccharides (as *Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*) (David et al. 2013). Moreover, the gut microbiota of adults has the ability to return to its original composition after short-term dietary changes (Durbán et al. 2013).

A large study of mammals and their gut microbiota shows that phylogeny and host diet have influenced significantly the structure of the gut microbiota along their co-evolution time. A same type of diet (herbivore, omnivore or carnivore) has leaded to a microbial assembly with common features between different phylogenetic groups (Ley et al. 2008a).

1.3.1.4. MICROBIAL INTERACTIONS

Microbial interactions are important determinants of intestinal niche's ecology and contribute to increase the diversity and the variability between individuals (Day et al. 2003, Freter 1983, Savage 1977). For example, the interspecific cooperation that occurs during metabolism of dietary fiber and the subsequent fermentation of the monosaccharides involve diverse bacterial groups that are metabolically connected in a complex way, contributing to determine the assembly of the intestinal microbiota of individuals. Also, microbial competition through different mechanisms as the production of toxic metabolites or antimicrobial compounds (as bacteriocins), induces complex population dynamics that generate different patterns of diversity between individuals (Dethlefsen et al. 2006, Flint 2004, Fons et al., 2000). Moreover, interactions (as cooperation or competence) between microorganisms in the gut ecosystem can complicate the responses to external factors as dietary changes or medication, leading to greater inter-individual variability.

1.3.1.5. STOCHASTIC EVENTS

The gut microbiota of healthy adults is claimed to be stable in the absence of disturbances, mainly because the temporal variability is smaller than the inter-individual differences, but stochastic processes as colonization history, bacteriocin production or phage dynamics, may explain the differential dynamics of some bacterial groups among individuals (Dethlefsen et al. 2006).

The order of colonization and establishment of the gut after birth is a probabilistic process that depends on the environmental origin of the microbial sources, as well as the retention in the colon via attachment to mucus, or to particles in the lumen (Freter et al. 1983, Sonnenburg et al. 2004). Since there is a wide repertoire of bacteria adapted to live in the gut environment and a high functional redundancy, different assemblies are possible to persist over time (Dethlefsen et al. 2006).
An important source of variability along life are bacteriocins and phages, that can cause rapid shifts by depleting well-established members of the community, changing temporarily the dynamic of the gut ecosystem or even leading to evolutionary changes in the gut microbial populations (Brüssow & Kutter 2005, Czárán et al. 2002).

1.3.2. BIOLOGICAL FUNCTIONS OF THE HUMAN GUT MICROBIOTA

The genetic potential of the human gut microbiota has been deeply examined in recent years through "omics" approaches as metagenomics, metatranscriptomics or metaproteomics (Arumugam et al. 2011, Gill et al. 2006, Gosalbes et al. 2011, Kolmeder et al. 2012, Kurokawa et al. 2007, Qin et al. 2010, Turnbaugh et al. 2010, Verberkmoes et al. 2009).

As we previously mentioned, a great variation in bacterial composition calls into question the existence of a core microbiota, but a great number of shared genes between individuals allow to identify a core microbiome, which implies that a high functional redundancy is a property of the human gut microbial community (Lozupone et al. 2012, Qin et al. 2010, The Human Microbiome Project Consortium 2012, Turnbaugh et al. 2010).

In the study of Qin and colleagues, a total of 3.3 million of non-redundant microbial genes on human faeces, 99% of them from bacteria, were found. The authors identified a set of functions necessary for living in a gut context (minimal gut genome) and genes involved in the homoeostasis of the whole ecosystem encoded across different phylogenetic groups (minimal gut metagenome). The first group includes the house-keeping genes of main metabolic pathways, as amino acid biosynthesis, nucleic acid processing, or central carbon metabolism, and also specific genes for inhabit the gut environment as those involved in adhesion to the host proteins, or in catabolism of globoseries glycolipids. In addition, the minimal gut metagenome group comprises genes involved in the biodegradation of dietary complex sugars and glycans that are indigestible

for the host, as well as synthesis of some essential amino acids and vitamins, or detoxification of xenobiotics. These functions are important for the maintenance of the equilibrium on the gut microbial ecosystem and for host-bacterial interactions, being most of them not only present, but also enriched in our gut microbiome (Gill et al. 2006, Kurokawa et al. 2007, Qin et al. 2010) (Figure 1.3).

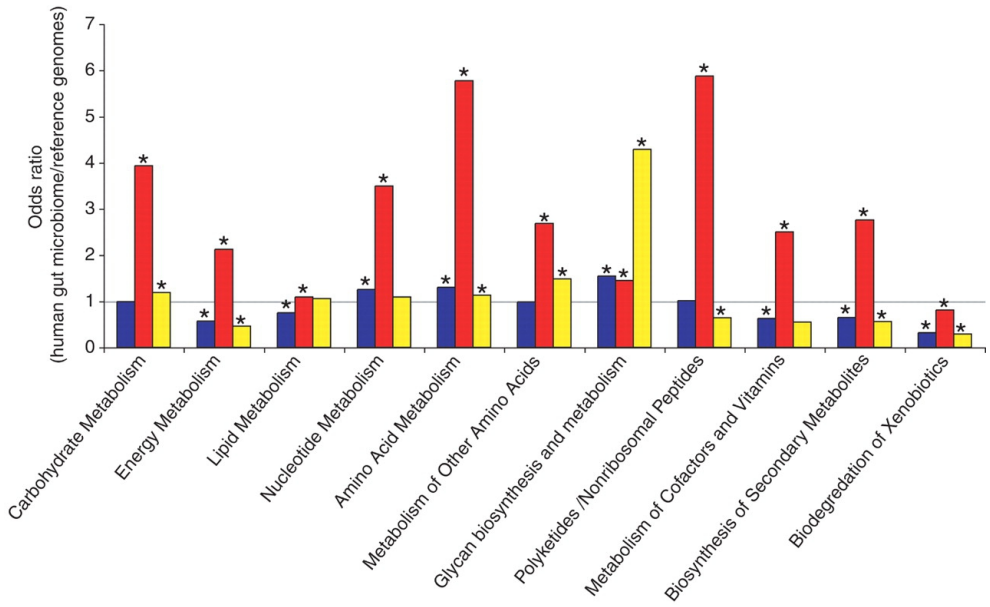


Figure 1.3. KEGG pathways reveal metabolic function enriched or underrepresented in the human gut microbiome. The samples were compared with all sequenced bacterial genomes in KEGG (blue), the human genome (red), and all sequenced archaeal genomes in KEGG (yellow). Asterisks indicate significant enrichment (odds ratio > 1) or underrepresentation (odds ratio < 1) (Adapted from Gill et al. 2006).

Metatranscriptomic and metaproteomic studies confirmed that some of the potential functions identified on human gut metagenomes are actually being expressed in the gut ecosystem, highlighting carbohydrates metabolism, energy production and vitamin synthesis (Gosalbes et al. 2011, Kolmeder et al. 2012, Verberkmoes et al. 2009).

Other reservoir of functions are encoded on genetic elements related to gene transfer, such as phage, plasmids, transposons, and others mobile genetic elements. These elements of the human microbiota constitute the mobile microbiome or mobilome. Since molecular techniques to isolate and handle with these type of genetic elements are so far complicated and can give biased results, little is known about its nature and roles in the human gut (Marchesi 2011). Some studies have found a mobilome core in the gut with certain roles that benefit the host, as butyrate production or bile salt deconjugation, but also with large proportion of genes of unknown function (Jones 2010). Nevertheless, the develop of new approaches as the *in silico* plasmid identification from metagenomic data will allow a great increase in the knowledge of those mobile genetic elements and their putative function in a short period of time (Jørgensen et al. 2014).

1.3.2.1. METABOLIC FUNCTIONS

The gut microbiota plays an important role in obtaining energy from the food. In fact, germ-free mice (born and raised under sterile conditions) require 30% more energy in their diet to survive (Wostmann 1981).

One of the main constituents of human diet are carbohydrates, which essentially are plant derived polysaccharides (cellulose, hemicellulose, and pectin), starches and sugars. Human cells are able to hydrolyze some disaccharides and absorb monosaccharides as glucose or galactose, but they cannot break down most of the polysaccharides, which as a consequence pass to the distal gut.

In contrast, many colonic living bacteria contain a large repertoire of enzymes for the digestion of fiber of which the human cells lack (Gill et al. 2006, Hooper et al. 2002, Qin et al. 2010). Thus, some bacterial groups are specialized in degrading a wide range of dietary carbohydrates undigested by the human. For example, species of the *Bacteroides* genus are very successful in the gut because they are able of degrade and ferment many types of polysaccharides (Bäckhed et al. 2005, Cho & Salyers 2001, Kurokawa et al. 2007, Xu et al. 2003, Zocco et al. 2007).

Members of the gut microbiota have evolved the capacity to degrade not only dietary carbohydrates, but also a variety of host-derived glycoconjugates (glycans), including chondroitin sulfate, mucin, hyaluronate and heparin (Bäckhed et al. 2005, Hooper et al. 2002, Kurokawa et al. 2007, Mahowald et al. 2009).

The microbial fermentation of all these compounds generates SCFAs (mainly acetate, propionate and butyrate) that are absorbed by the host and provide around the 10% of calories extracted by the daily diet (Leser & Molbak 2009, Turroni et al. 2008). Butyrate is considered the preferred source of the colon epithelial cells and 70% of their energy is derived from the oxidation of these acids (Bik 2009, Fitch & Fleming 1999, Leser & Molbak 2009, Montalto et al. 2009).

Acetate is taken by peripheral tissues as skeletal and cardiac muscle and is also utilized by adipocytes for lipogenesis (Bergman 1990, Cummings & Macfarlane 1977). The SCFAs have others beneficial effects on the gut physiology such as stimulate intestinal blood flow, participating in absorption of water and ions due its anionic nature, stimulating the human immune system and participating in the inflammatory response (Hooper et al. 2002, Lawley & Walker 2013, Montalto et al. 2009, Mortensen et al., Russell et al. 2013).

In addition to its role in caloric uptake through polysaccharides fermentation, the gut microbiota regulates fat storage in the host if there is no regular access to food. It has been demonstrated that the introduction of gut microbiota into germ-free mice is associated with increased hepatic lipogenesis and fat storage (Bäckhed et al. 2004, 2005, Vrieze et al. 2010).

The intestinal microbiota also contributes to the host health with the synthesis of vitamins, mainly K and B12, biotin, folic acid and pantothenate (Montalto et al. 2009, Nelson 2011, Sumi et al. 1977, Wostmann 1981). Also essential amino acids are provided by the gut microbiota. Indeed, from 1% to 20% of circulating plasma lysine and threonine in adults is derived from intestinal bacteria metabolism (Metges 2000, Nelson 2011). Other role carried by the gut microbiota is the bile salt transformation, allowing its re-absorption across the colonic epithelium (Ridlon et al. 2006). Otherwise, its accumulation would trigger health issues, as it has been associated with the pathogenesis of colon cancer and other GIT diseases (McGarr et al. 2005, Montalto et al. 2009, Nagengast et al., Nelson 2011). Finally, the gut microbiota is also involved in xenobiotic detoxification and its relation with the efficiency of drug metabolism (Björkholm et al. 2009, Russell et al. 2013).

1.3.2.2. GUT EPITHELIAL PROLIFERATION, DIFFERENTIATION AND HOMEOSTASIS

Germ-free animals have been used to study how the microbiota affects different processes of host physiology. Regarding the intestinal epithelium, it has been found that cell turnover is twice faster in conventional animals that in those germ-free, as well as the number of secretory goblets and enteroendocrine cells is increased in conventional animals (Bates et al. 2006, Savage et al. 1981). This is due to the gut microbial action in cell proliferation and differentiation. Beneficial members of the gut microbiota such as *Bacteroides thetaiotaomicron* are involved in regulating the mucosal barrier function, as well as in the maintenance of the epithelial barrier integrity (Hooper & Gordon 2001, Schiffrin & Blum 2002). Also, the interactions between microbiota and immune cells stimulate the renewal of epithelium, being even able of repairing damaged mucosal barriers (Pull et al. 2005).

SCFAs are not only important energy sources for the colonic epithelium, but they also regulate the epithelial cell growth and differentiation (Fung et al. 2012, Matsuki et al. 2013, O'Keefe 2008, Wong et al. 2006). Besides its role in cell differentiation,

butyrate participates in prevention of colonic cancer by terminating cell cycle progression and promoting apoptosis of transformed colonocytes (Comalada et al. 2006, Fung et al. 2012, Yu et al. 2012).

1.3.2.3. DEVELOPMENT AND HOMEOSTASIS OF THE IMMUNE SYSTEM

Bacterial colonization of the gut is essential for the development of innate and adaptive immune responses of the host, being the gut microbiota one of the most important factors driving the process of immune education (Atarashi et al. 2011, Chow & Mazmanian 2010, Clarke et al. 2010, Clemente et al. 2012, Kelly et al. 2004, 2007; Mazmanian et al. 2005, Tourneur & Chassin 2013). Thus, a healthy mucosal defense system protects against pathogens, but tolerates the indigenous microbiota (Cebula et al. 2013, Edwards 2009). This acquired tolerance to several microbial antigens reduces allergies and inflammatory processes over life (Cebra 1999, Montalto et al. 2009, Nicholson et al. 2005).

Strong regulation of the innate and adaptive immune system operates through intercellular signalling between lymphoid cell types that occur within germinal centers and secondary lymphoid follicles associated with the gut-associated lymphoid tissues (GALT). The development of the GALT, which is one of the main components of the immune system, is largely due to their interactions with the gut microbiota (Bauer et al. 2006, Fagarasan et al. 2010, Kelly et al. 2007).

The microbiota induces the host mucosal immune system to produce immunoglobulin A (IgA), as well as some antimicrobial compounds, two protective mechanisms that limit colonization and penetration of harmful bacteria through the epithelial layer (Kunisawa & Kiyono 2013, Macpherson et al. 2005, Müller et al. 2005, Satoh-Takayama et al. 2008, Suzuki et al. 2010, Zelante et al. 2013). Moreover, commensal members of the gut community stimulates the mucin secretion, which is component of the mechanical mucosal barrier that also protects against pathogen invasion (Burger-van Paassen et al. 2009, Leser & Molbak 2009, Szentkuti et al. 1990). Some SCFAs produced by the microbiota as acetate and butyrate also play a role in controlling inflammation and also in the protection against pathogens by stimulating the immune system (Fukuda et al. 2011, Fung et al. 2012, Kamada & Núñez 2014, Maslowski et al. 2009).

1.3.2.4. PROTECTION AGAINST PATHOGENS

The gut microbiota is able of blocking pathogen colonization and the consequent infection from the beginning. The mechanism whereby the gut microbiota protects against harmful microorganisms infection is called colonization resistance (Servin 2004).

Regarding the colonization resistance capacity of the gut microbiota, multiple levels of defence have been proposed (Figure 1.4). It works through direct interactions between microorganisms, and also via indirect mechanisms by stimulating the host immune system (known as immune-mediated colonization resistance) (Buffie & Pamer 2013, Lawley & Walker 2013, Stecher & Hardt 2011).

In vivo and *in vitro* studies have demonstrated that interactions between microorganisms are a key component of colonization resistance. These microbe-microbe interactions inhibit pathogen colonization by different ways. First, the resident microbiota competes with invaders for niches (as the intestinal mucosa) or available nutrients (Freter et al. 1983, Juge 2012, Lawley & Walker 2013, Wilson & Perini 1988). Thus, the direct competition limits the required densities of pathogens within the lumen to invade the epithelial surface and deeper tissues (Falkow 1997, Lawley & Walker 2013).

A second mechanism of colonization resistance is the metabolic exclusion. Specific products of the metabolism of the indigenous microbiota such as SCFAs create conditions that inhibit growth and virulence of pathogens (Duncan et al. 2009, Gantois et al. 2006, Shin et al. 2002, Veiga et al. 2010). Also, the metabolic activity of the gut microbiota reduces free oxygen, thus slowing the growth rate and virulence of intestinal pathogens, as for example, some members of the Enterobacteriaceae

that are facultative anaerobes (Altier 2005, Marteyn et al. 2011).

The third system is based on directly attack the harmful microorganisms by releasing different types of antimicrobial compounds, highlighting the bacteriocins that are peptides with narrow or broad-spectrum bactericidal activity (Dobson et al. 2012, Gong et al. 2010, Rea et al. 2010).

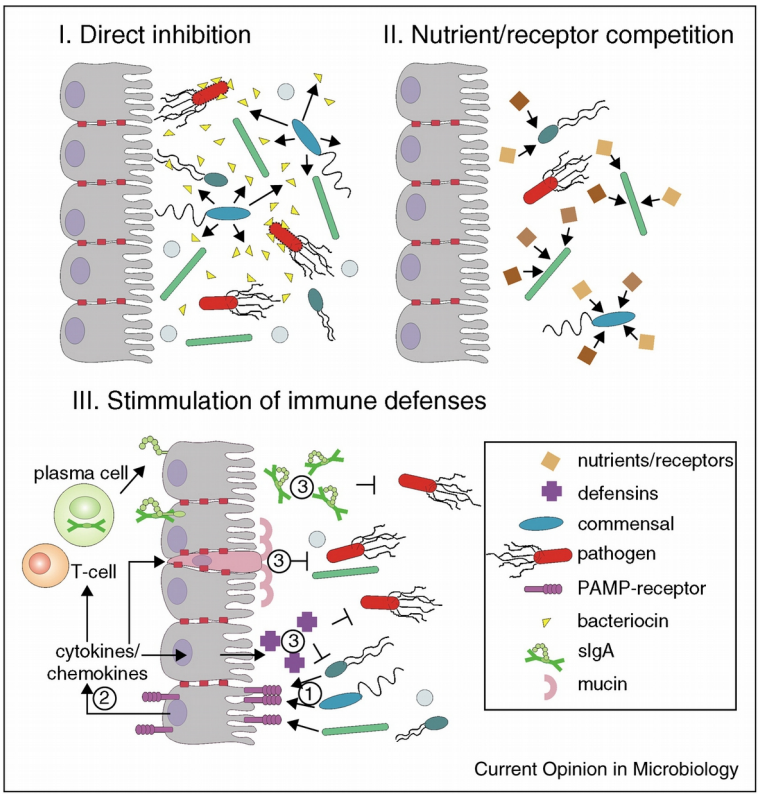


Figure 1.4. Three mechanisms of intestinal microbiota-mediated colonization resistance against enteric pathogens. (1) Direct inhibition of the pathogen by antimicrobial molecules (bacteriocins, metabolic by-products). (2) Competition for nutrients establishes colonization resistance by shutting down all potentially available nutrient niches for the pathogen. (3) Indirect inhibition by stimulating the host defence system. The microbiota releases microbial patterns (LPS, peptidoglycan) which are sensed by the host's epithelial cells, triggering antibacterial mechanisms such as release of epithelial-derived defensins, mucin secreted by goblet cells, and secretory IgA produced by lamina propria plasma B-cells (Adapted from Stecher and Hardt, 2011).

On the other hand, host-commensal interactions promote resistance to pathogen infections. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NODs) are key in recognizing pathogens and trigger the host defense and inflammation. The interactions between the TLRs with the commensal microbiota are required to maintain the intestinal homeostasis and regulation of this system (Buffie & Pamer 2013, Kawai & Akira 2009, Rakoff-Nahoum et al. 2004, Vaishnava et al. 2008). Besides this, the microbiota is involved in the regulation of the activity of T cells (lymphocytes involved in immunity) in the intestine, which is important for fighting pathogen invasion, as well as for protecting against exuberant inflammation and mucosal injury (Barnes & Powrie 2009, Buffie & Pamer 2013, Lawley & Walker 2013, Round & Mazmanian

2010, Sokol et al. 2008). Moreover, SCFAs produced by beneficial bacteria of the gut play a role in colonization resistance, because they participate in lowering inflammation, by counteracting the damaging effects of neutrophils (Kamada & Núñez 2014, Maslowski et al. 2009, Vinolo et al. 2011).

1.4. ANTIBIOTICS AND GUT MICROBIOTA

Disturbance has been defined as an event or process (physical or biological) that causes drastic structural changes to community composition. In fact, it is the main source of spatial and temporal heterogeneity in natural communities (Sousa, 1984). The type of disturbance and its characteristics (intensity, frequency, and duration) determine which specific organisms and properties of a community are selected over time, as well as the specific features of adaptation that they possess (Relman 2012). Properties of the ecosystems as resistance, resilience or functional redundancy, also contribute to define the response to perturbations (Allison & Martiny 2008) (Figure 1.5).

Aspects of modern lifestyles such as medications (mainly antibiotics), dietary changes or topical use of detergents, are interfering in the co-evolved human-microbiota interactions. Many of these human activities occur at a frequency and intensity higher than those at the microbial ecosystem are able to adapt, thus increasing the incidence of some diseases associated to an altered microbiota (Relman 2012).

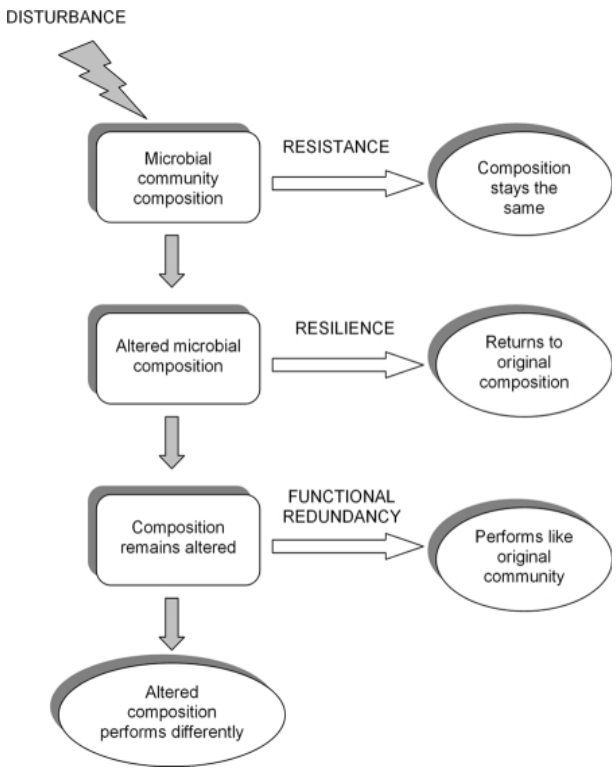


Figure 1.5. A schematic of the states and possible responses of microbial communities during disturbance (resistance, resilience, functional redundancy) (Adapted from Allison & Martiny, 2008).

Antibiotics are probably one of the most important discoveries in the history of medicine. Since their introduction into clinical practice in the 1940s, the majority of infectious diseases that plagued human history for many centuries have been successfully treated. Antibiotics are now one of the most common disturbance in human associated communities since on any given day approximately 1 to 3% of people in the developed world are exposed to antibiotics (Goossens et al. 2005).

Although some antibiotics target specific pathogenic populations, most of the administered antibiotics have broad-spectrum activity and are used to treat many infections (Nathan 2004). Thus, not only the specific pathogens, but also related members of the human microbiota are affected by antibiotic therapies.

Moreover, as microorganisms establish a complex network of co-dependence for the provision of nutrients, secondary metabolites or removal of toxic waste products, antibiotics also affect the microorganisms that are functionally connected to them (Figure 1.6) (Belenguer et al. 2006, Samuel & Gordon 2006, Willing et al. 2011). For example, this "chain reaction" effect was found in a study of mice treated with vancomycin, after which some Gram-negative bacteria were depleted, despite the antimicrobial activity of this agent is restricted to Gram-positive microorganisms (Robinson & Young 2010).

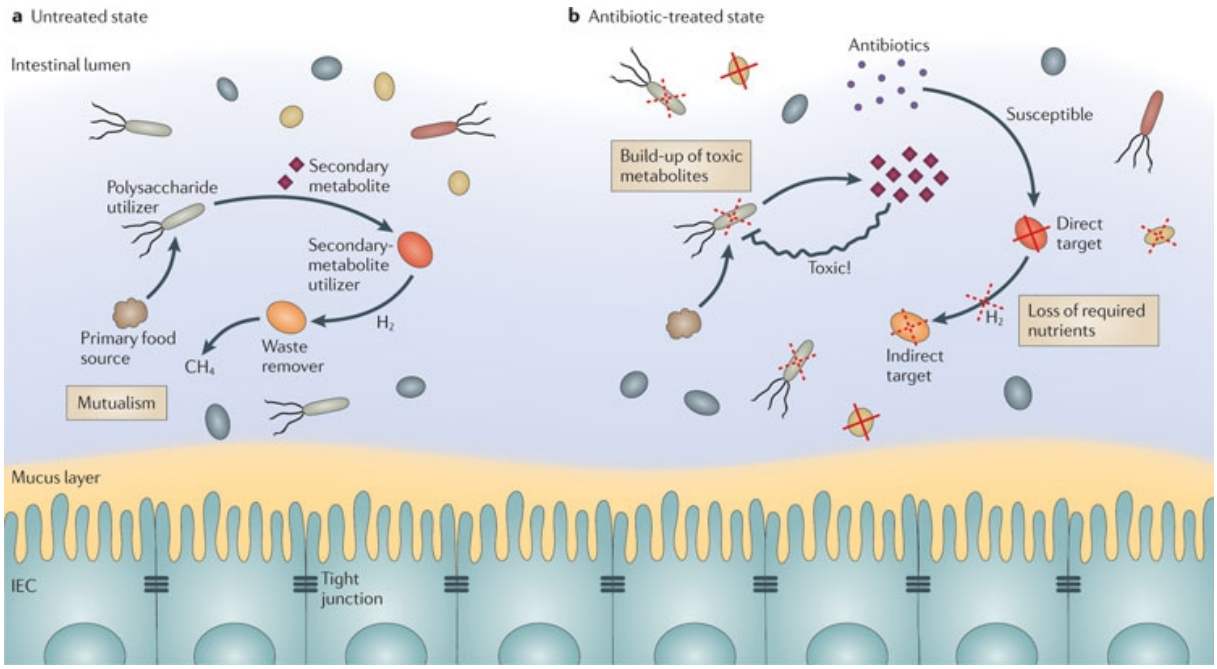


Figure 1.6. Direct and indirect effects of antibiotics on the gut microbiota. (a) Mutualism exists between gut symbionts and the host in the absence of antibiotics. Co-dependence among symbionts is exemplified in the figure. (b) When antibiotics are introduced, mutualistic traits of the symbionts that are directly targeted (solid crosses) by the antibiotic are eliminated, and the microorganisms participating in those mutualistic associations are indirectly affected (dashed crosses). IEC„ intestinal epithelial cell (Adapted from Willing et al. 2011).

Besides the spectrum of the agent, other features such as dosage and duration of treatment, route of administration or pharmacokinetic and pharmacodynamic properties influence the microbial community antibiotic-associated changes (Jernberg et al. 2010, Looft et al. 2012).

Alterations of the gut microbiota affect the beneficial functions they performed, leading to negative effects on several

aspects of the host health, such as immune system homeostasis, energy metabolism or colonization resistance (Brandl et al. 2008, Buffie et al. 2012, Dessein et al. 2009, Donskey 2006, Ng et al. 2013, Romick-Rosendale et al. 2009, Willing et al. 2011, Yap et al. 2008). Moreover, antibiotic use also promotes the increase of antibiotic-resistant strains, turning the intestine in a reservoir for resistance genes (Jernberg et al. 2010, Löfmark et al. 2006, Modi et al. 2013, Sommer et al. 2009).

1.4.1. ANTIBIOTIC EFFECTS ON THE MICROBIAL DIVERSITY AND COMPOSITION OF THE GUT MICROBIOTA

Due to its importance for public health most of the research about the detrimental effect of antibiotics has been focused on the emergence and spread of resistant strains. Recent studies have addressed the impact of antibiotics on the structure of the intestinal microbiota, mainly through DNA sequencing-based approaches (Antonopoulos et al. 2009, Buffie et al. 2012, Dethlefsen & Relman 2010, Dethlefsen et al. 2008, Jakobsson et al. 2010, Jernberg et al. 2007, O'Sullivan et al. 2013, Panda et al. 2014).

Most of the studies on human and animal models have found an association of antibiotics with a reduced microbial diversity (Antonopoulos et al. 2009, Dethlefsen & Relman 2010, Dethlefsen et al. 2008, Jernberg et al. 2007, Panda et al. 2014). However, a reduction of the total biomass seems to depend on the type and doses of the antibiotic (Panda et al. 2014). In fact, antibiotic-associated changes are more related to altered taxon abundance than to the gain or loss of bacterial members (Dethlefsen & Relman 2011).

Studies of the effect of antibiotics on the gut community have covered different types of them as fluoroquinolones, lincosamides, beta-lactams, oxazolidinones, among others. In most of them, shifts in relative proportions of certain populations are observed since human-associated microbial communities exhibit low resistance to antibiotic perturbations (Jernberg et al. 2010, Panda et al. 2014, Robinson & Young 2010).

Several antibiotics are especially active against anaerobic bacteria that are dominant in the human gut microbiota. One example is clindamycin that has been shown to have a large negative effect on the anaerobic fraction of the gut microbiota (Löfmark et al. 2006, Nyberg et al. 2007). Also, amoxicillin treatment has a strong effect on specific groups of the gut environment, among aerobic and anaerobic species, leading a decrease in *Clostridium* and *Eubacterium* and an increase in Enterobacteriaceae, *Bacteroides* and *Prevotella* taxa (Barc et al. 2004, Sullivan et al. 2001). The commonly used treatment for *H. pylori* gastritis, which is a triple therapy with clarithromycin, metronidazole and omeprazole, perturbs dramatically the composition of the gut microbiota, decreasing mainly beneficial members of Clostridia and Bifidobacteria classes (Jakobsson et al. 2007, 2010).

The gut microbiota, as a whole, is considered relatively resilient to antibiotic challenge and after short-term therapy it returns to the pre-treatment state (De La Cochetière et al. 2005). However, it is clear that antibiotic therapies have long-term effects in the community structure, with a lower abundance of specific bacterial groups after the therapy, as well as an increase in the risk of antibiotic resistance genes and its transference to pathogens (Dethlefsen & Relman 2011, Dethlefsen et al. 2008, Francino 2013, Jakobsson et al. 2010, Jernberg et al. 2007).

For instance, a treatment with clindamycin resulted in lower diversity of the *Bacteroides* genus, in an enrichment of resistant *Bacteroides* clones, and also in an increase in the abundance of the resistant *erm*-genes up to two years after treatment (Jernberg et al. 2007, Löfmark et al. 2006).

Long-term effects of metronidazole and clarithromycin on the faecal human microbiota up to four years after treatment have been also described, with increased persistence of the macrolide resistance gene *ermB* (Jakobsson et al. 2010).

The two longer follow-up studies about antibiotic effects on human gut diversity and composition so far, were carried out by Dethlefsen and colleagues. They studied the effects of the commonly used antibiotic oral ciprofloxacin, on the faecal microbiota of different individuals by sampling their microbiota before, during and after the antimicrobial therapy (Dethlefsen & Relman 2011, Dethlefsen et al. 2008). For both studies, ciprofloxacin administration correlated with a reduction of taxon

richness, diversity and evenness and the individuals showed different responses and recovery periods. Interesting, in one of the studies, individuals were treated with a second round of ciprofloxacin and responded differently respect to the first course. This phenomenon is likely, since repeated perturbations are "recorded" by the microbiota, even when the community seems to have recovered from the initial perturbation (Dethlefsen & Relman 2011).

On the other hand, the absence of clinical symptoms on the individuals of the study could be explained by the functional redundancy of the gut inhabitants, at least in some functions related to diet processing as the carbohydrate metabolism. However, other functions as colonization resistance to pathogens or regulation of host immunity, may be restricted to a subset of the community. Therefore, every antibiotic course could contribute to displace a mutualist with a strain that may provide or not the same benefit, having an accumulative detrimental effect on the beneficial functions of the gut microbiota (Dethlefsen & Relman 2011).

1.4.2. ANTIBIOTIC EFFECTS ON THE FUNCTIONAL PROFILE OF THE GUT MICROBIOTA

The effect of antibiotics on the functions carried by the human gut microbiota has been poorly addressed. Most of the knowledge about the antibiotic-related changes of the biological functions of the gut microbiota have been carried on animal models. For instance, a metagenomic study of antibiotic-associated shifts on the functional potential of the swine gut microbiota showed that genes encoding virulence, gene-transfer and energy production and conversion are positively selected by the antibiotics. Due to their roles, those genes could promote the stability and spread of antibiotic resistance genes in the gut ecosystem (Looft et al. 2012).

The metabolic profiles have been studied in mice under different antibiotic regimens and a common feature with treated humans is the reduced production of SCFAs (Willing et al. 2011, Woodmansey et al. 2004). Some mice studies have also shown that the microbial fermentation of carbohydrates is disrupted by the antibiotics, since high concentrations of oligosaccharide and low concentrations of amino acids and SCFAs were observed in treated mice. Interesting, as for the microbial diversity, the levels of some metabolites were not recovered weeks after the therapy (Yap et al. 2008). Antibiotic treatments have a profound impact on the mice intestinal metabolome affecting the levels of more than the 87% of all the metabolites, being decreased pathways critical for host health as bile acid, eicosanoid and steroid hormone synthesis (Antunes et al. 2011).

In a culture-based study of metabolic activities of the gut microbiota in young adults and in elderly subjects (treated and non-treated with antibiotics), Woodmansey and colleagues showed that antibiotic-treated individuals presented a high proteolytic species diversity (fusobacteria, clostridia and propionibacteria) (Woodmansey et al. 2004). Increased levels of protein break-down and amino acid fermentation result in the formation of toxic bacterial metabolites (Macfarlane et al. 1989). Also, the presence of bacterial fermentation products that are essential for host health, specifically SCFAs (mainly butyrate, propionate, acetate) was lower in antibiotic-treated elderly.

Recently, a chemostat model consisting of a defined consortium model (14 species) of the most common cultivable and saccharolytic and amino acid fermenting bacteria of the gut was used to study the effects of two broad spectrum antibiotics (metronidazole and ampicillin) on the bacterial composition and metabolic activities (Newton et al. 2013). The two antibiotics showed great differences on their effect on the microbiota model, but for both agents, the production of SCFAs decreased, while the production of some hydrolytic enzymes was dependent of the antibiotic-resistant populations that survived.

1.4.3. ANTIBIOTIC THERAPY AND RESISTANCE GENES IN THE GUT ENVIRONMENT.

The antibiotic usage is the major risk factor for the spread of resistant bacteria and resistance genes in the human intestinal ecosystem. Other factors include the capacity of resistant strains to colonize the gut, their relative fitness, mutation rates and

efficiency of horizontal gene transfer (Jernberg et al. 2010).

The gut environment has good conditions for efficient transmission of resistance genes, since it comprises high numbers of bacteria and a stable diverse ecosystem (rich in nutrients and with relatively constant chemical parameters). After the resistance selection process by the antibiotic therapy, the resistance genes can be transferred to other bacteria, including pathogens. For instance, the transfer of a plasmid carrying a beta-lactamase gene from a resistant *Escherichia coli* strain to a susceptible one was demonstrated in an ampicillin-treated child (Karami et al. 2007). Thus, antibiotics not only select for resistance bacteria but also increase the chance of transfer events from the increased resistant microorganisms.

Recently, it has been postulated that when the selective pressure of antibiotics is no longer present, the resistant strains could have a lower fitness compared with the non-resistant ones, but there are mechanisms such as the acquisition of compensatory mutations, by which these resistant bacteria can reduce the fitness costs (Andersson & Hughes 2011, Chewapreecha 2014, Snitkin et al. 2013).

Some studies have shown the impact of antibiotic therapy on long-term persistence of antibiotic resistance genes. In a study about the effect of clyndamicin in the faecal microbiota it was found a significant increase in *erm* resistance genes (*ermF*, *ermG* and *ermB*) ,that could be still detected two years after the antibiotic course (Jernberg et al. 2007). Two other studies investigated the effect of clarithromycin, showing the presence of highly resistant enterococci that carried the *ermB* gene, one year after the antibiotic administration, as well as the presence of the macrolide-resistant *Staphylococcus epidermis* up to four years after therapy (Sjölund et al. 2003, 2005).

Sommer and colleagues characterized the antibiotic resistance reservoir in the human microbiota of individuals who had not been exposed to antibiotics for at least one year, finding an immense diversity of resistance genes in their microbiome (Sommer et al. 2009). Most than half of the resistance genes harboured by the gut microbiota were distantly related to resistance genes detected in pathogenic isolates. This could imply that the resistance genes of our gut are difficult to access or infrequently exchanged with human pathogens. However, the resistance genes identified were functional in *E. coli*, suggesting that if there is a barrier between commensal bacteria and pathogens for gene transfer, it must due to processes different of functional compatibility. On the other hand, nearly half of resistance genes of cultured aerobic gut isolates were identical to resistance genes from human pathogens. Some examples are the genes encoding for tetracycline efflux pumps (*TetA*), two classes of aminoglycoside-modifying enzymes (*AAC(3)-II* and *AAC(6)-Ib*) and three classes of beta-lactam-inactivating enzymes (*TEM*, *AmpC* and *CTX-M*). Besides this, in a metagenomic study of antibiotic resistance genes of a large human cohort (162 individuals) more than 1000 resistance genes were identified, being highly abundant those conferring resistance against tetracycline. In addition, the levels of antibiotic resistance genes were significantly higher in the gut microbiome on average (0.266%) compared with environmental data sets (0.008-0.171%) (Hu et al. 2013). Moreover, a study of antibiotic effects on the swine gut microbiome showed that antibiotics use causes a significant increase in the abundance of diverse resistance genes, including genes that do not confer resistance against the administered antibiotic, which suggests an indirect mechanism of selection, as co-occurrence on mobile elements, to explain this event (Looft & Allen 2012).

Interestingly, a recent study of the phageoma (total resident phages) of the murine gut under antibiotic pressure has shown that they are enriched in a wide range of antibiotic resistances after drug perturbation. This observed cross-resistance can be mediated by drug-specific inactivators as well as multidrug-resistance exporters (Modi et al. 2013).

On the other hand, some bacteria that originate from ingested food are only transient inhabitants of the intestine during digestion, even so they can play a role in the transfer of resistance genes during their passage through the intestine (Andremont 2003, Finley et al. 2013, Salyers et al. 2004). Therefore, the use of antibiotics in agriculture might be contributing to the emerging resistance problem in clinical environment, since cultivated soils are large global resistance reservoirs (Aubry-Damon et al. 2004, Lipsitch et al. 2002). In fact, it has been described in soils resistance cassettes against five classes of antibiotics (beta-lactams, aminoglycosides, amphenicols, sulfonamides and tetracyclines) that are identical to genes from diverse human pathogens, demonstrating the exchange (horizontal gene transfer) of resistance genes of soil bacteria with human pathogens (Forsberg et al. 2012).

Resistance genes, resistant bacteria and antibiotics flow between different areas of use (human, animal, soil, water etc...) promote the emerging, enrichment and spread of resistant bacteria between hosts and environments. Figure 1.7 represents the resistant bacteria and antibiotics flows between different areas of use and environments (Andersson & Hughes 2011). The

antibiotic therapies are often administered in humans, animals and agriculture at high concentrations. Thus, about half of all antibiotics are excreted in an active form in humans and animals urine into the environment (waste water, manure) where they finally reach aquatic environments (lakes, rivers) and soils (Bryskier 2005).

It is difficult to assess the relative contributions of environments and selection to the spread of resistances and it is commonly assumed that hospitals are a significant source because of the high use of antibiotics (strong local selection) and the large host population that promote the spread of resistant bacteria (Andersson & Hughes 2011). However, it is noteworthy that weaker selective pressures as a whole (lakes, rivers, soils) are equally important for the emerging of resistances (Baquero 2001a,b).

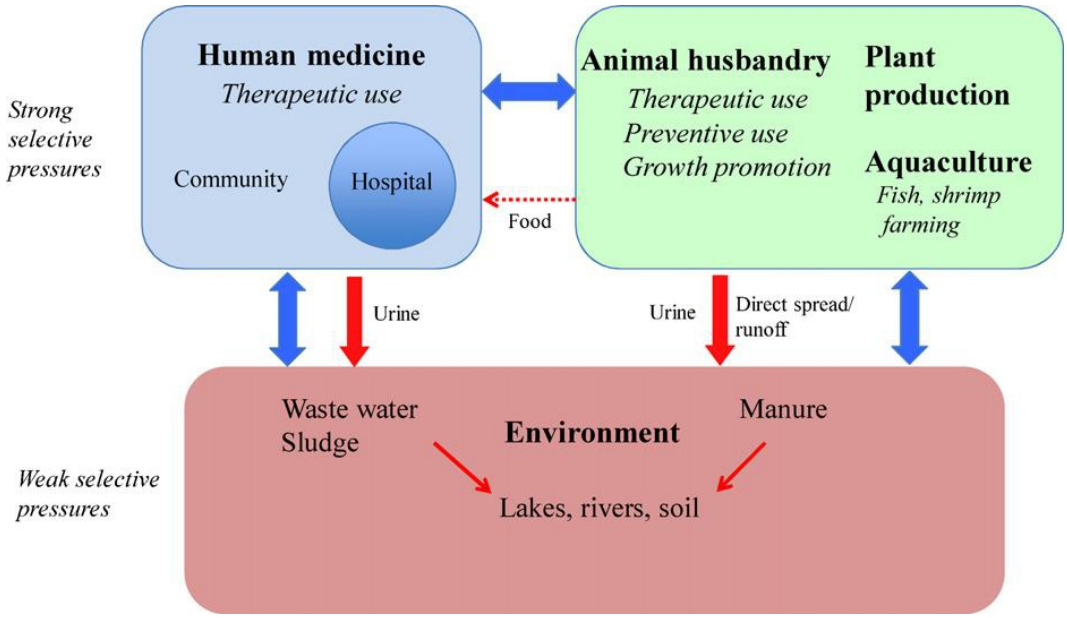


Figure 1.7. Schematic representation of flows of resistant bacteria (blue) and antibiotics (red) between different environments and areas of use (Andersson & Hughes, 2012).

1.4.4. RELATION OF ANTIBIOTIC USE AND HUMAN PATHOLOGIES.

As stated before, the commensal gut microbiota plays a major role in the homeostasis of the human host. Hence, disruption of the gut microbiota by antibiotics increase the chance of disease and have long-term consequences for host health. A dysbiotic microbiota is defined as one that contains an imbalance in structure and function compared with that found in healthy individuals and it has strong effects on host physiology and disease processes. Additionally, a dysbiotic microbiota can harbour bacteria that exacerbate intestinal inflammation or manifest systemic disease (Pham & Lawley 2014). In fact, a dysbiotic microbiota has been associated to several intra and extra-intestinal conditions as inflammatory bowel disease, irritable bowel syndrome, antibiotic-associated diarrhea, obesity, cancer and pathogen infection, HIV infection, among others (Figure 1.8) (Francino 2013, Kassinen et al. 2007, Macfarlane 2014, Macfarlane et al. 2009, McGarr et al. 2005, Morgan et al. 2012, Mutlu et al. 2014, Turnbaugh et al. 2008, Vázquez-Castellanos et al. 2014, Willing et al. 2011).

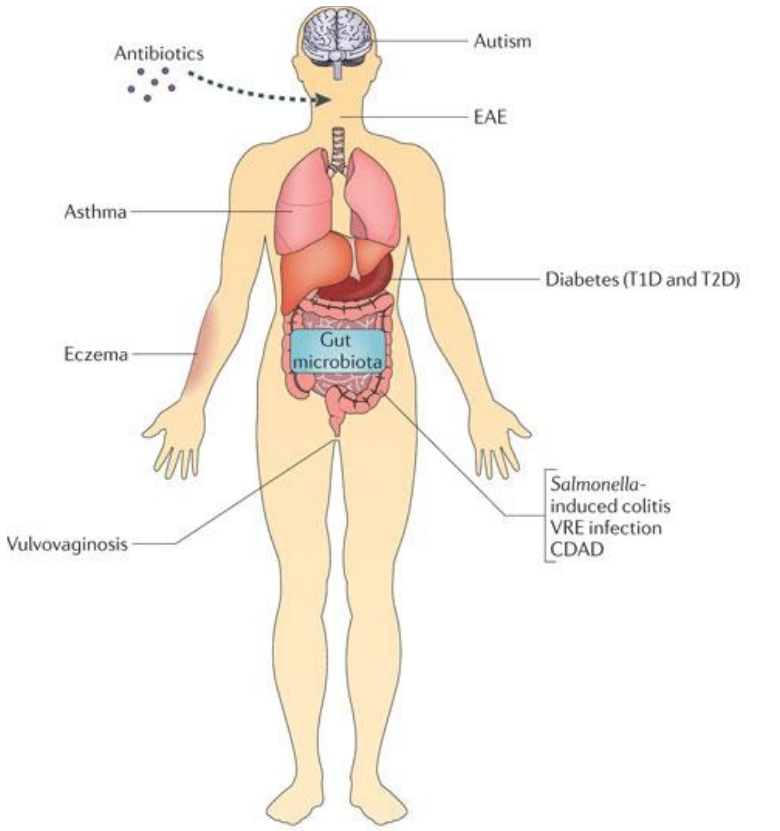


Figure 1.8. Oral antibiotics have been associated with changes in numerous intra- and extra-intestinal diseases. CDAD, *Clostridium difficile*- associated diarrhoea; EAE, experimental autoimmune encephalopathy; T1D, type 1 diabetes; T2D, type 2 diabetes; VRE, vancomycin-resistant *Enterococcus* (Willing et al. 2011).

A common side effect of antibiotic treatment is the appearance of diarrhea (antibiotic-associated diarrhea or AAD). It is not necessary an etiologic agent for AAD as the drugs cause the dysfunction *per se*. For instance, if the bacteria responsible for breaking down diet fiber in the colon are eliminated by the antibiotic, an osmotic diarrhea may appear. This is because the normal digestion of these compounds prevent this type of diahrrea (Young & Schmidt 2004). On the other hand, some opportunistic pathogens can cause disease during therapy, including *Salmonella* spp., *Clostridium perfringens*, *Klebsiella oxytoca, Candida albicans* or *Clostridium difficile*, being this last the most common cause of pathogen-associated AAD (15%-25%) (Walk & Young 2008).

As we previously explained, the gut microbiota has an important role in the colonization resistance against pathogens. Thus, the disruption of the microbiota by antimicrobial therapies results in increased susceptibility to infection by pathogens. For example, the vancomycin-resistant *Enterococcus* (VRE), which can cause serious systemic infections, is a strain able to colonize effectively those mice treated with antibiotics. It has been proposed that the colonization of this pathogen is possible due to a reduced production of the antimicrobial peptide REG3γ as a consequence of microbial signals loss (Brandl et al. 2008). Moreover, in a study of patients undergoing allogeneic hematopoietic stem cell transplantation, which received antibiotics during their hospitalization, it was found an intestinal domination (>97%) by VRE previous to bloodstream infection (Ubeda et al. 2010). Recently, Ubeda and colleagues found that intestinal colonization with commensal anaerobic bacteria from *Barnesiella* genus confers resistance against an overgrowth in the intestine and bloodstream infection with VRE (Ubeda et al. 2013).

Using a mouse-model, Buffie and colleagues showed that even a single dose of oral clindamycin promotes the infection and persistence of *C. difficile*, whose overgrowth leads to a high risk for pseudomembranous colitis (Bartlett 2002, Buffie et al. 2012). Also in a mouse-based study it was found that some antibiotics as clindamycin and piperacillin-tazobactam increase the abundance of carbapenemase-producing *Klebsiella pneumoniae* (KPC), which produces principally pneumonia, and decrease the presence of some anaerobic bacteria, suggesting the importance of anaerobes for protection against KPC colonization (Perez et al. 2011).

Germ-free mice have not only intestinal but also extra-intestinal problems indicating that the host-microbiota interactions have systemic implications. Over recent years there has been an increase in the presence of immunological disorders in the developed world, supporting what it is called the "hygiene hypothesis" (Guarner et al. 2006, Wills-Karp et al. 2001). This hypothesis proposes that insufficient exposure to microorganisms, parasites and infectious agents alters the normal immune development, increasing the susceptibility to allergic diseases (Willing et al. 2011). An extension of this hypothesis is the "microflora hypothesis" since epidemiological studies have associated early antibiotic use with an increase in the risk of atopic diseases. Thus, the overuse of antibiotics during childhood alters the microbiota and inhibits the normal maturation of the immune system, fuelling some diseases as allergies and asthma (Blaser 2011, Francino 2013).

1.5. *CLOSTRIDIUM DIFFICILE* INFECTION.

The recent increase in the incidence and virulence of *C. difficile* infection (CDI), its association with antibiotic administration and the present interest in the role of the human microbiome in health and disease have led to a renewed attraction to investigate the relation between *C. difficile* and the human gut microbiota.

1.5.1. CLINICAL FEATURES ABOUT *C. DIFFICILE* INFECTION.

C. difficile is a Gram-positive, spore-forming anaerobic bacillus that is commonly found in the gut of mammals and is the agent that causes CDI (Bartlett 1994). The first confirmed case of CDI was reported in 1977 and its incidence has continued rising since then, and at present is the most frequent cause of nosocomial diarrhea worldwide (Kuijper et al. 2007). The symptoms of CDI range from mild diarrhea to severe pseudomembranous colitis and often occur after broad-spectrum antibiotic therapies (Dawson et al. 2009). Other common clinical symptoms are abdominal pain and cramping, fever and leucocytosis. Pseudomembranous colitis in the distal colon and rectum is fatal in 6%-30% of cases (Kuijper et al. 2007). However, the infection with *C. difficile* could remain asymptomatic probably due to different factors as the microorganism level of virulence, the degree of disruption of the normal microbiota, or the host immune response (Gerding 2012). Several routine laboratory tests are applied for CDI diagnosis such as enzyme immunoassays to detect toxins A and B, real-time PCR to detect the toxigenic genes, anaerobic culture for toxigenic *C. difficile* strains, culture cytotoxicity assay and enzyme-linked immunosorbent assay (ELISA) of the toxins A and B (Hookman & Barkin 2009).

The major risk factor for CDI is antibiotic exposure, particularly the broad-spectrum antibiotics with activity against anaerobes (Denève et al. 2009). Other factors are hospitalization, immunocompromised status and old age (>65 years). In fact, old debilitated patients in hospitals are highly vulnerable to CDI with a reported colonization rate of 73% (Barbut et al. 1996). Other risk factors are treatments with proton-pump inhibitors, H2 antagonists and methotrexate, as well as the presence of some gastrointestinal diseases as inflammatory bowel diseases (Dial et al. 2005).

The pathogenesis of *C. difficile* starts generally with the antimicrobial therapy which disrupts the normal gut microbiota, thus reducing the colonization resistance capacity. Then, pathogen spores of endogenous or exogenous origin germinate and the vegetative forms multiply. Then, *C. difficile* penetrates the mucus layer with the aid of flagella and proteases and adheres to the

enterocytes through multiple adhesins. These steps belong to the first phase of the pathogenic process, "the colonization" (Denève et al. 2009) (Figure 1.9).

The second phase of the pathogenesis is the production of toxins A and B encoded by the genes *tcdA* and *tcdB*. Those genes are located in the pathogenicity locus PaLoc with the *tcdE* gene encoding a putative holin and the genes *tcdR* and *tcdC* encoding regulatory proteins (Dupuy et al. 2008, Tan et al. 2001). The toxins TcdA and TcdB modify the actin cytoskeleton of epithelial cells via the covalent glucosylation of Rho, Rac and Cdc42 proteins, which regulate actin, leading to cytoskeleton disorganization, inhibition of cell division and membrane trafficking, resulting in the destruction of the intestinal epithelial cells (Jaffe & Hall 2005, Just et al. 1995a,b; Ridley 2001) (Figure 1.9).

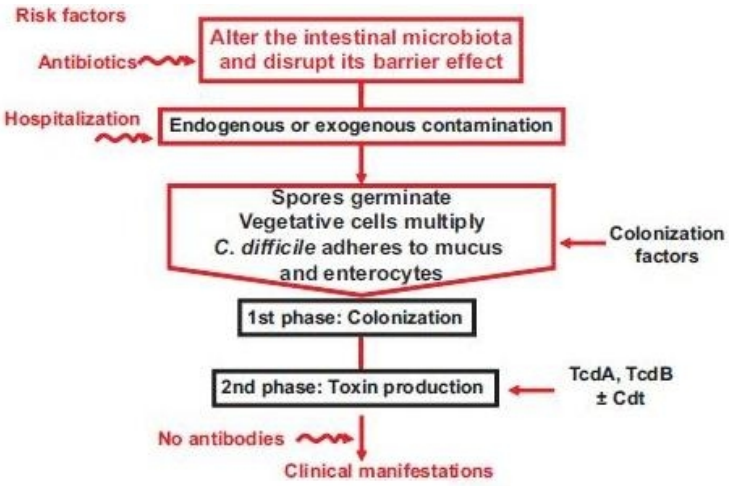


Figure 1.9. Pathogenesis of *Clostridium difficile* (Dèneve et al. 2009).

Few strains (6% of *C. difficile* isolates) present a binary toxin called CDT (encoded by *CdtA* and *CdtB* in the *Cdt* locus) which consists of a binding and enzymatic component with actin-specific ADP ribosyl transferase activity that leads to cytoskeleton disorganization (Perelle et al. 1997). This toxin is considered as an additional virulence factor since it potentiates the toxicity of TcdA and TcdB, causing a more severe disease (Barbut et al. 2005). In the last years the epidemiology of CDI has changed radically with a five-fold increase in its incidence in the population of North America, eight-fold increase in the elderly, as well as an increase in its incidence in European countries but associated with specific outbreaks (Kuijper et al. 2007, Pépin et al. 2004). In Spain, the incidence is similar to the surrounding European countries but with a trend to increase due to the high frequency of prescription of antibiotics (Asensio & Monge 2012). Other recent epidemiological changes are an increase in severity of the disease associated with septic shock, toxic megacolon, intestinal perforation, mortality, treatment failure plus several relapses and spread to low-risk populations (McFarland 2008, Musher et al. 2005, Pépin et al. 2005). These changes in the pattern of the pathology of CDI worldwide have been associated with the emergence of a hypervirulent strain named *C. difficile* NAP1/027/BI strain. This epidemic strain produces higher levels of the TcdA (16-fold) and TcdB (23-fold) toxins due to a truncated and inactive TcdC protein which is a negative regulator of toxin genes (Dupuy et al. 2008, Warny et al. 2005). Besides, *C. difficile* NAP1/027/BI strain possesses the locus *Cdt* encoding the binary toxin CDT which potentiates the toxicity of the TcdA and TcdB toxins.

The most effective initial treatments for CDI are oral antibiotic therapies, mainly vancomycin and metronidazole (Miller 2007). However, a high number of patients (between 20-35%) develop recurrent illness several days to weeks after the initial "success" of the antibiotic therapy (McFarland et al. 2002, Wilcox 1998).

An alternative method for the maintenance and restoration of the gut microbiota after antibiotic therapies involves the use of prebiotics and probiotics. Probiotics as bifidobacteria and lactobacilli seem to counteract AAD and relapsing CDI, but

some studies on probiotics to treat CDI have shown conflicting results (Hookman & Barkin 2009, Macfarlane 2014). For instance, a study of probiotic treatment with three types of probiotics (*Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG, and probiotic mixture) showed that only *S. boulardii* was effective in treating the recurrent disease (McFarland 2006). Other alternative therapies are the use of a novel neutralizing monoclonal antibody against *C. difficile* toxins, anion-binding resins that neutralize clostridial toxins, vaccination against the pathogen and its toxins, or immunoglobulin based therapies, but most of these treatments are in early stage of development (Jank et al. 2008, McPherson et al. 2006, Peterfreund et al. 2012, Sougioultzis et al. 2005, Taylor et al. 2008). A promising approach for patients with recurrent CDI involves the transplantation of defined microbial communities or the entire faecal microbiota from healthy individuals (bacteriotherapy). The faecal microbiota transplantation has been effective against recurrent CDI and restoring the intestinal microbial diversity in up to 95% of patients (Bakken et al. 2011, Borody et al. 2004, Gough et al. 2011, Khoruts et al., Lawley et al. 2012, Petrof et al. 2013). Further studies will be required to understand how the implantation of these microbial communities are able to suppress the CDI and other consequences that they may have on human health (Britton & Young 2014).

1.5.2. INFLUENCE OF ANTIBIOTICS IN THE GUT MICROBIOTA AND ITS RELATION WITH *C. DIFFICILE* INFECTION.

As we previously mentioned, the main risk for developing CDI is antibiotic use, mainly clindamycin, cephalosporins, penicillins and more recently fluoroquinolones (Bartlett 2010, Britton & Young 2012). Also, several antibiotic exposures over time increase the risk of developing disease, since an accumulative detrimental effect shifts the normal gut microbiota that loses its colonization resistance capacity (Stevens et al. 2011). Besides this, it has been demonstrated that antibiotic use and the exchange of mobile elements between intestinal bacteria lead to a rapid evolution of the epidemic *C. difficile* strain (ribotype 027) (He et al. 2013).

The development of animal models for studying CDI in the basis of microbial ecology has led to advance in the relation between the microbiota and the development of the infection and its complications. Several studies in animals and humans have shown that antibiotics have strong and long-lasting effects on the community structure of the microbiota, affecting the biomass, composition and functions, thus reducing resistance to CDI (Britton & Young 2014) (Figure 1.10).

In mice, a variety of antibiotics have been associated to the loss of resistance to *C. difficile* colonization (Buffie et al. 2012, Chen et al. 2008, Reeves et al. 2011, Theriot et al. 2011). For example, a study showed that a single dose of clindamycin markedly reduced the diversity of the intestinal microbiota and the inoculation of *C. difficile* to these mice resulted in development of diarrhea, colitis and a high mortality rate (40-50%) (Buffie et al. 2012).

A similar study on *C. difficile* colonization of antibiotic treated mice described a high abundance of Proteobacteria (mainly Enterobacteriaceae) and a low abundance of normal members of the microbiota as Lachnospiraceae (Firmicutes) in the ill animals compared with the healthy controls (Reeves et al. 2011). The same group investigated, also in mice, the role of strains of both groups (Enterobacteriaceae and Lachnospiraceae) in colonization resistance against *C. difficile* (Reeves et al. 2012). They found that only the Lachnospiraceae strain was able to partially restore the colonization resistance because Lachnospiraceae-inoculated mice presented decreased *C. difficile* colonization, lower intestinal cytotoxin levels, less severe clinical signs and colonic histopathology. Moreover, a recent study showed that six phylogenetically diverse species of gut normal bacteria administered together were able to restore colonization resistance and prevented the chronic carriage of *C. difficile* in mice (Lawley et al. 2012).

Microbiome-based studies of patients with CDI have provided data that correlated with some findings from animal studies, specifically a decrease in biodiversity and a differential abundance of some beneficial microbial groups, as a lower abundance of some Clostridiales members (mainly Lachnospiraceae and Ruminococcaceae) compared with healthy individuals (Antharam et al. 2013, Chang et al. 2008, Hopkins & Macfarlane 2002, Vincent et al. 2013).

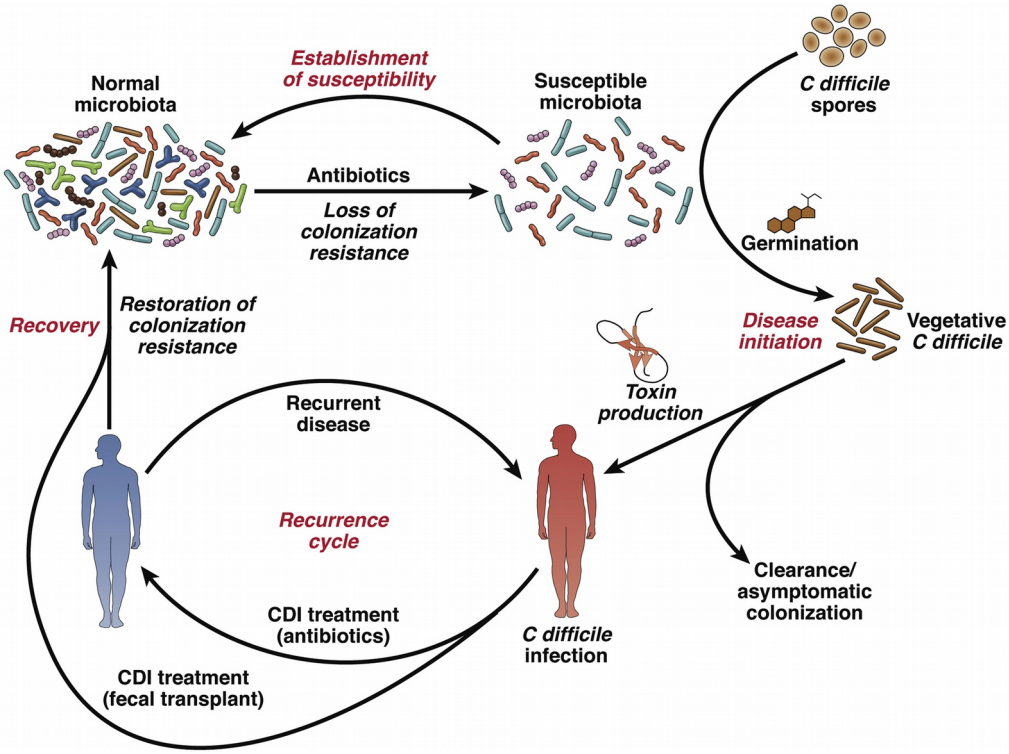


Figure 1.10. Cycle of CDI. Antibiotic administration alters the indigenous intestinal microbiota, producing an environment that permits germination of *C. difficile* spores and expansion of the pathogen. *C. difficile* produces toxins that cause colitis and resulting symptoms. Returning the microbiota to a state of colonization resistance cures CDI. However, if the microbiota is unable to restore resistance to colonization by *C difficile*, then patients have recurring CDI. In certain cases, repeat courses of anti-*C. difficile* antibiotic therapy can eradicate the pathogen. In other cases, therapeutic restoration of a diverse microbiota via faecal microbiota transplantation is required to overcome CDI (Britton & Young, 2014).

Also, Chang and colleagues profiled the gut microbiota of patients with antibiotic-associated diarrhea due to *C. difficile* in both, initial and recurrent episodes using 16S rRNA-encoding gene clone libraries. They found a highly variable bacterial composition and a very low diversity in the recurrent patients. Patients with initial infections showed an intermediate diversity pattern between recurrent cases and controls (Chang et al. 2008).

A different study examined the microbiota associated to CDI in elderly by pyrosequencing of the 16S rRNA gene amplicons, finding a marked reduction in microbial diversity at genus level in infected patients compared with non-infected (Rea et al. 2011). The infected patients showed a higher abundance of Lactobacillaceae and Enterococcaceae and a lower abundance of *Bacteroides*, *Prevotella* and Bifidobacteria.

A lower diversity prior to episodes of CDI was observed in a similar comparative study of patients under antibiotic therapy. Bacteria from Bacteroidaceae and Clostridiales were depleted in patients compared with controls, whereas patients were enriched in Enterococcaceae family (Vincent et al. 2013). The higher abundance of Enterococcaceae members could be explained by its opportunistic nature that, similar to *C. difficile*, could take advantage of a low bacterial diversity to overgrow.

A recent research compared the structure of the microbiota (based on 16S rRNA gene pyrosequencing) of three groups of individuals: *C. difficile* infected (CDI) subjects with diarrhea, *C. difficile*-negative nosocomial diarrhea (CDN) and healthy controls. Significant alterations in the microbiota were associated to CDI and CDN compared with controls. Bacteria producing butyrate and other SCFAs mainly from Ruminococcaceae and Lachnospiraceae families were depleted in CDI and CDN,

suggesting a main role of these bacteria in colonization resistance against the pathogen and in the maintenance of the ecosystem equilibrium (Antharam et al. 2013).

1.6. METHODS FOR STUDYING THE HUMAN MICROBIOTA.

The gut microbiota is an ecosystem of great interest because of its essential role for the host health. For a long time, the research on the gut microbial community has been based on isolation and culture of the microorganisms in the laboratory. Over the last years, the development of new molecular techniques and high-throughput sequencing technologies have promoted an exponential increase of the amount of data related to the intestinal microbiota.

1.6.1. CULTURE TECHNIQUES FOR INTESTINAL MICROBIOTA CHARACTERIZATION

The first insights into the gut microbiota composition have been obtained from isolating organisms from faecal or intestinal samples by culturing them. The majority of the cultivated representatives of the gut were discovered after the introduction of anaerobic cultivation technologies that are now standard tools (Arank et al. 1969). Bacteria that have been cultivated from the gut include a variety of butyrate-producing bacteria from Firmicutes phylum and other abundant bacteria from Bacteroidetes (Bakir et al. 2006, Barcenilla et al. 2000, Hayashi et al. 2007, Pryde et al. 2002, Sakamoto et al. 2007) . Also, the use of alternative carbon sources has allowed the discovery of species of minority phyla such as Verrucomicrobia or Lentisphaerae (Derrien et al. 2004, Zoetendal et al. 2003). Nevertheless, a great number of microorganisms will remain under-represented among cultured bacteria, due to they have developed an intimate and highly dependent relation with the host and with other members of the community along evolution, being almost impossible to grow into pure culture (Zoetendal et al. 2008). Despite its limitations, microbial cultivation is still essential to define bacteria-bacteria and bacteria-host interactions (Walker et al. 2014).

1.6.2. CULTURE-INDEPENDENT METHODS TO STUDY THE GUT MICROBIOTA

It is well known that the major part of gut bacteria has not been described by cultivation techniques. This fact, together with the development of high-throughput sequencing technologies, have promoted the implementation of culture-independent methods, such as sequencing of the 16S small subunit (SSU) rRNA gene and meta-"omics" approaches, to expand our knowledge about the structure, functions and ecology of the human gut microbiota.

1.6.2.1. 16S rRNA BASED APPROACHES TO EXPLORE THE DIVERSITY OF THE GUT ECOSYSTEM.

The use of the ribosomal 16S rRNA gene for phylogenetic classification focused on the sequence diversity of this gene to study the diversity of microbial communities (Woese 1987, Woese et al. 1990). In fact, 16S rDNA sequencing has become the gold standard for microbial communities characterization, and around 75% of microbes described in the gut has been detected only through 16S rRNA sequencing (Petrosino et al. 2010, Zoetendal et al. 2008).

The first studies of SSU rRNA sequencing of GIT were performed by cloning 16S rRNA gene amplicons and sequencing them with Sanger technology. The application of this method allowed to describe a significant fraction of the bacteria so far

unknown from different regions of GIT such as colon, ileum, oral cavity or human faeces (Eckburg et al. 2005, Hold et al. 2002, Paster et al. 2001, Suau et al. 1999, Wang et al. 2003, Zoetendal et al. 1998). Sanger sequencing projects of 16S rDNA amplicons showed the predominance of two phyla, Bacteroidetes and Firmicutes in the gut ecosystem, as well as the presence of other less abundant phyla as Actinobacteria, Proteobacteria, Verrucomicrobia or Fusobacteria (Bäckhed et al. 2005, Ley et al. 2006, Zoetendal et al. 2006).

The high-throughput sequencing technology revolutionized the microbial ecology since it allows to deep sequencing the total 16S rRNA genes of an environment, thus revealing low-abundance species (named the rare biosphere) and giving a more complete picture of the whole community (Huse et al. 2008, Marchesi 2010, Sogin et al. 2006). The high-throughput 16S rRNA gene sequencing has become the most widely used method to characterize the structure of the human gut microbiota under different conditions, as well as the influence of internal and external factors in its composition and diversity. For instance, Andersson and colleagues used 454-pyrosequencing of a hyper-variable region of the 16S rRNA gene to compare microbial communities of the human throat, stomach and faeces (Andersson et al. 2008). This approach has been applied to examine the human gut microbiota under several illness conditions as CDI , inflammatory bowel diseases, irritable bowel syndrome or Crohn's disease. The gut microbial differences associated to factors as age, diet, or antibiotic usage have been also addressed in this manner (Antharam et al. 2013, Claesson et al. 2011, Dethlefsen & Relman 2011, Durbán et al. 2012b, Filippo et al. 2010, Li et al. 2012, Rea et al. 2011, Walker et al. 2011, Willing et al. 2011).

Issues of 16S rRNA gene sequencing are the biases introduced by the nucleic acid isolation (different molecular methods) or PCR (number of cycles, gene region to amplify) that makes difficult to compare data from different studies (Youssef et al. 2009, Zoetendal et al. 2004). Other problem associated to PCR-based surveys is the underestimation of the low-proportion taxa of the microbial communities and that its detection depends on the sequencing effort (Gonzalez et al. 2012).

Despite these limitations, some advancements including a higher sequencing resolution at lower cost and the development of analytical tools for multiple related samples (temporal and spatial series) have contributed to maintain a central role for 16S rRNA in microbial ecology (Tringe & Hugenholtz 2008).

A group of techniques that have been successfully performed to monitor the dynamics of microbial communities are those based on fingerprinting of the 16S rRNA gene. The fingerprinting techniques are based on 16S rRNA gene PCR and their results represent profiles of sequence diversity of ecosystems, but the principles and procedures vary between them. For example, Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE) and Temporal Temperature Gradient Gel Electrophoresis (TTGE) are based on sequence specific melting behaviour of amplicons, Single Strand Conformation Polymorphism (SSCP) on the secondary structure of single strand DNA and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) on specific target sites for restriction enzymes (Zoetendal et al. 2004). DGGE, TGGE and TTGE have been the most widely applied to monitor and characterize the GIT microbial communities and have resulted in knowledge about factors that affect the microbiota such as perturbations, physiological states or differences between GIT locations (La Cochetière et al. 2008, Lepage et al. 2005, Zoetendal et al. 2001).

The disadvantages of these approaches are that they do not provide quantitative information and that they are not able to identify lower abundance bacterial groups. However, other methods also based on the 16S rRNA gene have been applied to obtain quantitative information such as the real-time PCR approach that is nowadays one of the most used to quantify the bacterial load of GIT on humans and animal studies (Buffie et al. 2012, Matsuki et al. 2004).

A different quantitative method to analyze environmental samples is the fluorescent *in situ* hybridization (FISH) using 16S rRNA targeted oligonucleotide probes (Amann et al. 1995). It combines the probe hybridization with microscopy or flow cytometry for detection and quantification of individual cells. The major disadvantage of FISH is that it depends on the availability of probes and that only a few probes can be used simultaneously per analysis (Zoetendal et al. 2004).

Other probe based method that has been used to study the microbial diversity of the gut is the DNA microarrays that can be hybridized with DNA or RNA. The DNA microarrays have been applied in several ecological studies in which not only SSU rRNA genes but also others genes, as antibiotics resistance genes, have been used as targets (Call et al. 2003, Peplies et al. 2003). The limitation of this method is also the bias introduced by the nucleic acids isolation and the subsequent PCR (Zoetendal et al. 2008).

The flow-cytometry is a powerful approach since it is possible to sort uncultured bacteria and study specific bacterial

groups (Wallner et al. 1997). It has been used to describe the diversity of active microbial fractions compared with the whole community from human faecal samples (Peris-Bondia et al. 2011).

Since growing bacteria have greater amounts of rRNA, targeting 16S rRNA rather than 16S rDNA allows to identify bacteria that are metabolically more active (Itoh et al. 2013, Kemp et al. 1993, Lee et al. 2008, Moeseneder et al. 2005, O'Sullivan 2000). This approach is based on retrotranscription of the total RNA to generate double-strand cDNA for sequencing. The differences in copy number of rRNA operons between bacteria is an issue even worse for ecological analysis at level of RNA respect to DNA, being the bacteria with low copy number, less represented than those with high copy number. However, bioinformatics methods have been developed to correct the copy number and make more accurate inferences about microbial diversity and abundance based on 16S sequence data (Kembel et al. 2012).

1.6.2.2. "OMICS" APPROACHES TO STUDY THE FUNCTIONS OF THE GUT MICROBIOTA

The SSU rRNA-based approaches are useful for describing the composition of microbial communities, but the results are limited only to describe the microbial diversity, since the potential functions of the microbes present cannot be extracted from these kinds of data. A recent computational approach called PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) is able to predict the functional composition of a sample using a marker gene as the 16S rRNA and a database of reference genomes (Langille et al. 2013). However, the most realistic way to gain insights into the functions and activities of microbes without cultivation is by applying metagenomics and other community-based approaches. Metagenomics, which studies all the genomes from an ecosystem, can be used to determine the phylogenetic, physical and functional properties of microbial communities (Handelsman 2004). The first golden standard was the use of metagenomic libraries by cloning DNA from metagenomes in fosmids, cosmids or bacterial artificial chromosomes (Lepage et al. 2013). The next generation sequencing technologies, mainly pyrosequencing, SOLiD or Illumina, have enabled to sequence the clones from metagenomic libraries, but also the direct sequencing of the total DNA content from an ecosystem, deeply, rapidly and in a cost-effective manner (Niedringhaus et al. 2011). Screening of gut metagenomic libraries has allowed to identified carbohydrate active enzymes that are of interest in human nutrition, bile salt hydrolases, or bacterial genes involved in colorectal carcinoma cell line proliferation (Gloux et al. 2007, Jones et al. 2008, Tasse et al. 2010).

Overall, metagenomic studies have shed light on the effect of different factors as host health, age, or diet on the functions of the gut microbial community (Arumugam et al. 2011, Gill et al. 2006, Kurokawa et al. 2007, Qin et al. 2010). For instance, the first human metagenomic study showed a significant enrichment of the gut microbiome of healthy adults in metabolic pathways related to metabolism of glycans, amino acids and xenobiotics (Gill et al. 2006). A different metagenomic study revealed an enrichment of carbohydrate transport and metabolism favouring milk intake on infants, while the adult gut microbiome was enriched in energy harvest from diet (Kurokawa et al. 2007).

The larger catalogue of microbial genes from the human gut was obtained by sequencing the total DNA from 33 faecal samples with Ilumina Genome Analyser technology (Qin et al. 2010). The catalogue highlighted functions important for bacterial survival in the intestine and for the homeostasis of the gut ecosystem, as well as the existence of a functional core in spite of the high inter-individual variability of the microbial composition. A latter study carried out by Arumugam and colleagues enabled to distinct "types" of gut compositon (named as enterotypes) that also differ in some metabolic capabilities (Arumugam et al. 2011). Metagenomic sequencing has also allowed to identify signatures that differentiate obesity, inflammatory bowel disease and diabetes from healthy status (Greenblum et al. 2012, Karlsson et al. 2013, Qin et al. 2010).

Although the importance of metagenomic contribution to the human microbiome characterization is out of doubt, it has, however, some important limitations. For example, the methods for total DNA extraction and storage that are crucial for the representativeness of the metagenomic DNA respect to the original microbial community is not standardized (Yu & Morrison 2004). Other problem is the high proportion of data that cannot be functionally assigned due to a lack of reference in databases (Qin et al. 2010, Walker et al. 2014). In spite of that metagenomics is a powerful tool for exploring the genes of a microbial ecosystem, it does not provide information about the actual activity or gene expression of the microorganisms that

are present. Other meta-"omics" approaches focus on messenger RNA (metatranscriptomics), proteins (metaproteomics) or metabolites (meta-metabolomics) (Figure 1.11) and provide information about the activity of microbial ecosystems (Lepage et al. 2013, Zoetendal et al. 2008).

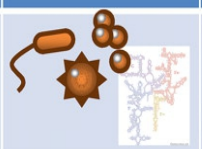
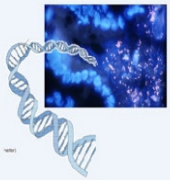
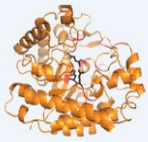
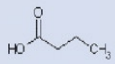
Meta-omics		Molecule	Knowledge	Limits	Clinical implications
Phylogeny		16S rDNA	▶Bacterial composition & diversity	▶ No information on bacterial functions ▶Except archaea	▶ Composition dysbiosis ▶Healthy or disease specific species
Metagenomics		Chromosomal genomic DNA	▶ High resolution Microbiota profiling ▶Genes contents from uncultivated microbes	▶ No information on microbial expressed functions	▶ Functional dysbiosis ▶Healthy or disease specific microbial genes ▶Toward diagnostics functional based studies
Metatranscriptomics		Messenger RNA/ cDNA	▶High resolution gene expression profiling ▶Differential microbial gene expression various physiological/ environmental conditions	▶ Poor stability of bacterial mRNA ▶Representativity unknown / Mutliple purification steps needed ▶No unique protocol	▶ Functional Dysbiosis ▶Microbial activity kinetics ▶Expressed genes at specific time and location ▶Specific monitoring of active bacteria
Metaproteomics		Proteins/ Peptides	▶High resolution protein mapping and profiling ▶Differential microbial proteins production under various physiological/ environmental conditions	▶ Many unknown proteins in databases ▶Heterogeneous stability ▶No unique protocol	▶Function confirmed: genome annotation improvement ▶Eucaryotes-procaryotes analogs identification ▶Biomarkers
Metabolomics		Metabolites	▶ Microbial and host Metabolic profiling ▶Easy to perform on very low amount of material feces/serum/urine	▶ Many unknown metabolites in databases ▶Strict Identification of compound tedious ▶No unique protocol ▶Combination of host and microbial molecules	▶New pathways confirmed or identified ▶High throughpout metabolomic screening of biomarkers ▶Easy translation to clinical setting

Figure 1.11. Integrated meta-omics. Different levels of analyses are represented from phylogeny to metabolomics (Adapted from Lepage et al. 2013).

Frias-Lopez *et al* were pioneers in applying metatranscriptomics to characterize the gene expression of microbial communities of ocean surface waters. They found that the most expressed genes were not the most represented on the metagenome, suggesting the combination of these two approaches to better address the dynamics of ecosystems (Frias-Lopez et al. 2008). Since them, metatranscriptomics has been applied to investigate the gut microbiota under different conditions (Booijink et al. 2010, Maurice et al. 2013, McNulty et al. 2011, Turnbaugh et al. 2010). Our group carried out one of the first metatranscriptomic studies of the human gut microbiota analyzing the total gene expression of 10 healthy individuals. The mRNA characterization revealed a uniform functional pattern, being carbohydrate metabolism, energy production and synthesis of cellular components the main roles (Gosalbes et al. 2011, 2012).

Some limitations of metatranscriptomics are the purification of high-quality total RNA and the enrichment in mRNA depleting the 16S rRNA and 23S rRNA, which are the most abundant types of RNA (Sorek & Cossart 2010, Stewart et al. 2010, Walker et al. 2014). Also, the short half-life of mRNAs makes difficult to detect short-term responses of the microbial community to environmental changes (Simon & Daniel 2011).

Other challenging approach to be combined with metagenomics is metaproteomics. As in the metatranscriptome, the human faecal metaproteome presents a skewed distribution relative to the metagenome, with more proteins for translation, energy production and carbohydrate metabolism (Verberkmoes et al. 2009). The combination of these two approaches allowed comparing the gut microbial community between an obese and a lean adolescent. The two gut communities maintained largely similar gene repertoires and functional profiles, but for example a higher abundance of proteins involved in B12 synthesis and butyrate production were observed in the obese gut, whereas bacteria from lean gut seem to be more engaged in vitamin B6 synthesis (Ferrer et al. 2013). A different follow-up study showed that the adult fecal metaproteome is subject specific and stable over time and also identified core functions as carbohydrate metabolism and transport (Kolmeder et al. 2012). An important limitation of this approach is the large gaps in databases that limit the functional annotation of the proteins (Walker et al. 2014).

Finally, meta-metabolomics is at present the most used of the meta-"omics" approaches to study the human microbiota also combinable with metagenomics (Turnbaugh et al. 2008). It has been used to investigate several digestive disorders as colorectal cancer, inflammatory bowel disease, irritable bowel syndrome, or obesity, as well as to address the consequences of antibiotic treatment on the metabolic status of the human gut microbiota (Ferrer 2013, Lepage et al. 2013).

The application of these innovative exploratory approaches and especially their integration could reveal new information about the gut ecosystem, its functioning and dynamics as well as its relation with the human health.

1.6.2.3. BIOINFORMATICS METHODS

The recently developed sequencing technologies as those commercialized by 454 Life Sciences/Roche Applied Sciences (454), Illumina Incorporated (Solexa) or Applied Biosciences (SOLiD) has increased the depth of sequencing by orders of magnitude compared to traditional Sanger sequencing (Mardis 2008, Robinson et al. 2010, Shendure & Ji 2008).

The studies based on these techniques have generated huge amount of data difficult to analyse, since it is time-consuming and usually required several gigabytes of memory to store and multiple processors to deal with the sequences. However, many programs, pipelines websites and databases have been developed or improved in the last years to store and analyse the generated data. In big databases as the National Center for Biotechnology Information (NCBI) or the European Bioinformatics Institute (EMBL-EBI), among others, the amount of information has significantly increased over these years. At the same time, various specific databases have been created, especially for the analysis of the 16S rRNA data. For instance, the Ribosomal Database Project (RDP), SILVA or greengenes are comprehensive rRNA gene databases used for the taxonomic affiliation of the 16S rRNA gene sequences (Cole et al. 2009, DeSantis et al. 2006, Pruesse et al. 2007). Moreover, databases containing functional information such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) or TIGRFAMs are continuously improved and updated (Haft et al. 2003, Kanehisa & Goto 2000).

Many of the programs and pipelines are able of process the information (sequences) and to perform most of the ecological and statistical analysis from the raw data. Some of the most currently used in microbial ecology are the software packages Mothur and Quantitative Insights Into Microbial Ecology (QIIME) that can process the 16S rRNA sequences (as trimming, denoising, aligning, etc...) and to perform ecological analysis (estimators of richness, diversity, similarity, etc...) (Caporaso et al. 2010, Robinson et al. 2010; Schloss et al. 2009).

Regarding the functional and taxonomic analysis of metagenomes or metatranscriptomes, other software as Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST), or the web server for metagenomic analysis (WebMGA) have also been developed (Meyer et al. 2008, Wu et al. 2011). Additionally, to graphically represent data and results, as well as to perform several types of ecological and statistical analysis, the use of The R Project for Statistical Computing (R)

has been standardized in recent years (mainly for statistics) (R Development Core Team 2011). Several packages of this software for statistical and ecological analysis (bioconductor, vegan, etc.), as well as for graphic data representation (lattice, ggplot2) are continually released (Gentleman et al. 2004, Oksanen et al. 2011, Sarkar 2008, Wickham 2009).

2

OBJECTIVES

2. OBJECTIVES

The human gut microbiota represents a very complex ecosystem involved in essential functions for the host physiology. Since the antibiotics are one of the strongest disturbing agents of its equilibrium, their uses exert a great impact on human health. The study of microbial communities has changed dramatically since the development of culture-independent approaches, in combination with high-throughput sequencing technologies. The emerging of "omics" technologies as metagenomics, metatranscriptomics, meta-metabolomics and metaproteomics have proven effective in characterizing the structure, activities, functions and interactions of the human gut microbiome.

The general aim of this thesis was to gain insight into the effect of antibiotics on the human gut microbiota and its implications in human health through the application of these ecological approaches. This has been achieved by three specific objectives:

The first objective was to study in-depth the evolution of the human faecal microbiota under antibiotic therapy through the combination of multiple "omics" approaches, since each of them provides information about a different biological level. The specific aim was to characterize the structure (diversity and microbial composition of the total and active fraction) and activity (genes, mRNAs, proteins and metabolites) of the microbial ecosystem in response to stress induced by beta-lactam antibiotics. This proof-of-concept study corresponds to the original article: "Gut microbiota disturbance during antibiotic therapy: a multi-omic approach" (Chapter 3.1).

The second objective was to study the effect of different classes of antibiotics in the human intestinal microbiota. Specifically, we wanted to test if differences in spectrum, mode of action and antimicrobial effect are reflected in the respective changes of the total and active microbiota and in the genetic potential of the gut ecosystem. We also wanted to characterize the evolution of resistance genes (resistome) during therapy and its relation with the type of antibiotics. The main results of this research are included in the original article "Differential effects of antibiotic therapy on the structure and function of human gut microbiota" (Chapter 3.2).

The third goal consisted in to better understand the development of CDI and its relation with an antibiotic-associated altered microbiota. First, we wanted to evaluate the structural and functional changes of the intestinal microbiota due to infection by the pathogen. Moreover, we were interested in identifying microorganisms and metabolic functions associated with infection and other bacteria and functions that might be involved in colonization resistance against *C. difficile*. This work is included in the original research article: "Structural and functional changes in the gut microbiota associated to *Clostridium difficile* infection" (Chapter 3.3).

3

PUBLICATIONS

3.1

"GUT MICROBIOTA DISTURBANCE DURING ANTIBIOTIC THERAPY: A MULTI-OMIC APPROACH"

AUTHORS: Ana Elena Pérez Cobas, María Jose Gosalbes Soler, Anette Friedrichs, Henrik Knecht, Alejandro Artacho, Kathleen Eismann, Wolfgang Otto, David Rojo, Rafael Bargiela, Martin von Bergen, Sven Neulinger, Carolin Däumer, Femke-Anouska Heinsen, Amparo Latorre, Coral Barbas, Jana Seifert, Vitor Martins dos Santos, Stephan Ott, Manuel Ferrer, Andrés Moya.

Published in Gut, 62(11): 1591-1601. November, 2013

RELATED PUBLICATIONS OF THE AUTHOR

Metatranscriptomic approach to analyze the functional human gut microbiota. PLoS One. 6(3):e17447.

Metagenomics of human microbiome: beyond 16s rDNA. Clinical Microbiology and Infection. 18(s4):47–49

Functional consequences of microbial shi″s in the human gastrointestinal tract linked to antibiotic treatment and obesity. Gut Microbes. 4(4):306–15

OPEN ACCESS

ORIGINAL ARTICLE

Gut microbiota disturbance during antibiotic therapy: a multi-omic approach

Ana Elena Pérez-Cobas,[1,2] María José Gosalbes,[1,2] Anette Friedrichs,[3,4] Henrik Knecht,[3] Alejandro Artacho,[1] Kathleen Eismann,[5] Wolfgang Otto,[5] David Rojo,[6] Rafael Bargiela,[7] Martin von Bergen,[5,8,9] Sven C Neulinger,[10] Carolin Däumer,[11] Femke-Anouska Heinsen,[3] Amparo Latorre,[1,2] Coral Barbas,[6] Jana Seifert,[5] Vitor Martins dos Santos,[12,13] Stephan J Ott,[3,4] Manuel Ferrer,[7] Andrés Moya[1,2]

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For numbered affiliations see end of article.

Correspondence to
Professor Andrés Moya, Cavanilles Institut on Biodiversity and Evolutionary Biology, University of València, José Beltrán 2, 46980 Paterna, Valencia, Spain; andres.moya@uv.es

Dr. Manuel Ferrer, CSIC-Institute of Catalysis, Marie Curie 2, 28049 Madrid, Spain; mferrer@icp.csic.es

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ABSTRACT

Objective Antibiotic (AB) usage strongly affects microbial intestinal metabolism and thereby impacts human health. Understanding this process and the underlying mechanisms remains a major research goal. Accordingly, we conducted the first comparative omic investigation of gut microbial communities in faecal samples taken at multiple time points from an individual subjected to β-lactam therapy.

Methods The total (16S rDNA) and active (16S rRNA) microbiota, metagenome, metatranscriptome (mRNAs), metametabolome (high-performance liquid chromatography coupled to electrospray ionisation and quadrupole time-of-flight mass spectrometry) and metaproteome (ultra high performing liquid chromatography coupled to an Orbitrap MS^2 instrument [UPLC-LTQ Orbitrap-MS/MS]) of a patient undergoing AB therapy for 14 days were evaluated.

Results Apparently oscillatory population dynamics were observed, with an early reduction in Gram-negative organisms (day 6) and an overall collapse in diversity and possible further colonisation by 'presumptive' naturally resistant bacteria (day 11), followed by the re-growth of Gram-positive species (day 14). During this process, the maximum imbalance in the active microbial fraction occurred later (day 14) than the greatest change in the total microbial fraction, which reached a minimum biodiversity and richness on day 11; additionally, major metabolic changes occurred at day 6. Gut bacteria respond to ABs early by activating systems to avoid the antimicrobial effects of the drugs, while 'presumptively' attenuating their overall energetic metabolic status and the capacity to transport and metabolise bile acid, cholesterol, hormones and vitamins; host–microbial interactions significantly improved after treatment cessation.

Conclusions This proof-of-concept study provides an extensive description of gut microbiota responses to follow-up β-lactam therapy. The results demonstrate that ABs targeting specific pathogenic infections and diseases may alter gut microbial ecology and interactions with host metabolism at a much higher level than previously assumed.

Significance of this study

What is already known on this subject?
- Changes in gut microbiota have been shown to be associated with antibiotic (AB) usage.
- Broad-spectrum ABs result in a significant reduction in Bacteroidetes and a concurrent increase in Firmicutes.
- The understanding of the dynamics and mechanisms underlying functional changes in the microbiome in response to AB treatments remains limited.

What are the new findings?
- AB treatment provoked apparently oscillatory population dynamics with major changes at the level of gut microbiota metabolism and total and active microbial fraction compositions at days 6, 11 and 14, respectively, after the initiation of the therapy.
- ABs have ecological implications related to the energy metabolism of colonic bacteria, which partially improved at day 6 to cope with an intermittent nutrient supply and AB stress but decreased at later stages and after treatment cessation.
- Although no abundant bacteria can be very active at a given moment in the presence of ABs, minor community members play a significant active role in overall gut metabolism and host interactions.

How might it impact on clinical practice in the foreseeable future?
- The treatment of patients with ABs targeting specific pathogenic infections and diseases might influence the global metabolic status of gut microbes in such a way that could affect human biology. Integrative omics approaches represent a promising strategy for preventing metabolic diseases associated with AB uptake during therapeutic and clinical interventions.

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INTRODUCTION

The human colon harbours a vast ensemble of microbes that carry out vital processes for human physiology and nutrition, and the microbes in this complex ecosystem are defined as the microbiota. Accordingly, the human colon can be viewed as an

anaerobic bioreactor in which trillions of microorganisms add a vast catalogue of genes to the genetic resources of the host to provide complementary metabolic pathways for energy harvest, food digestion, detoxification, the production of bioactive compounds and the assimilation of otherwise inaccessible dietary nutrients.[1–5] Recent studies have shown that each individual harbours a unique and relatively stable gut microbiota, generally dominated (over 90%) by the Bacteroidetes and Firmicutes, in addition to minorities of Actinobacteria, Proteobacteria and Verrucomicrobia, the majority of which have not yet been isolated or characterised.[6] However, this well engaged bacterial machinery can be disrupted by external factors such as antibiotic (AB) treatment.[7–9] AB therapy has been successfully used for many years to treat bacterial infections, but the emergence of AB-resistant bacteria has caused enormous public health problems. Moreover, resistant species can persist in the human gut for years.[7 10–12]

AB therapies affect not only the target microorganism but also the host-associated microbial communities, particularly those in the intestine. In recent years, the effects of distinct ABs on the gut microbiota have been evaluated, primarily by 16S rRNA analysis.[7 10 11 13–16] The patterns of microbial diversity and gene-encoded functions are highly intricate,[11] and contradictory reports on the compositions of microbial communities have complicated the identification of functional and molecular hotspots associated with AB therapy in humans. Recent experiments have revealed that treatment with broad-spectrum ABs affects the microbiota composition, resulting in a significant reduction in Bacteroidetes and a concurrent increase in Firmicutes.[13] Moreover, different studies have reported bacterial resilience following AB treatment.[11 14 15 17]

The understanding of the dynamics and mechanisms underlying functional changes in the microbiome in response to AB treatments remains limited, primarily because most research to date has relied on indirect evidence from DNA-based approaches that fail to provide information on actual gene expression, protein synthesis and metabolite composition and variation. Moreover, no integrated metatranscriptomic, metaproteomic or metametabolomic surveys on AB-treated human microbiota have been performed. Specifically, in the gut ecosystem, few studies have focused on metatranscriptomics. Turnbaugh *et al*[18] focused on gene expression analysis in faecal samples from a monozygotic twin pair, whereas Booijnk *et al*[19] and Gosalbes *et al*[20] studied the faecal metatranscriptomes of healthy volunteers using cDNA amplified fragment length polymorphism and pyrosequencing, respectively. Proteomic platforms have also been used to separate and identify thousands of proteins in faecal samples from healthy individuals,[21–24] but these analyses have not uncovered the effect of ABs on the stability or expression of core proteins or their functions. Finally, metabolic changes in mouse—but not human—gut microbiota following treatment with the broad-spectrum AB enrofloxacin were recently evaluated using nuclear magnetic resonance based metabolomics.[25] However, this study yielded limited information because the faecal samples were only analysed for changes in eight metabolites, including increased levels of amino acids and urea caused by the loss of microbial proteases and ureases, and reduced levels of acetate, butyrate and propionate generated by lactate-using bacteria.

In this study, we provide the first report of AB-treatment related changes in the faecal microbiota, including the total microbiota, active microbiota, metagenome, metatranscriptome, metametabolome and metaproteome. This multi-omics approach yielded a global picture of the microbial community structure and the metabolic status of the gut ecosystem, which is paramount to understanding the total effect of a given AB and to establishing correlations with host physiology. This study of one patient constitutes a proof of concept for this approach.

MATERIALS AND METHODS

Fresh faecal samples were collected from a patient who had not taken ABs within the previous 3 months. The patient (a 68-year-old man) was admitted to the Department for Internal Medicine at the University Hospital Kiel (Germany) due to an infected cardiac pacemaker. A clinical examination identified inflamed skin and subcutaneous tissue around the pacemaker, and laboratory findings revealed an elevated C-reactive protein level (CRP 19.7 mg/litre; normal value <8 mg/litre) and a full blood count within normal ranges. The patient did not present any intestinal disorders. The pacemaker had been placed to treat the patient's sick sinus syndrome, first diagnosed in 1994. His cardiovascular risk factors were arterial hypertension and non-insulin-dependent diabetes mellitus. The patient was regularly taking amlodipine, ramipril, hydrochlorothiazide and glimepiride on a daily basis and Marcumar according to his international normalised ratio values. AB therapy was initiated with a combined intravenous therapy of ampicillin/sulbactam and cefazolin on the day of admission as a single dose and continued with intravenous cefazolin alone for the next 14 days. The patient's CRP level returned to normal within 1 week after the beginning of AB therapy. Faecal samples were collected on the day of admission, prior to AB treatment (day 0, FS-0), on days 3, 6, 11 and 14 of AB treatment (FS-3, FS-6, FS-11 and FS-14, respectively) and 40 days after AB therapy (FS-40). Fresh faeces were collected, frozen immediately and stored at −80°C until further processing. Informed consent was obtained from the patient, and the study was approved by the Ethical Board of the Medical Faculty of the Christian-Albrecht-University, Kiel, Germany. The patient provided written informed consent. Full descriptions of the materials and methods used for the following are available in the Materials and Methods in the online supplement: nucleic acid and RNA extraction; 16S rDNA and 16S rRNA sequencing; metagenome sequencing; mRNA purification, amplification and sequencing; metagenomic and metatranscriptomic analysis; protein extraction, separation and identification and data processing; and metabolite extraction, separation and identification and data processing. All sequences have been entered in the European Bioinformatics Institute database, under accession number ERP001506.

RESULTS

Total and active faecal microbiota

We used 16S rDNA and 16S rRNA analyses to characterise the total bacteria (16S rDNA) and metabolically active bacteria (16S rRNA), respectively, in each faecal sample.[26–28] As shown in figure 1, there were large fluctuations in the relative abundances of the various bacterial taxa in the total and active microbiota throughout the follow-up study. In the first days of treatment, the majority of the total microbiota comprised species from the phylum Firmicutes, with the exception of FS-11 (11 days after AB treatment), which exhibited a remarkable shift towards Bacteroidetes (*Parabacteroides* and *Bacteroides* genera) and a significant increase in Betaproteobacteria (figure 1 and figure 1 in online supplement). The Lachnospiraceae and Ruminococcaceae families constituted the most abundant taxa on days 3 and 6 of AB treatment. Meanwhile, the Firmicutes represented the most abundant active phylum in most samples. However, the shift

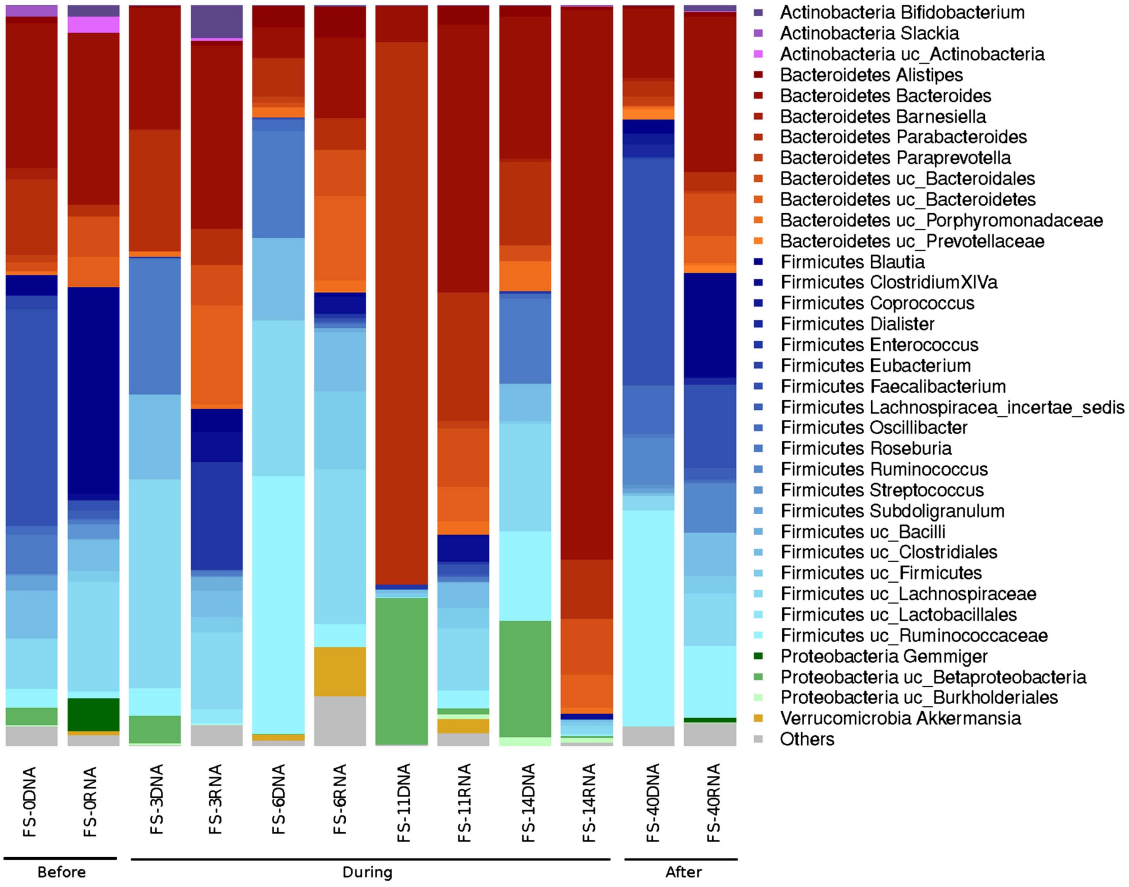

Figure 1 Total and active bacterial composition based on 16S rDNA and 16S rRNA analyses, respectively, in the follow-up study. Samples FS-0, FS-3, FS-6, FS-11 and FS-14 correspond to the materials collected on days 0, 3, 6, 11 and 14 of antibiotic (AB) treatment, respectively. The FS-40 sample corresponds to the materials collected 40 days after cessation of the AB treatment.

towards the Bacteroidetes was even more distinct and occurred later, at the 14th day (FS-14) of AB treatment, among the active bacteria, with the *Parabacteroides* genus (74%) being the predominant taxa.

As shown in figure 1, there was a general tendency towards the restoration of the original (day 0) untreated total and active bacterial composition at 40 days after cessation of the AB treatment. However, certain taxa, such as Actinobacteria (*Slackia* and *Bifidobacterium* genera), Betaproteobacteria (*Gemmiger* genus), Streptococcaceae (*Streptococcus* genus), Lachnospiraceae (*Roseburia* genus), Porphyromonadaceae (*Barnesiella* genus) and Clostridiales (*Eubacterium* and *Subdoligranulum* genera) were lost. The heat map and clustering analysis (see figure 1 in online supplement) results support the restoration of the microbiota because untreated samples clustered together regardless of whether we considered the total bacterial composition or the active bacterial composition. The results of a correspondence analysis of the relative abundance of each taxon in the total and active microbiota fractions are shown in figure 2 in the online supplement. Approximately 55.90% of the total variation can be bundled in two axes (CA1 and CA2), each of which contributed a similar degree of variation. CA1, bundling 23.81% of the variation, indicates how divergent a given sample is from the average abundance of taxa, whereas CA2, bundling 22.09% of the variation, clearly differentiates the composition of the total (DNA) and active (RNA) microbiota. Moreover, the active microbiota clearly behaved in a more homogeneous manner than the total microbiota, and samples FS-40 and FS-11 exhibited the most divergent microbiota among the samples analysed.

As shown using the Chao1 richness estimator (table 1), the diversity of the total bacterial community decreased during AB treatment and reached a minimum value after 11 days of AB treatment (FS-11). At this time point, the microbiota also exhibited a lower Shannon index value, indicating that there were fewer and more heterogeneously distributed bacterial families in the FS-11 sample compared with the other samples (figures 1, and figures 1 and 2 in online supplement). Interestingly, the biodiversity evenness and richness of the active bacteria remained essentially uniform until the 14th day of treatment (FS-14), at which time there was a marked decrease in bacterial taxa and richness.

Table 1 Biodiversity measures of total and active microbiota

Genetic material	Sample	N	Shannon	Chao1	SD
DNA	FS-0	41	3.5	46.9	5.4
	FS-3	17	2.7	21.8	5.6
	FS-6	21	2.8	23.0	3.8
	FS-11	13	1.2	14.5	0.3
	FS-14	21	3.2	21.5	1.4
	FS-40	38	3.1	43.9	6.8
RNA	FS-0	30	3.3	38.3	9.5
	FS-3	31	3.6	38.4	7.6
	FS-6	39	3,9	50.7	1.9
	FS-11	32	3,3	45.1	12.0
	FS-14	18	1.5	26.1	9.0
	FS-40	35	3.7	47.8	13.3

The number of observed taxa (N), the biodiversity index value (Shannon) and the richness estimator (Chao1) are shown, with the SD.

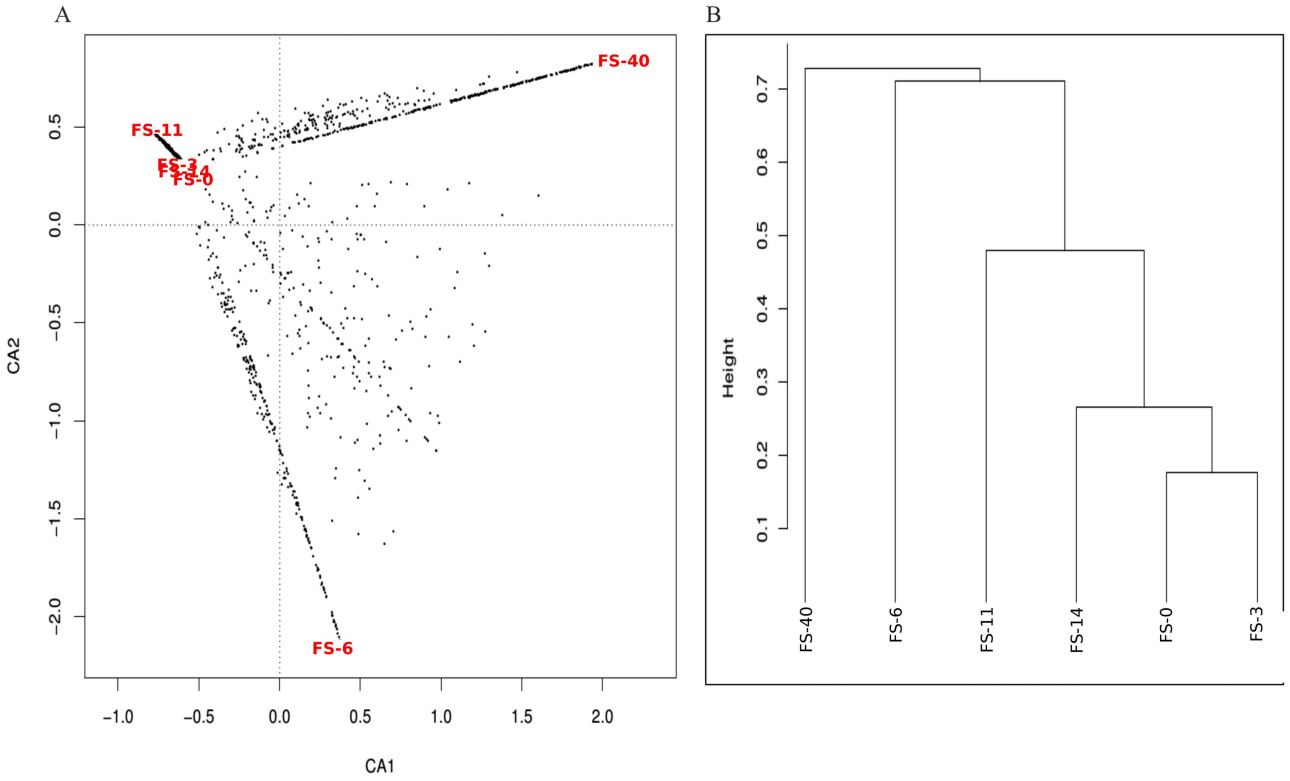

Figure 2 (A) Correspondence analysis of the expressed genes in each sample. (B) Clustering of the samples based on the type and abundance of expressed genes, applying the Bray–Curtis distance.

At the species level, the principal component analysis (PCA) showed that the total bacterial community (16S rDNA) profiles of samples FS-0 and FS-40 differed from those of the other samples (see figure 3 in online supplement). Together, the two axes projected 69% of the total variance in the data. Fourteen operational taxonomic units (OTUs) responsible for the differences between the samples were identified by the lengths of their vectors in the PLS-DA (see figure 4 in online supplement). Before and after AB treatment, OTUs with homology to *Faecalibacterium prausnitzii* and *Blautia wexlerae*, belonging to the phylum of Firmicutes, were highly abundant. However, *Enterococcus durans*, an abundant OTU in FS-3, FS-6 and FS-11, was absent in the FS-0 and FS-40 samples. Furthermore, we observed an increase in OTUs with homology to different species of *Bacteroides* and *Parabacteroides* such as *B fragilis*, *P merdae*, *B dorei*, *P distasonis, B uniformis* and *B ovatus* in the FS-11 and FS-14 samples. Thus, during AB therapy, the relative abundance of Bacteroidetes increased, whereas OTUs with homology to Firmicutes regained their dominance after cessation of the AB.

Abundance and diversity of gene transcripts by metatranscriptome analysis

To identify the major regulated pathways and processes under AB pressure, we evaluated the microbiota-regulated genes (mRNAs) (see table 1 in online supplement).[29 30] Figure 2A shows the correspondence analysis for the expressed genes in each sample, independent of the gene functions. The two axes, CA1 and CA2, bundle 39.91% and 33.12% of the total observed variation, respectively, which represents a substantial percentage (73.03%) of the total variation. The mRNA transcript contents of samples FS-6 and FS-40 were clearly distinct (figure 2B). Moreover, at the expression level, a drastic shift occurred on the 6th day of AB therapy, and the initial profile was recovered on the 14th day.

Using a self-organising map package,[31] the gene expression profiles were analysed and yielded six groups (figure 5 in online supplement). The genes included in clusters 0 and 1 exhibited increased expression only on the 3rd day following AB treatment and returned to basal levels at later stages of the treatment. These genes were functionally categorised to have roles in protein transport and binding (potassium uptake protein, TonB-dependent receptor, nitrile hydratase propeptide microcin bacteriocin system ATP-binding cassette transporter, peptidase/ATP-binding protein), toxin production and resistance (resistance-nodulation-cell division superfamily and hydrophobe/amphiphile efflux-1 transporters), detoxification (heavy metal efflux and CzcA permeases), mobile and extrachromosomal elements (phage lambda tail tape measure protein) and protein fate determination (TolC type I secretion outer membrane protein). Genes belonging to clusters 2, 3 and 4 (DNA metabolism/DNA replication, recombination and repair, protein fate/protein and peptide secretion and trafficking and protein synthesis/tRNA and rRNA base modification) presented a more uniform profile before, during and after AB treatment. The genes in cluster 5 remained constant but exhibited a relatively higher expression level on the 14th day following the beginning of AB treatment, returning to the basal level after the discontinuation of AB treatment; these genes functioned in molecule renewal and transport (proteins, peptides, amino acids, polysaccharides, purines, purine ribonucleotides, pyrimidines and glycopeptides (bio)synthesis, secretion, trafficking, transport, binding and/or degradation) and DNA metabolism/DNA replication, recombination, and repair and cellular processes related to sporulation and germination.

As illustrated in figure 3, the taxonomic assignment of expressed genes showed that the compositions of the FS-6 and

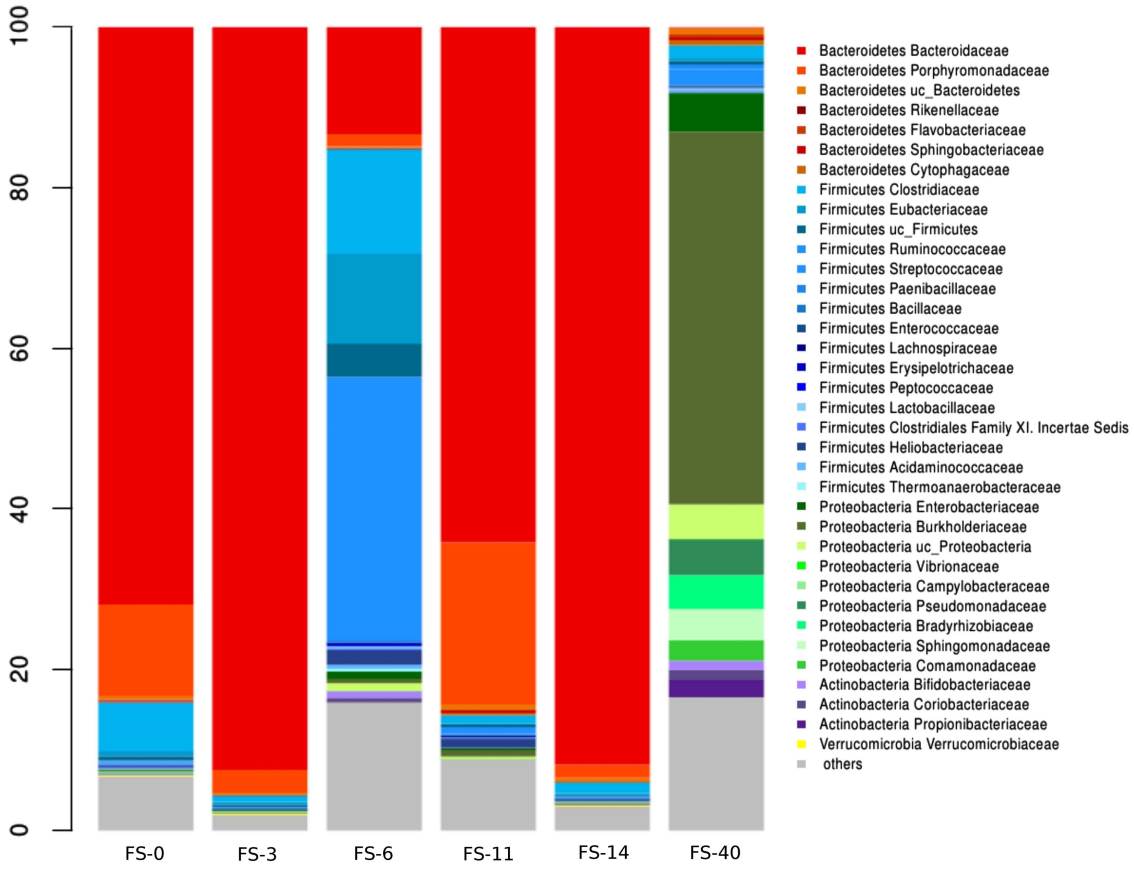

Figure 3 Taxonomic assignments of mRNAs for each sample according to the lowest common ancestor algorithm.

FS-40 samples were clearly different. The FS-6 sample contained primarily Streptococcaceae (27%), Clostridiaceae (13%) and Bacteroidaceae (13%). In the FS-40 sample, Burkholderiaceae (46%) (phylum Proteobacteria) were the most abundant, despite the low abundance of 16S rRNA from these organisms (figure 1). However, in the other samples (FS-0, FS-3, FS-11 and FS-14), the Bacteroidaceae family was the major taxon responsible for the observed gene expression. This family contains genera, such as *Parabacteroides* and *Bacteroides*, that are resistant to ampicillin and cephalosporins, as previously reported.[32] These results account for the three clusters shown in figure 2B.

We statistically evaluated the changes in gene expression following treatment according to various functional categories by applying a regression analysis in the ShotgunFunctionalizeR package.[33] The categories that demonstrated significant changes in expression during treatment are shown in figure 6 in the online supplement. Samples FS-6 and FS-40 exhibited major differences, whereas the rest of the samples were more closely related. This observation was further supported by the correspondence analyses (see figure 7 in online supplement), in which the first two axes, CA1 (56.72%) and CA2 (34.13%), accounted for 90.85% of the total variation.

Abundance and diversity of metabolites by metametabolome analysis

The metabolite contents (type and abundance) of samples FS-6 and FS-40 were clearly different (figure 4), consistent with the correspondence analysis of the observed gene expression (figure 2). Of the 382 different features identified (29 being common), the FS-40 sample revealed the greatest number (280) of associated mass features, followed by the FS-6 sample, with 234 features (see figures 8 and 9 in online supplement); FS-14 (185), FS-11 (121) and FS-0 (139) showed fewer features. According to the probability of match with the isotopic pattern shown in table 2 in the online supplement, 49 distinct common features were tentatively identified. Based on their abundance level, five groups of molecules were established (see figure 12 in online supplement). Most long-chain fatty acids and peptides exhibited increased abundance on the 6th day (sample FS-6) as well as after the discontinuation of AB treatment (FS-40); they included two masses corresponding to putative sphingolipid-related compounds, such as C_{17} sphinganine and dihydroceramide C_2, six unsaturated fatty acyls, two fatty acid amides, a lysophosphatidic acid and a tri-peptide formed by Asp/Ile/Phe, Asp/Leu/Phe or Phe/Glu/Val. Most of the putative glycerol(lyso)phospholipids (including five putative glycerophospholipids and fatty acid carnitines) exhibited increased abundance on the 11th and 14th days, but significantly decreased (from 4 to 20 000 fold) after the discontinuation of AB treatment. Finally, nine putative human-associated derivatives of cholesterol, the cholesterol-precursor vitamin D, bile acids, prostaglandins and sterol lipids appeared only after AB treatment (FS-40), with production levels of up to six orders of magnitude higher than that of samples before and during AB therapy.

Abundance and diversity of proteins by metaproteomic analysis

A total of 3011 proteins (1359 common) were unambiguously quantified (see table 3 and figure 10 in online supplement). Considering a threshold of at least 1.5 and −1.5 $\log_2$ ratios of abundance levels, we observed that AB treatment reduced the number of highly abundant proteins compared with the control

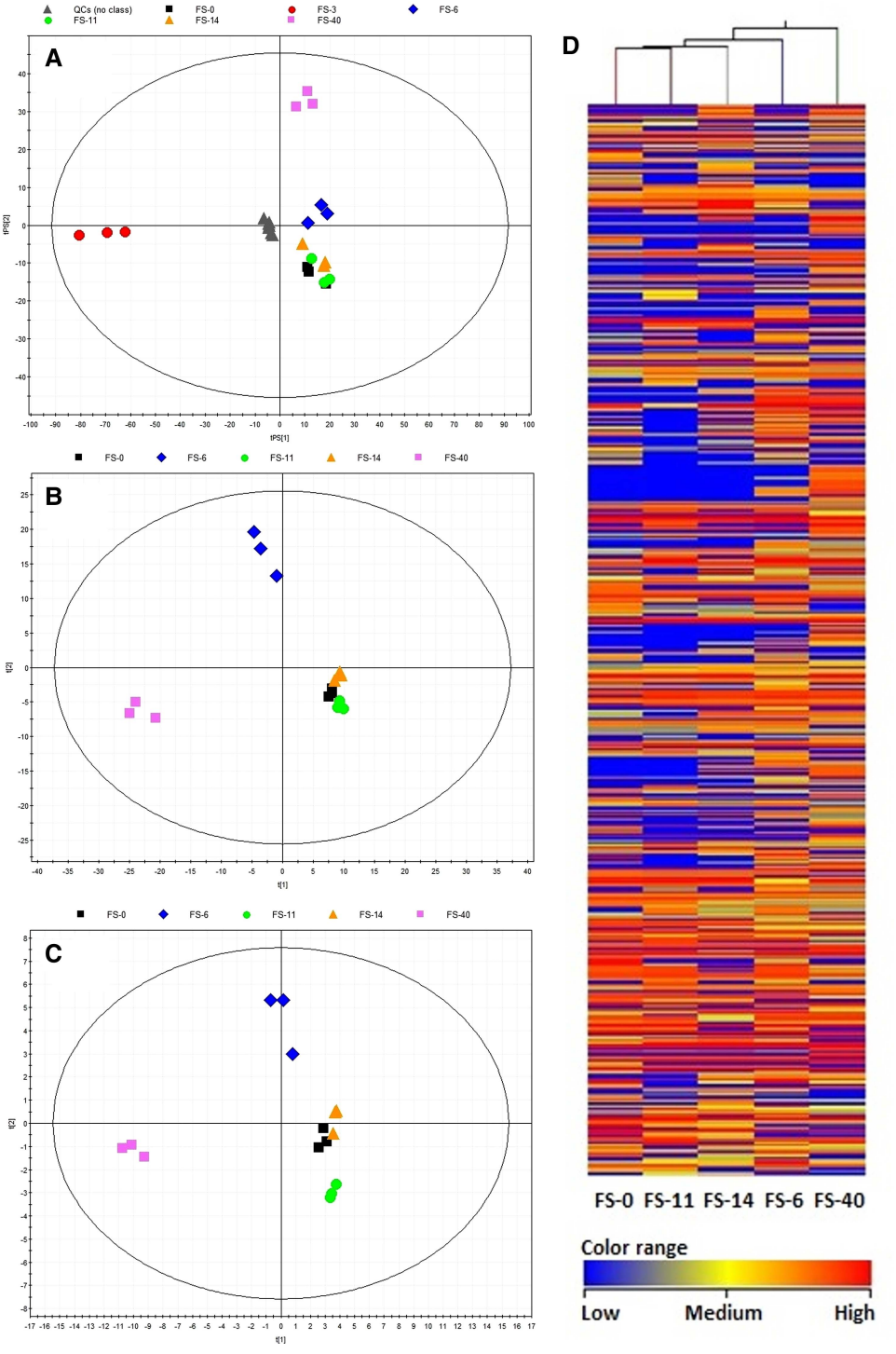

Figure 4 Partial least-squares discriminant analysis score plots and clustering analysis of metabolite profiles after different comparisons. (A) The whole dataset (8600 features) with the prediction for quality control (QC) samples, seven components, R^2=0.989, Q^2=0.670; the robustness of the analytical procedure was demonstrated by the tight clustering of the QC samples. (B) Discriminant variables identified by comparing samples in a pairwise fashion (382 discriminant features from 988 of the 4349 initial variables that were present in all three replicates of samples from any group), four components, R^2=0.978, Q^2=0.928. (C) Statistically significant variables identified in the METLIN database (49 features), four components, R^2=0.968, Q^2=0.915. (D) The effect of antibiotics on the human gut microbiota, as determined by a two-way hierarchical clustering analysis of the metabolite profiles. Hierarchical clustering was performed with a matrix of the total masses that passed the filtering and statistical treatments for each sample. Less abundant masses in a given community are shown in blue, whereas more abundant masses are shown in red. Note: sample FS-3 was discarded from the analysis due to the presence of faecal material in the cell extracts.

sample (FS-0) over time but promoted the number of low-abundance proteins (figure 5), with the FS-40 sample containing the lowest number of high-abundance proteins and the highest number of low-abundance proteins. Samples FS-11 and FS-40 exhibited clearly different protein expression profiles based on the correspondence (figure 6A) and clustering (figure 6B) analysis of differentially expressed proteins (figure 6A), which was corroborated by the corresponding functional analysis (see figure 11 in online supplement). These results suggest the restoration of the microbiota because untreated samples (FS-0 and FS-40) clustered together, indicating that the initial profile was recovered at the end of the treatment despite the drastic shift that occurred on the 11th day (see table 1 in online supplement), consistent with the analysis of the total and active bacterial compositions (figure 1).

According to the lower and over-representation of functional gene categories (clusters of orthologous groups; COGs), we found a rather stable distribution between the samples, with significantly different contributions from only 29 out of 494 COGs. As shown in figure 7, we observed specific differences that clearly indicated drastic shifts on day 6 of AB therapy (FS-6) and after treatment cessation (FS-40) of proteins assigned to 23 distinct COGs within the functional categories of glycolysis, pyruvate and glutamate metabolism, iron uptake and translation

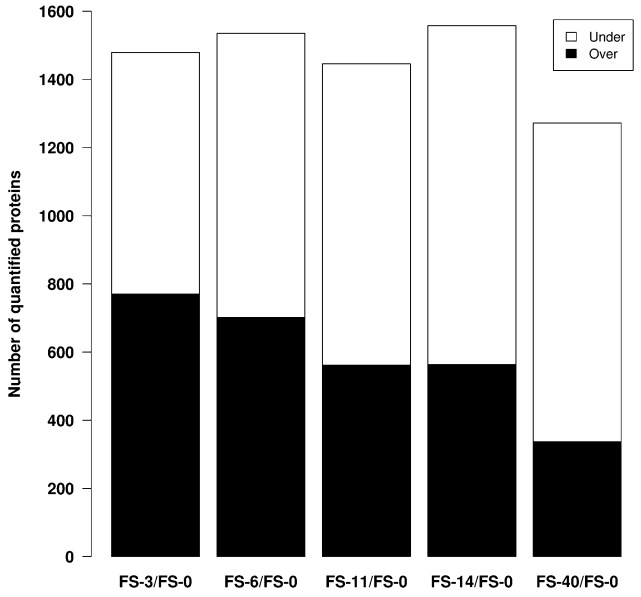

Figure 5 Number of quantified proteins showing either high (black bars) or low (white bars) abundance levels relative to the proteins identified in sample FS-0. Only proteins with values ≥1.5 or ≤-1.5 $\log_2$ ratios were considered.

(figure 7A–C); a transient increase in expression at day 6 was followed by depletion during and after the follow-up period. AB-treated samples were also characterised by a striking depletion of the translation elongation factors required for protein synthesis, such as GTPases, which facilitate the release of nascent polypeptide chains; this depletion was accentuated after treatment cessation (figure 7B). By contrast, samples taken during AB treatment were characterised by an enrichment of antimicrobial peptide transporters and multidrug efflux pumps that peaked on the 3rd day of AB treatment (figure 7D) and were absent in untreated samples (FS-0 and FS-40). Additional key proteins, such as aerobic CobN cobaltochelatases (COG1429) essential for the biosynthesis of vitamin B_{12}, were also found to be depleted (see table 3 in the online supplement) in samples FS-3, FS-11 and FS-14 compared with untreated samples.

DISCUSSION

The effects of ABs and the mechanisms underlying the connection between AB treatment and microbial gut metabolism require clarification, which can only be achieved through an integrated approach that goes well beyond the 16S DNA analysis that has been the cornerstone of previous research in this area. Thus, the aims of this study were as follows: to provide a proof of concept for an integrated workflow to assess the nature of such changes in the intestines of patients undergoing AB treatment at the structural and functional levels; and to evaluate whether there is a relationship between these types of changes. For instance, changes in the total composition at a given time point may also be associated with changes in the AB-resistant bacteria, although not necessarily at the same moment or during the same interval. Two major active factors that modulate changes in the microbiota should be considered. First, it is important to understand how AB treatment determines the emergence of bacterial species that are resistant to β-lactams (such as ampicillin and cephalosporins) and how the microbiota recovers once ABs have been removed. Second, the speed at which such changes are observed according to the different levels under consideration should be carefully evaluated.

In our study, we demonstrated that the greatest change in the active microbial fraction occurred later (day 14) than that in the total microbial fraction, which reached a minimum biodiversity and richness on the 11th day of AB treatment (figure 1). Further, oscillatory population dynamics were observed (at both the DNA and RNA levels). An early reduction in Gram-negative bacteria at day 6 and an overall collapse in diversity was followed by possible colonisation of the upper gut by naturally resistant Bacteroidetes by day 11, a consequent increase in the colonisation of the lower gut with dominance at 11–14 days and eventual re-growth of the Gram-positive bacteria at day 14. Various studies based on 16S rDNA analysis revealed important variability in the recovery of the baseline bacterial composition after AB therapy depending on the individual and the AB used (type and dose).[11 14 15] The large fluctuations in the relative abundances of the various bacterial taxa for the total and active microbiota throughout the follow-up study were most likely associated with an additive effect of ampicillin/sulbactam and

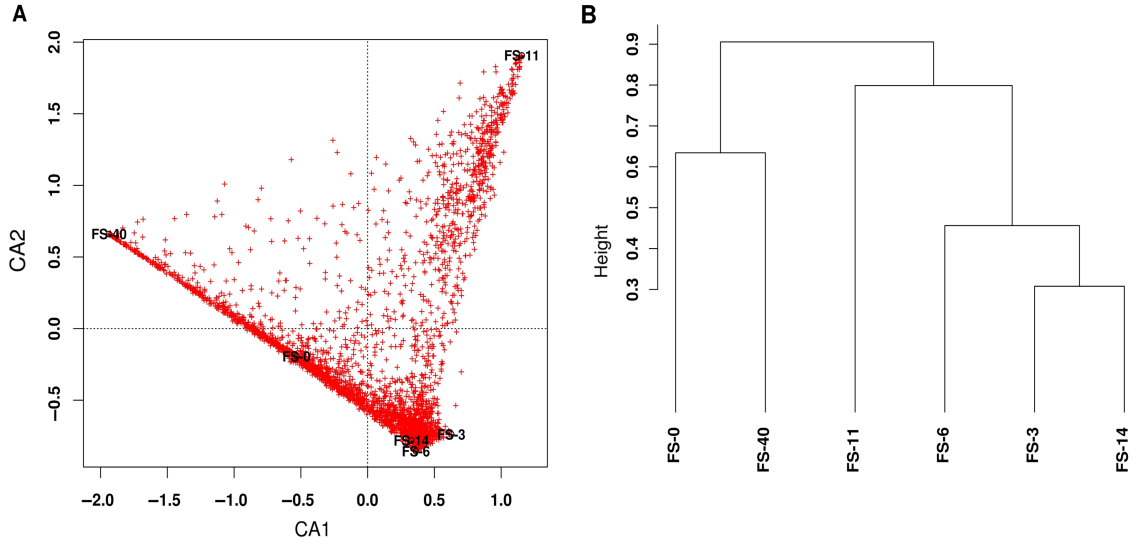

Figure 6 (A) Correspondence analysis of the expressed proteins in each sample. (B) Clustering of the samples based on the type and abundance of expressed proteins, with Pearson's correlation applied to calculate the distances. The two axes, CA1 and CA2, in (A) bundle 33% and 28% of the total observed variation, respectively.

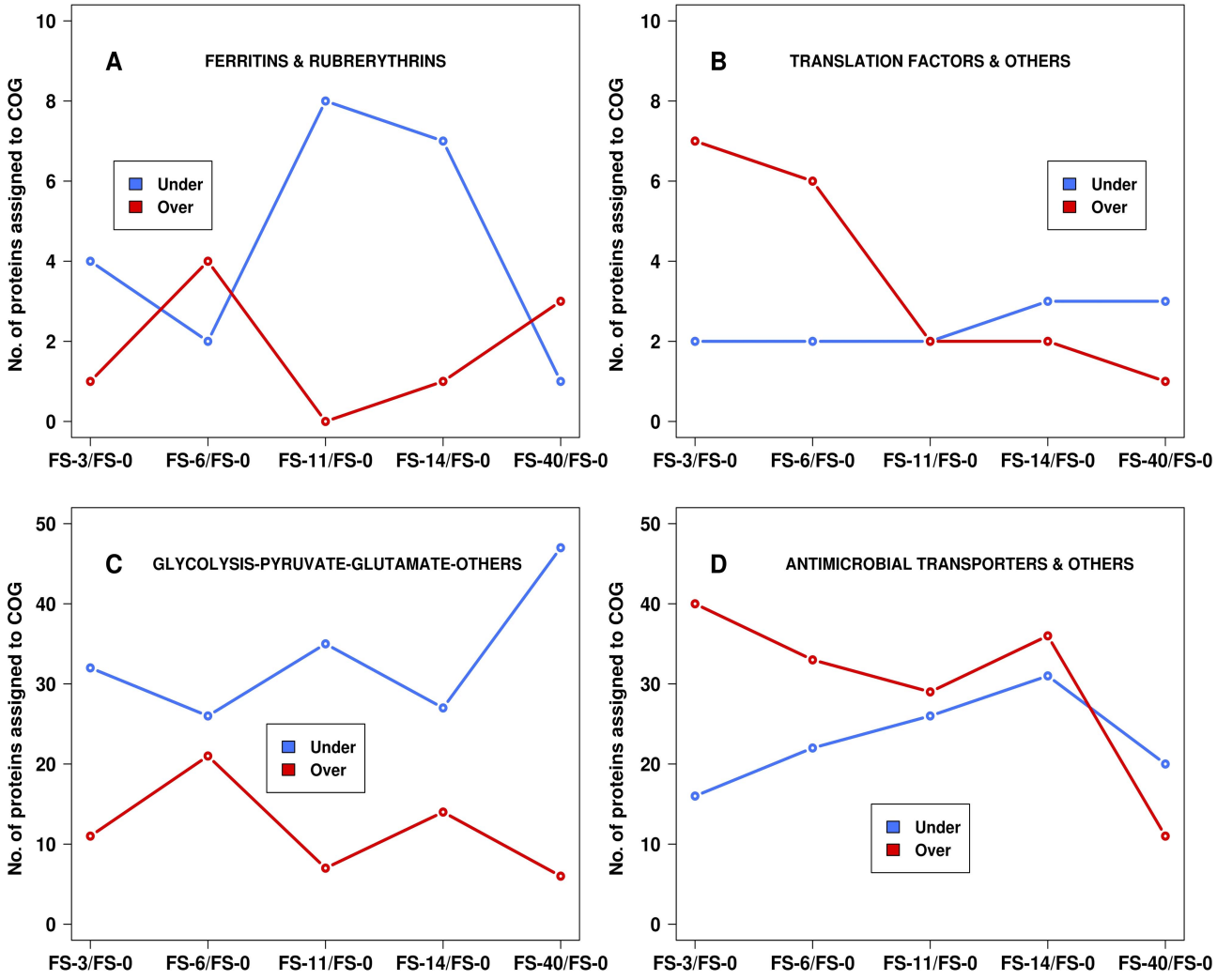

Figure 7 Graphical representation of the high-abundance and low-abundance proteins in different pathways, according to the clusters of orthologous group (COG) number assigned to each protein. (A) Rubrerythrin/ferritin COGs: COG1592 (rubrerythrin) and COG1528 (ferritin-like protein). (B) Translation factor and translation enzyme COGs: COG0193 (peptidyl-tRNA hydrolase), COG0264 (translation elongation factor Ts), COG0050, COG0532 (GTPases—translation elongation factors), COG0216 (protein chain release factor A), COG0480 (translation elongation factors (GTPases)) and COG0231 (translation elongation factor P/translation initiation factor 5A). (C) Glycolysis, pyruvate, glutamate and other related COGs: COG0126 (3-phosphoglycerate kinase), COG0205 (6-phosphofructokinase), COG0148 (enolase), COG0076 (glutamate decarboxylase and related proteolipid protein-dependent proteins), COG1830 (DhnA-type fructose-1,6-bisphosphate aldolase and related enzymes), COG0334 (glutamate dehydrogenase/leucine dehydrogenase), COG1053 (succinate dehydrogenase/fumarate reductase, flavoprotein subunit), COG0588 (phosphoglycerate mutase 1), COG0479 (succinate dehydrogenase/fumarate reductase, Fe-S protein subunit), COG0191 (fructose/tagatose bisphosphate aldolase), COG0149 (triosephosphate isomerase), COG0166 (glucose-6-phosphate isomerase), COG0057 (glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase) and COG0469 (pyruvate kinase). (D) Antimicrobial transporters, multidrug efflux pumps and other transporter COGs: COG0841 (cation/multidrug efflux pump), COG2825 (outer membrane protein), COG3292 (predicted periplasmic ligand-binding sensor domain), COG3264 (small-conductance mechanosensitive channel), COG1538 (outer membrane protein) and COG1629 (outer membrane receptor proteins). Only proteins with values ≥1.5 or ≤-1.5 $\log_2$ ratios were considered.

the first-generation cephalosporin cefazolin as well as the widespread development of β-lactamases (for details, see the Discussion in the online supplement).[13]

The apparent oscillations in the population dynamics were shown to further influence the biodiversity and richness of metabolites and active proteins; these changes, some of which may play essential roles in protection against ABs (for details, see the Discussion in the online supplement), may also have important ecological implications. In our study, we observed a drastic shift 6 days after the onset of AB treatment, at which time the predominantly active taxa were mainly members of the Streptococcaceae, Clostridiaceae and Bacteroidaceae, and at 40 days after the end of AB treatment, when the most abundant active bacteria were members of the Burkholderiaceae (Proteobacteria phylum). Thus, these bacteria may have contributed to the distinct functional profiles and metabolic statuses of colonic bacteria during the follow-up therapy.

A notable finding in this study was that protein expression appeared to decrease as a consequence of AB treatment; furthermore, the production of proteins needed for glycolysis, pyruvate decarboxylation, the tricarboxylic acid cycle, glutamate metabolism, iron uptake, GTP hydrolysis and translation termination were enhanced at the initial stages of AB treatment (day 6), most likely to cope with an intermittent nutrient supply and the stress caused by the ABs, but decreased at later stages and after treatment cessation. Together, these results suggest for first time that AB treatment may 'presumptively' negatively affect the

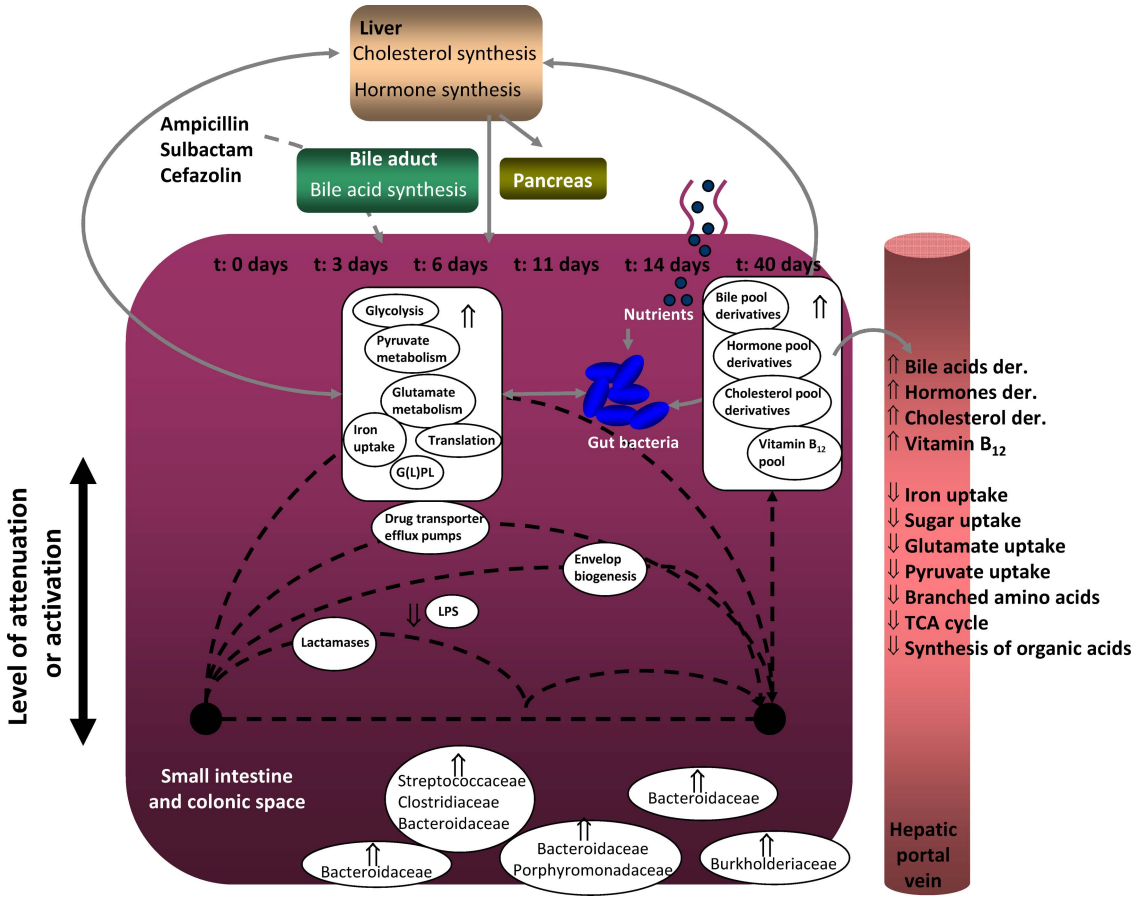

Figure 8 The 'presumptive' model related to the follow-up effect of antibiotics (ABs) on the microbial and metabolic composition of the human gut. The model is based on the combination of experimental multi-omics data. The biliary excretion of ABs triggers a cascade of metabolic events. At the earlier stages of AB therapy, the bacteria respond by promoting systems to avoid the antimicrobial effects of the drugs (expressing beta-lactamases, antimicrobial peptide transporters and multidrug efflux pumps and producing glycero(lyso)phospholipids—G(L)PL) and to cope with an intermittent nutrient supply while decreasing polysaccharides and lipopolysaccharide (LPS) production. Genes involved in cell envelope biosynthesis and the degradation of peptidoglycan-like components are increasingly expressed until the end of AB treatment but with a time delay compared with other drug-detoxifying mechanisms. Finally, the bacterial metabolism of the bile acid, hormones and cholesterol synthesised in the liver and pancreas is attenuated by AB therapy, thus possibly affecting entero-hepatic recirculation and systemic lipid metabolism, that is, the emulsification, absorption and transport of dietary fats; however, after treatment cessation, the metabolism of these factors improved significantly. Similarly, the pool of vitamins that are directly synthesised by gut bacteria was significantly improved after treatment cessation. The nutrient supply mechanisms, such as glycolysis, pyruvate decarboxylation, tricarboxylic acid (TCA) cycle, glutamate metabolism, and iron uptake, that are induced at earlier stages (day 6) become attenuated during the late stages of the therapy and become significantly attenuated after treatment cessation, suggesting that the entero-hepatic recirculation system may contain a lower amount of iron, sugars, branched amino acids, short organic acids and pyruvate produced or transported by colonic bacteria. At the active bacterial structure level, an apparently oscillatory population dynamic was further observed, with the initially predominant active Bacteroidaceae becoming replaced by Burkholderiaceae after treatment cessation. The broken line indicates the overall trend in each of the gut bacteria components during the follow-up treatment.

overall metabolic status of the colonic space (for an example, see the Discussion in the online supplement), although further studies may be required to further confirm this hypothesis. Additionally, the expression levels of all the genes belonging to the 'mobile and extrachromosomal element functions' category were decreased during treatment, and all of them were associated with clustered regulatory interspaced short palindromic repeats (CRISPRs). These genes encode a system that functions as a type of bacterial adaptive 'immune' response.[34] Specifically, the genes that belong to the CRISPR/Cas system are involved in protecting cells from invasion by foreign DNA (viruses and plasmids) through an RNA-interference-like process.[35] Thus, the decreases in the expression of these genes may render the bacteria more susceptible to the acquisition of foreign DNA. This could provide an advantage in an AB-containing environment because it increases the likelihood of obtaining resistance genes by horizontal gene transfer.

Of major metabolic significance was the observation that the production of metabolites that are known to be produced by the host and further metabolised by colonic bacteria, such as derivatives of bile acids, cholesterol and hormones, was altered during the AB treatment and was significantly improved after treatment cessation. In essence, this finding suggests that AB treatment altered the continual interplay between the liver/pancreas and bacterial enzymes operating in the colonic space and that AB therapy may have a positive long-term effect in human biology. This result is consistent with the finding that the biological production of host-beneficial molecules such as vitamin B_{12} and the uptake of key metals such as Co^{2+} by colonic bacteria were affected by AB therapy because the expression of genes and proteins associated with those functions was restored after treatment cessation. This is particularly important because it has been demonstrated that the microbiota from the distal guts of different individuals exhibit partial functional redundancy in

addition to clear differences in community structure in the absence of ABs.[36] By contrast, although further experimental evidence is required, our results suggest that the presence of ABs per se may have additional 'presumptive' collapse effects in key metabolic pathways independent of the community structure and that functional replacement events may be affected under AB stress.

Although, the investigation reported here was for a single patient and should generally be considered qualitative, it constitutes a proof of concept for an integrated, multi-omics approach towards unravelling the dynamics and mechanisms underlying the response of intestinal microbiota to AB treatment. A 'presumptive' model related to the follow-up effects of ABs on bacterial and metabolic composition is summarised in figure 8. These data may help to identify specific strains of gut microbiota with potential benefits in human health or to design specific therapies to decrease intestinal inflammation or normalise dysfunctions of the gut mucosa; for example, minor bacterial taxa such as Proteobacteria have been shown to play a significant, active role in overall gut metabolism and host interaction despite their low number. Further studies investigating different ABs and (un)related individuals are required to better ascertain the link between bacterial producers and the presence of particular proteins and molecules, and the metabolic consequences of AB treatment; this may serve as a promising focus for therapeutic interventions or the treatment of pathogenic infections and diseases.

Author affiliations
[1]Unidad Mixta de Investigación en Genómica y Salud del Centro Superior de Investigación en Salud Pública (CSISP) e Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universitat de València, Valencia, Spain
[2]CIBER en Epidemiología y Salud Pública (CIBEResp), Madrid, Spain
[3]Institute for Clinical Molecular Biology, Christian-Albrechts University, Kiel, Germany
[4]Department of Internal Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany
[5]Department of Proteomics, Helmholtz Centre for Environmental Research, Leipzig, Germany
[6]Center for Metabolomics and Bioanalysis (CEMBIO), Faculty of Pharmacy, Universidad CEU San Pablo, Campus Monteprincipe, Boadilla del Monte, Madrid, Spain
[7]CSIC-Institute of Catalysis, Madrid, Spain
[8]Department of Metabolomics, Helmholtz Centre for Environmental Research, Leipzig, Germany
[9]Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark
[10]Institute for General Microbiology at the Christian-Albrechts University, Kiel, Germany
[11]Institute of Medical Informatics and Statistics, Christian-Albrechts University, Kiel, Germany
[12]Chair of Systems and Synthetic Biology, Wageningen University, The Netherlands
[13]LifeGlimmer GmbH, Berlin, Germany

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SUPPLEMENTAL MATERIALS AND METHODS

DNA AND RNA EXTRACTION

For nucleic acid extraction, samples were resuspended in phosphate-buffered saline solution (PBS). The samples were then centrifuged at 2000 rpm at 4ºC for 2 minutes to remove faecal debris. The supernatant was then centrifuged at 13000 rpm for 5 min to pellet the cells. Total DNA was extracted from pelleted cells using the QIAamp® DNA Stool kit (Qiagen), according to the manufacturer's instructions. In the analysis of the species-level microbiota composition, the total DNA was purified from faecal samples as previously described [37].

Total RNA was extracted from pelleted cells using the RiboPure-Bacteria kit (Ambion) and then treated with DNAse I. To verify the removal of total DNA, a polymerase chain reaction (PCR) for each RNA sample was performed with universal 16S primers. The DNA and RNA extractions were verified by standard agarose gel electrophoresis and quantified with a Nanodrop-1000 spectrophotometer (Thermo Scientific). Total DNA was used for 16S rDNA biodiversity and metagenome determinations, whereas total RNA was used for 16S rRNA biodiversity and metatranscriptome determinations.

AMPLIFICATION OF THE 16S rDNA GENE AND METAGENOME DETERMINATION

For each sample, a region of the 16S rDNA (ssu gene) was amplified by PCR using theuniversal primers E8F (5'-AGAGTTTGATCMTGGCTCAG 3') and 530R (5'-CCGCGGCKGCTGGCAC 3'). For species-level analysis we used the primers 27F (5'-AGAGTTTGATCCTGGCTCAG 3') and 338R (5'-TGCTGCCTCCCGTAGGAGT 3'). The amplified region comprises the hypervariable regions and V1, V2 and V3 for the first pair ofprimers and V1, V2 for the second. In both cases we applied the sample-specific Multiplex Identifier (MID) for pyrosequencing. PCR was performed under the following conditions: 95ºC for 2 minutes, followed by 25 cycles of 95ºC for 30 s, 52ºC for 1 minute and 72ºC for 1 minute, and a final extension step at 72ºC for 10 minutes. Amplification was verified by electrophoresis in an agarose gel (1.4%). PCR products were purified using QIAquick gel extraction Kit (QIAGEN) or the NucleoFast® 96 PCR Clean-Up kit (Macherey-Nagel) and quantified with a Nanodrop-1000 spectrophotometer (Thermo Scientific) and with the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen). The pooled PCR products were directly pyrosequenced.

The metagenome was directly obtained by sequencing the total DNA using a Roche 454 GS FLX sequencer. Assembly was performed using Roche's Newbler assembler v. 2.5.3 with default parameters. We sequenced the metagenomes for each time sample, obtaining 17.37 Mb for FS-0; 245.33 Mb for FS-3; 22.74 Mb for FS-6; 12.07 Mb for FS-11; 23.22 Mb for FS-14; and 53.57 Mb for FS-40.

16S rRNA SEQUENCING AND mRNA EXTRACTION AND AMPLIFICATION

Total RNA was directly retrotranscribed to obtain the 16S rRNA (as a measure of active bacteria) as well as the rest of the RNA, particularly the mRNA. Prior to mRNA amplification, to remove the maximum amount of rRNA, rRNAs were first depleted with the MICROBExpress kit (Ambion), which captures and removes the rRNA (16S rRNA, 23S rRNA) by hybridization. [38] Second, we used the mRNA-ONLY Prokaryotic mRNA isolation kit (Epicentre), which uses a terminator 5'-phosphate-dependent exonuclease that specifically digests rRNAs due to the presence of 5' monophosphate groups. Finally, mRNA was linearly amplified using the MessageAmp II-Bacteria kit (Ambion), which adds poly(A) tails to the

mRNAs.

To retrotranscribe the total RNA and the amplified mRNA into single-stranded cDNA, we used the High-Capacity cDNA Reverse Transcription kit (Ambion). To synthesize doublestranded cDNA (ds-cDNA), we used standard procedures. The products were quantified with a Nanodrop-1000 spectrophotometer (Thermo Scientific) prior to sequencing.

All sequencing was performed by Life Sequencing (Valencia, Spain) with a Roche GS FLX sequencer and titanium chemistry.

BIODIVERSITY ANALYSIS

The 16S rRNA reads were retrieved from the total cDNA by comparing the total reads against the Small Subunit rRNA Reference Database (SSUrdb)[39] with BLASTN[40] and an e-value of 10^{-16}. For 16S DNA reads, we removed the sequences with low-quality scores (<20) and a short length (<200 bp). Sequences were checked for chimeras using MOTHUR software.[41] For species-level analysis sequences were clustered into Operational Taxonomic Units (OTUs) by 3% distance level based on the average neighbour algorithm.

The taxonomic information for the 16S rDNA and the 16S rRNA sequences was obtained by comparison against the Ribosomal Database Project-II (RDP).[28] We considered only annotations with a bootstrap cutoff value above 0.8 and terminated the assignation at the lowest phylogenetic category identified at or above this support level. For species identification, the taxonomical assignment was performed also with RDP and a bootstrap threshold of 60%. An additional web-based matching against type strain sequences was conducted with RDP Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). OTU abundances were Hellinger transformed[42] and a Principal Component Analysis (PCA) based on the covariance matrix of transformed OTU abundances was performed. To assess the number of interpretable axes within the PCA, a Kaiser-Guttman and broken-stick-model were computed (data not shown). Additionally, OTUs responsible for differences between the samples were identified in a scree plot based on their vector length in the PCA.

Two biodiversity parameters were calculated: Shannon and Chao1. The Shannon index is a measure of the degree of homogeneity of the microbiota,[43] and Chao1 is a richness estimator that also permits the assessment of the number of bacterial taxa in the samples.[44,45] To avoid a sequencing effort bias, both indices were calculated after subsampling with the multiple_rarefactions.py script of QIIME.[46]

Microbial communities were compared using heatmaps of taxa abundance and composition. Correspondence analyses and clustering by means of the Bray-Curtis distance were obtained by applying the statistical package R.[47]

METATRANSCRIPTOME ANALYSIS BY MAPPING THE METAGENOME

We sequenced metatranscriptomes for each time sample, obtaining 7.33 Mb for FS-0; 5.85 Mb for FS-3; 7.27 Mb for FS-6; 5.68 Mb for FS-11; 4.37 Mb for FS-14; and 2.58 Mb for FS-40. Our aim was to identify coding regions in the metagenomes and then map the corresponding transcriptomes to determine the relative expression of the genes. To identify the coding regions, two different strategies were used. First, our dataset was compared against the sequences belonging to the bacterial superkingdom in the NCBI-nr protein database using BLASTX[40] and an e-value of 10^{-3}. Second, we used Glimmer[48] to identify coding regions present in our samples but not in the NCBI-nr protein database. For coding regions identified using BLASTX, we performed taxonomic assignment by applying the Lowest Common Ancestor (LCA) algorithm.[49]

To obtain a relative measure of gene expression for each sample, we followed the methodology described by Turnbaugh *et al.*[18] Briefly, this methodology consists of adjusting the number of transcripts mapping to each coding region by dividing it by its associated copy number. To assign the copy number of each gene, we applied the BLASTX[40] coding regions against themselves with an e-value of 10^{-3}.

To functionally annotate the identified coding regions, we used HMMER against the database of prokaryotic models TIGRFAM with default parameters.[30] We used an integrative algorithm based on homology that enabled us to obtain a matrix containing the relative expression of the same coding regions in the different samples. To apply the copy numbercorrection to the functional and taxonomical annotation, each taxon or function must have an associated integer frequency to recover abundance matrices for each individual sample. To achieve this goal, we transformed the measure of relative expression to integer values by applying univariate standardization within each sample followed by logarithmic scaling. We performed a clustering analysis to classify the genes identified for each sample according to their expression profile with the SOM package (Self-Organizing Map).[31] This analysis applied a univariate scaling to the expression gene matrix to obtain all expression gene profiles with the same mean of 0 and standard deviation of 1. The scaled expression profiles were clustered by creating a Self-Organizing-Map. Using the information associated with each gene, we performed multivariate analyses, such as correspondence analysis or clustering. For this purpose, we used the package Vegan[50] in R.[47] We used different tools of the ShotgunFunctionalizeR[33] library of R[52] to perform the statistical comparisons. Specifically, we performed regression analyses at different functional levels for general roles and gene families based on a Poisson model to identify the functional levels that change significantly during treatment.

All of the analyses were integrated within a script written in the shell language of Linux that calls other scripts written mainly in R[47] and Perl (http://www.perl.org/).

PROTEIN EXTRACTION, SEPARATION AND IDENTIFICATION AND DATA PROCESSING

Protein extraction was performed by incubating 1.2 ml BugBuster Protein Extraction Reagent (Novagen) for 30 min at room temperature with the bacterial pellet obtained as described above. Faecal bacteria were further disrupted by mechanical lysis followed by sonication for 2.5 min on ice. The extract was then centrifuged for 10 min at 12000 rpm to separate cell debris and intact cells. The supernatant was carefully aspirated (to avoid disturbing the pellet) and transferred to a new tube, and protein concentrations were determined with the Bradford assay.[51] For 1-DE analysis, two 75-µg protein samples (technical replicates denoted by a or b) were precipitated with five-fold volumes of ice-cold acetone and separated on a 12% acrylamide separating gel with the Laemmli buffer system.[52] After electrophoresis, protein bands were stained by colloidal Coomassie Brilliant Blue G-250 (Roth, Kassel, Germany). Entire protein lanes were individually cut into six bands prior to performing in-gel tryptic digestion.[53] Tryptic peptides of each band were desalted with a C18 ZipTip prior to MS analysis.

Peptides were analyzed by UPLC-LTQ Orbitrap-MS/MS, as described in Bastida et al.[54] The peptides were eluted over 77 min with a gradient of 2 to 60% solvent (acetonitrile, 0.1% formic acid). Continuous scanning of the eluted peptide ions was performed at 300-1,600 m/z, automatically switching to MS/MS CID mode on ions exceeding an intensity of 2000. Raw data were searched using MaxQuantTM (version 1.2.18).[55] Each sample was measured with two technical replicates. The raw data obtained from peptide samples originating from the same lane on the 1-DE gel were searched together, and technical replicates were analyzed separately. The database that was searched against contained the metagenomic data obtained from samples FS-0 to FS-40. The settings for MaxQuant were the following: the peptide modifications included methionine oxidation as variable and cysteine carbamidomethylation as fixed; first search ppm of 20; main search ppm of 6; maximum number of modifications per peptide: 5; max. missed cleavages: 2; and a maximum charge for the peptide of 5. The parameters for identification included a minimum peptide length of 5 amino acids and a false discovery rate for peptides and proteins and a level of modification sites of 1%. A minimum of 2 unique peptides was required for protein identification. Apart from unmodified peptides, only peptides with oxidized methionine and carbamidomethylized cysteine were used for quantification. Only unique or razor peptides were chosen for use in quantification. Miscellaneous settings included “re-quantified”, “keep low scoring versions of identified peptides”, “match between runs” (time window of 2 min), “label-free quantification” and “second peptides”.

To analyze the data, the intensity attributed to each identified protein was divided by the number of peptides assigned to the protein. Normalization was then performed by dividing these corrected intensity values by the median of all corrected intensities from the same sample. The ratio of the normalized intensities was calculated for each protein by dividing the mean

of the normalized intensities from samples FS-3, FS-6, FS-11, FS-14 and FS-40, respectively, by the mean of the normalized intensities from sample FS-0. Ratio values were then calculated by taking the logarithms of the ratios to the base of 2. For up regulation of protein expression in the gut environment, a threshold of at least 1.5 for the ratio value was set, and for down regulation, a maximum value of -1.5 was set.

METABOLITE EXTRACTION, SEPARATION AND IDENTIFICATION AND DATA PROCESSING

Metabolite extraction was performed by adding 1.2 ml of cold (-80ºC) HPLC-grade methanol (MeOH) to the bacterial pellet obtained as described above. Samples were then vortex-mixed and stored at -80ºC for 60 min. Then, the samples were again vortex-mixed and sonicated for 30 seconds on liquid nitrogen and stored at -80ºC for 60 min. This protocol was repeated 5 times. The final pellet was removed by centrifugation at 16000 rpm for 10 min at 4ºC, and the supernatant was stored in a 20-ml penicillin vial at -80ºC. The methanolic extract was centrifuged at 13000 rpm and 4ºC for 20 min to precipitate any solid impurity. The supernatant was removed and transferred to analytical vials. Quality control (QC) samples
were prepared by pooling equal volumes of the supernatant from each of the 18 samples (6 samples x 3 replicates each). QC samples were analyzed throughout the run to provide a measurement of the system's stability and performance.

The HPLC-ESI-QTOF-MS system consisted of a degasser, two binary pumps and an autosampler (1200 series, Agilent). Ten µl of the sample was injected onto a reversed-phase column (Discovery HS C18 150x2.1 mm, 3 µm; Supelco) with a guard column (Discovery HS C18 20x2.1 mm, 3 µm; Supelco), both of which were maintained at 40ºC. The system was operated in positive ionization mode at a flow rate 0.6 ml/min. Solvent A was composed of water with 0.1% formic acid, and solvent B was composed of acetonitrile with 0.1% formic acid. The gradient was from 25% B to 95% B in 35 min, returning to initial conditions in 1 min, and re-equilibration was performed at 25% B for 9 min. Data were collected in positive ESI mode in separate runs on a QTOF (Agilent 6520) operated in full scan mode from 50 to 1000 m/z. The capillary voltage was 3000 V with a scan rate of 0.77 scan per second. The gas temperature was 330ºC, the drying gas flow was 10.5 l/min, and the nebulizer was 52 psi. The MS-TOF parameters were the following: fragmentor at 175 V, skimmer at 65 V and octupole radio frequency voltage (OCT RF Vpp) of 750 V. During the analysis, two reference masses were used: 121.0509 (C5H4N4) and 922.0098 (C18H18O6N3P3F24). These masses were continuously infused into the system to permit constant mass correction. Samples were analyzed in one randomized run, during which time they were kept in the LC autosampler at 4ºC.

Background noise and unrelated ions were removed from the resulting data file using the Molecular Feature Extraction (MFE) tool in the Mass Hunter Qualitative Analysis software (B.04.00, Agilent). Primary data treatment (filtering and alignment) was performed with MassProfiler Professional software (B.02.01, Agilent). The multivariate analyses were performed using SIMCAP+ software (12.0.1.0, Umetrics) to generate a PLS-DA model with all the variables, and QCs were predicted into this model (figure 4A). Data from samples P 3.1, P-3.3, P-3.4, P-3.5 and P-3.6 were then aligned and filtered by selecting features present in a minimum of 100 % of 1 of 5 groups. These data were then represented in a hierarchical condition tree (HCA) (figure 4D). Finally, t-tests were performed to compare samples FS-0/FS-6, FS-6/FS-11, FS-11/FS-14 and FS-14/FS-40 ($p \leq 0.01$) (figure 4B). The accurate masses of features representing statistically significant differences were searched against the METLIN database and represented in a PLS-DA model built using SIMCAP+ software (figure 4C).

SUPPLEMENTAL DISCUSSION

APPARENT OSCILLATORY DYNAMICS OF POPULATIONS

A remarkable increase in the abundance of Lachnospiraceae (28.25%), *Roseburia* (18.29%) and Clostridiales (11.43%) was

found at day 3 of treatment. Regarding this, some members of Bacteroidaceae family, as *Bacteroides*, that had an abundance of 16.37% showed a relative high gene expression (90%), noting that no abundant bacteria can be very active at a given moment in the gut. This result supports what was found in the active bacteria analysis where Bacteroidaceae family was very abundant among the active microbiota. At day 6 an increase in Ruminococcaceae family (34.81%) was detected, showing that the first days of treatment were characterized by a dominance of the phylum Firmicutes. In fact, Streptococcaceae, Clostridiaceae, and Eubacteriaceae, previously described as resistant to penicillin and other β-lactams[56,57], presented a relative gene expression of 22.87%, 11.27% and 11.06%, respectively at day 6 of treatment. At day 11 a significant shift to Bacteroidetes was found, specifically in the genus *Parabacteroides* (73.23%) that reached the highest abundance of all samples. The Bacteroidaceae family showed also a high level of gene expression (63.05%) and *Parabacteroides* genus, Porphyromonadaceae family, with a gene expression of 19.82%. Also, it was found a considerable increase in the abundance of Betaproteobacteria (19.82%). At day 14 there was homogeneity in abundances and in recovery of some taxa, being the most abundant *Bacteroides* (19.15%), Betaproteobacteria (15.75%), Lachnospiraceae (14.56%), Ruminococcaceae (12.01%), *Roseburia* (11.43%) and *Parabacteroides* (11.24%). The expression of genes was almost dominated by Bacteroidaceae family (89.6%). By temporal temperature gradient gel electrophoresis, De La Cohetière *et al.*[13] identified similar dominant taxa during a 5-day β-lactam (amoxicillin) treatment. After treatment, the microbiota was mainly constituted by *Faecalibacterium* (30.5%), Ruminococcaceae (29.24%) and *Bacteroides* (9.4%), being the abundance of Firmicutes phylum higher than before treatment. Interestingly, the taxon more active at gene level was Burkholderiaceae (circa 46%), a member of Proteobacteria phylum, highlighting the importance of groups that are present in low abundance that can have an essential role in some process or that are growing at that time.

The large fluctuations in the relative abundance of the various bacterial taxa for the total and active microbiota throughout the follow-up study may be most likely due to an additive effect of Ampicillin/Sulbactam and the first-generation cephalosporin Cefazolin. Both, Ampicillin and Cefazolin act on Gram-positives and Gram-negatives by cell wall lysis. Widespread development of beta-lactamases among Gram-negatives, e.g. due to PBP modification, results in a better antibiotic effect of Cefazolin against Gram-positives. Having said that, it should be also taken into consideration that Ampicillin/Sulbactam and Cefazolin act on the microbiota mainly because their biliar excretion, and accordingly their concentrations in the upper intestine (where Firmicutes are more abundant) should be higher than in the colonic space. Ampicillin/Sulbactam may increase the effect of Cefazolin on natural beta-lactamase producer populations of the gut (as many Bacteroidetes), and then, most of the time, the attribution of the observed effects may be only attributed to Cefazolin as a single drug. At the same time, Cefazolin may be probably degraded and extensively bound to proteins in the colonic space, reducing the effect on susceptible Gram-negatives at large. It is likely possible that once beta-lactamase producing organisms are selected by Cefazolin, the local amount of beta-lactamase could eliminate all locally available Cefazolin, thus allowing residual susceptible populations (including Gram-positives as Streptococci, but also other groups) to re-grow, even under therapy. Under this scenario the apparent oscillatory dynamics of populations (both in DNA and RNA) observed might be due to compartmental changes, with early reduction in Gram-negatives, and overall collapse in diversity, possible colonization of the upper gut by naturally-resistant Bacteroidetes, and consequent increased colonization of the lower gut with dominance at 11th -14th days, and then re-growth of Grampositives. Having said that, since β-lactams interfere with the synthesis of cell-wall peptidoglycans to prevent bacterial growth, the possibility that active fraction of the microbiota is represented by those microorganisms that were 'presumptively' resistant to the administered antibiotics cannot be ruled out; therefore, additional time of treatment would have been required to disrupt the composition of this group. Recently, Nakano et al.[32] demonstrated that intestinal Bacteroides and Parabacteroides genera carry resistance genes for several antibiotics, including β-lactams. Thus, OTUs belonging to *Bacteroides fragilis*, *Bacteroides dorei*, *Parabacteroides distasonis* and *Bacteroides ovatus* recovered in FS-11 and FS-14, when the effect of Ampicillin/Sulbactam stopped and only Cefazolin interacted on Gram-positive bacteria of the microbiota. The observed shift in the composition at the 11th day is compatible with the detection at day 6 of bacteria such as *Clostridium*, *Ruminococcus* and *Burkholderia*, which are 'presumptively' resistant to the applied antibiotics. Other potential facultative pathogenic bacteria, which may be possibly selected due to AB treatment, such as those belonging to the genera *Klebsiella*, *Escherichia* and *Salmonella*, were not found at any time of the treatment.

The identification of 3 beta-lactamases, likely attributed to single or several species of *Bacteroides* such as *B. fragilis*, which were significantly expressed (up to 27-fold) in FS-3, FS-6 and FS-14 metaproteomes as compared to FS-11 and FS-40

(table 3 in online supplement) may agree with the oscillatory dynamics of populations. Additionally, functional assignments and analysis of the predicted genes further evidenced the presence of 381 betalactamases in the consensus metagenome sequences; among them we found 231 being expressed in the faecal microbiota (mRNAs) at different levels before, during and after AB treatment. As shown in figure 13 in online supplement, an apparent oscillatory dynamics of the number of expressed proteins was also observed with early abundance of beta-lactamases being expressed (day 3), then a decrease with a minimum at day 11 and then re-expression at day 14. Only, 9 beta-lactamases were found to be expressed after treatment cessation. Although, beta-lactamases produced by different bacterial species may have quite a distinct substrate specificity and further studies may be required to ascertain their implication in AB resistance, the decrease of abundance of these expressed genes may be directly related to the decrease of abundance and variety of species during the follow-up treatment, namely at day 11 (table 1 and figure 1). The over-abundance of beta-lactamase sequences in the metagenome after treatment cessation (167 or 0.116% of open reading frames), but their low expression level may indicate these genes not being required in the absence of AB and that their overabundance might be a side-effect of the treatment. Binning analysis further evidenced that in the FS-0, FS-3, FS-11 and F-14 there are mainly Bacteroidaceae expressed beta-lactamases, whereas in FS-40 Burkholderiaceae are most abundant, in agreement with the contribution of these groups to the active community.

METAPROTEOMIC AND METAMETABOLOMIC CHANGES

Results suggest a “presumptive” lower capacity following AB therapy to feed the tricarboxylic acid cycle with intermediates to meet the demand for carbon skeletons for the synthesis of organic acids and amino acids. In addition, results highlight a ‘presumptive’ decrease in the use of glutamate because the expression of glutamate dehydrogenase (COG0334) and decarboxylase (COG0076) was reduced following AB treatment (figure 5). If the flux through glutamate to α-ketoglutarate and glutamate to gamma-aminobutyric acid decreases, it seems logical to presume that the flux through the entire pathway decreases as a consequence of AB treatment. The production of branched-chain fatty acids in the membrane is linked to the turnover of α-ketoglutarate to glutamate by glutamate dehydrogenase (COG0334), and we would expect them to be altered in concert.

A notable additional finding in this study was that it appears that additional AB treatment time is required to activate membrane permeability systems (day 14) as compared to detoxifying drug transport systems (day 3 and 6),58,59,60 and that antibiotic differentially affected cell wall components of gut bacteria with (lipo)polysaccharides being altered at earlier stages of the therapy while maintaining or improving the glycero(lyso)phospholipids, murein sacculus and peptidoglycan biosynthesis during the follow-up treatment. This is in agreement with the observed early activation in Gram-positive bacterial families, such as Streptococcaceae and Clostridiaceae, and the reduction in Gram-negatives.

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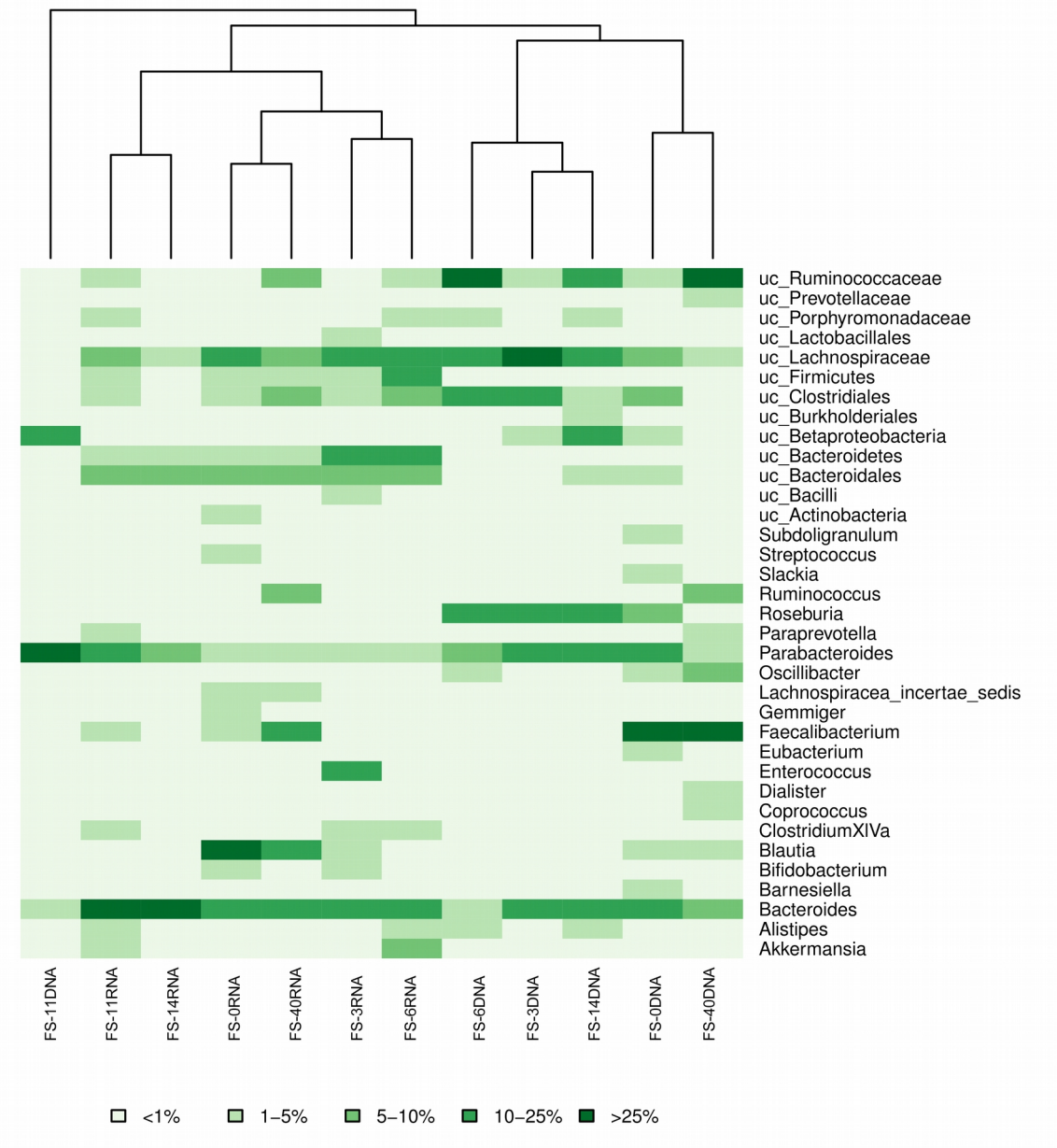
SUPPLEMENTAL TABLES

Supplemental Table 1 Summary of the metagenomic and metatranscriptomic data for each sample collection time.

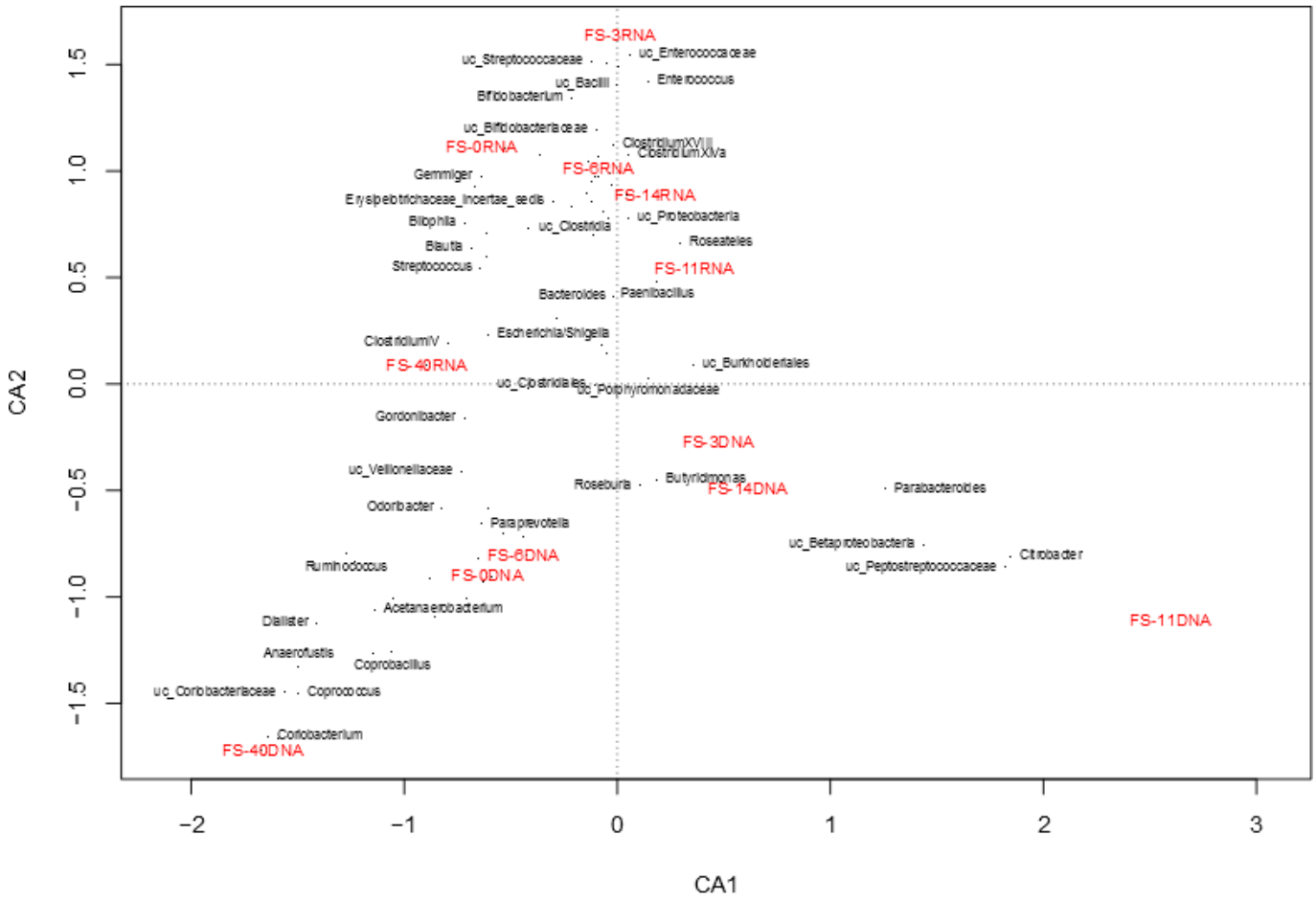
	FS-0	FS-3	FS-6	FS-11	FS-16	FS-40
Metatranscriptome (reads)	76374	60253	72691	66093	51349	5751
Metagenome (reads)	44653	519776	53893	29156	54901	126639
mRNA (reads)	40517	37107	50952	40785	28787	4635
TIGRFAM(reads)	29014	26120	13115	27461	19240	1968

NOTE: Supplemental tables 2 and 3 are included in the file Supplemental_tables_2_3.pdf in the CD due to their size.

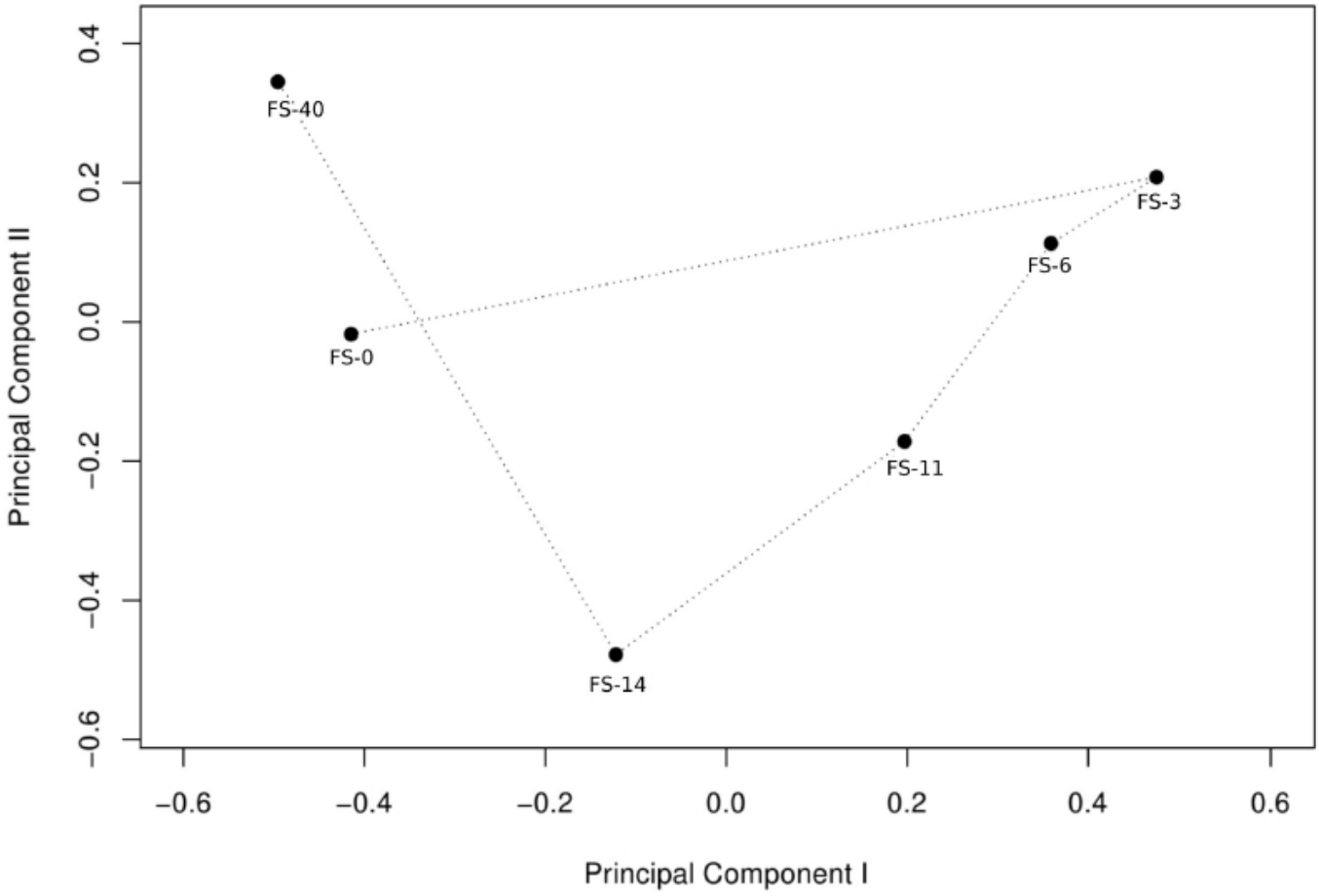
SUPPLEMENTAL FIGURES



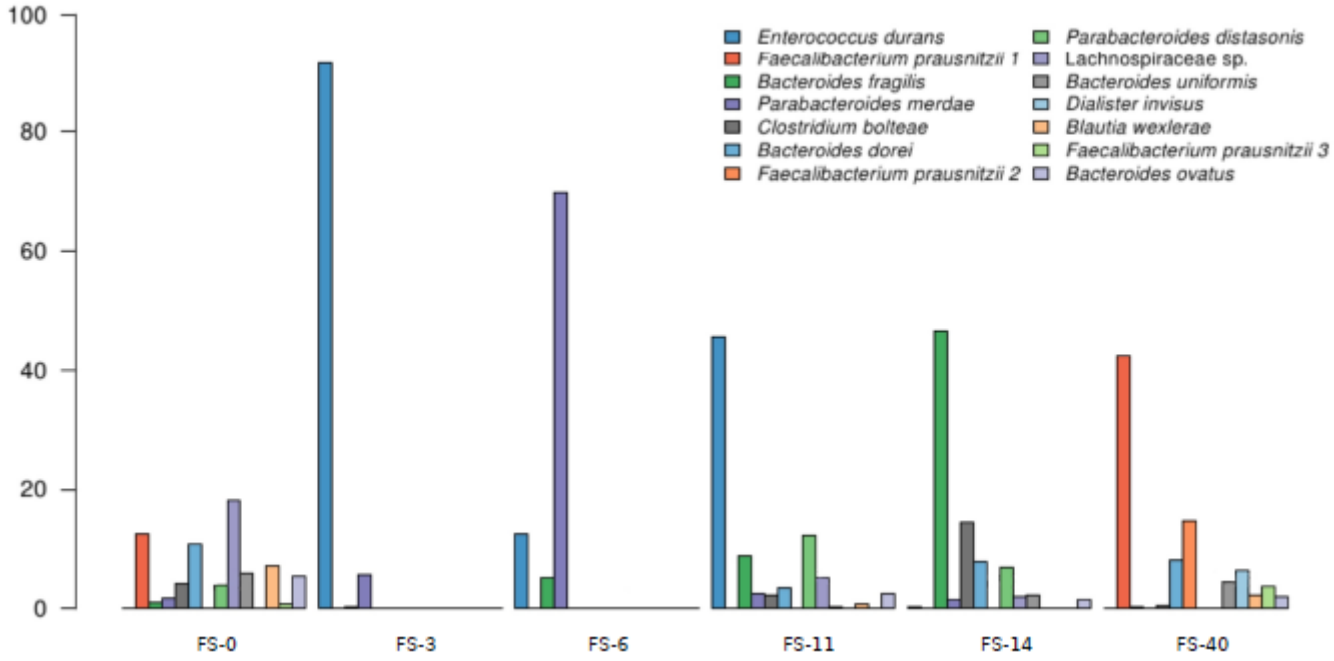
Supplemental figure 1 Heat map and hierarchical cluster based on the relative abundance of each bacterial taxon and the composition of the total (16SrDNA) and active (16SrRNA) microbiota. Colors depict the percentage ranges of sequences assigned to the main taxa (abundance >1% in at least one sample).



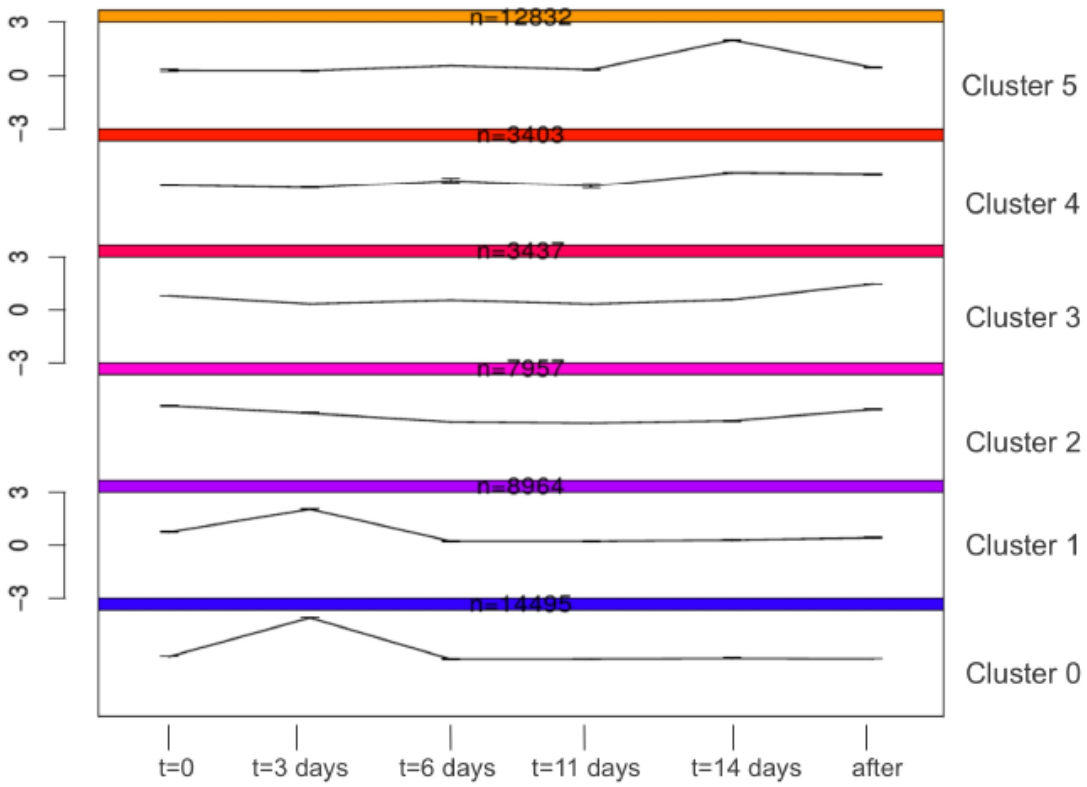
Supplemental figure 2 Correspondence analysis of total (DNA) and active (RNA) microbiota based on the relative abundance of each taxon per sample.



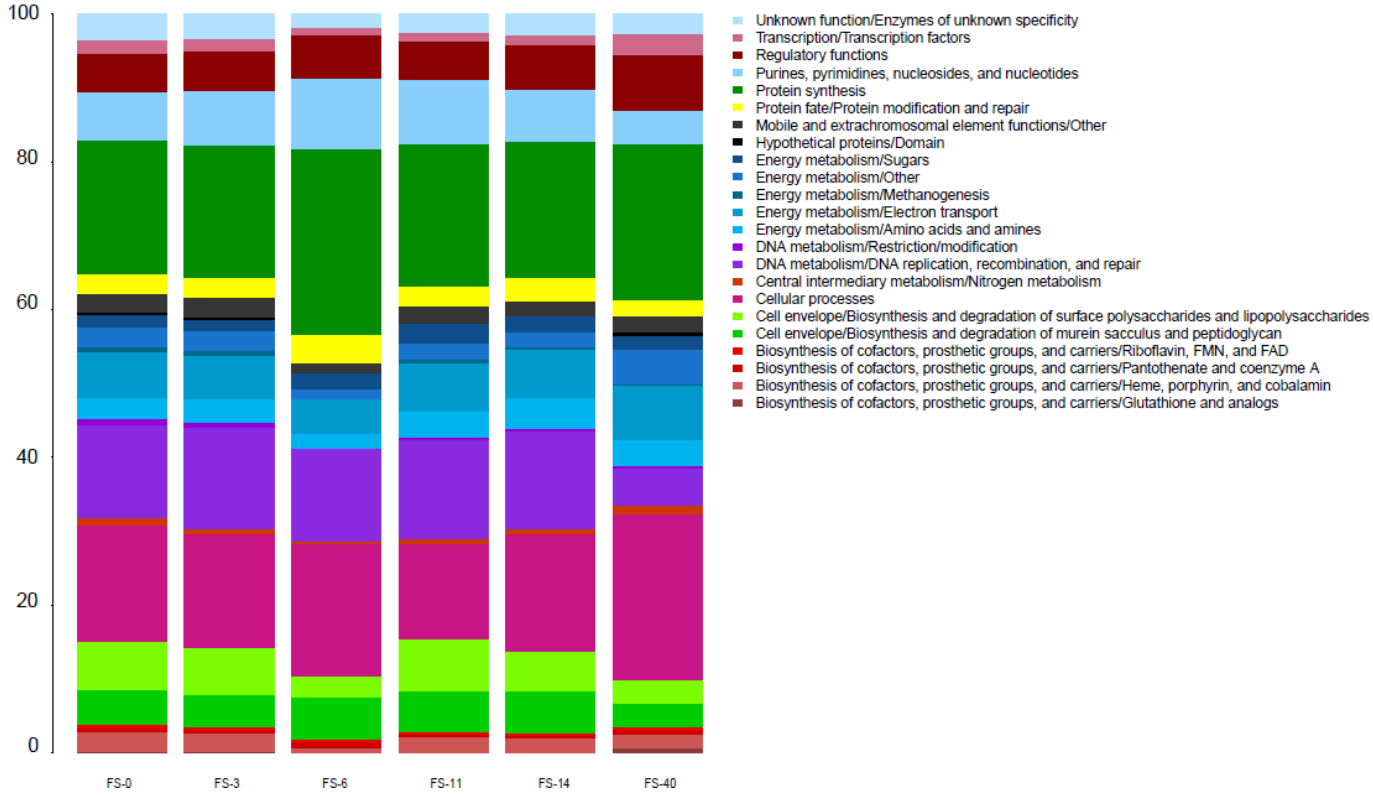
Supplemental figure 3 PCA of the Hellinger transformed OTU abundances for the samples FS-0 to FS-40.



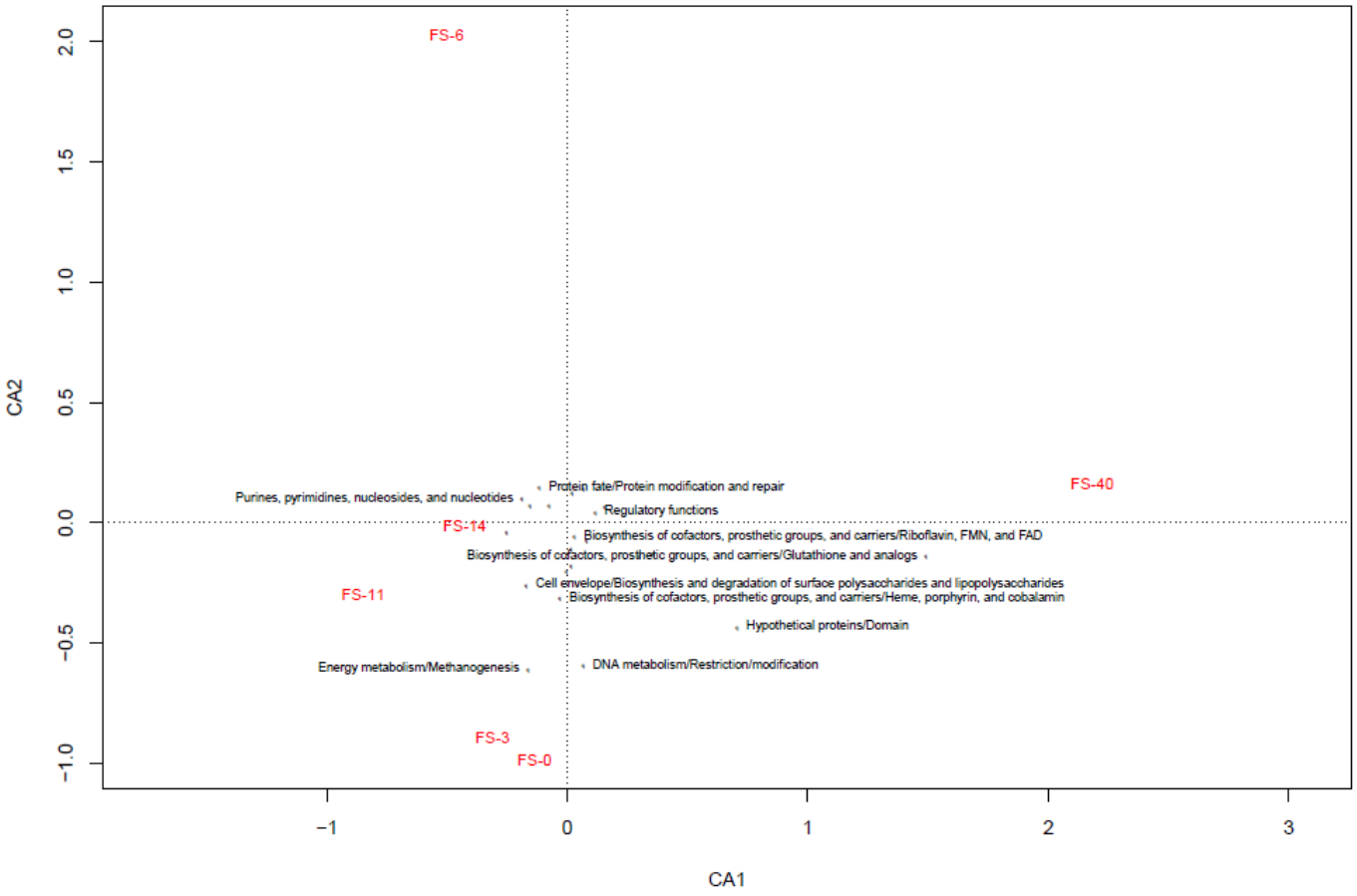
Supplemental figure 4 Distribution of prominent OTUs and their abundance identified by OTU vector length n the PCA.



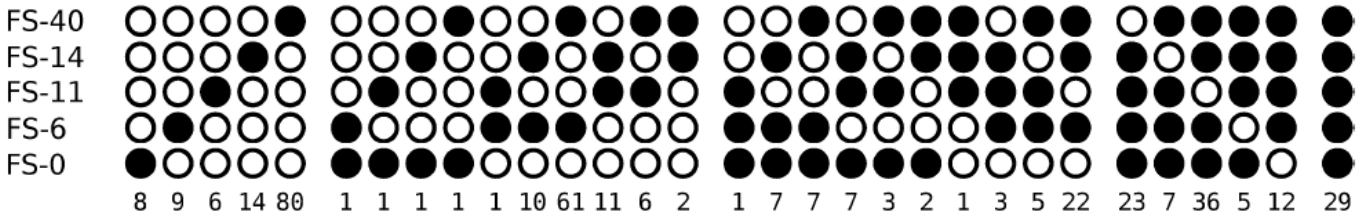
Supplemental figure 5 Clusters of genes based on their expression profile during antibiotic treatment. "n" corresponds to the number of genes included in each cluster.



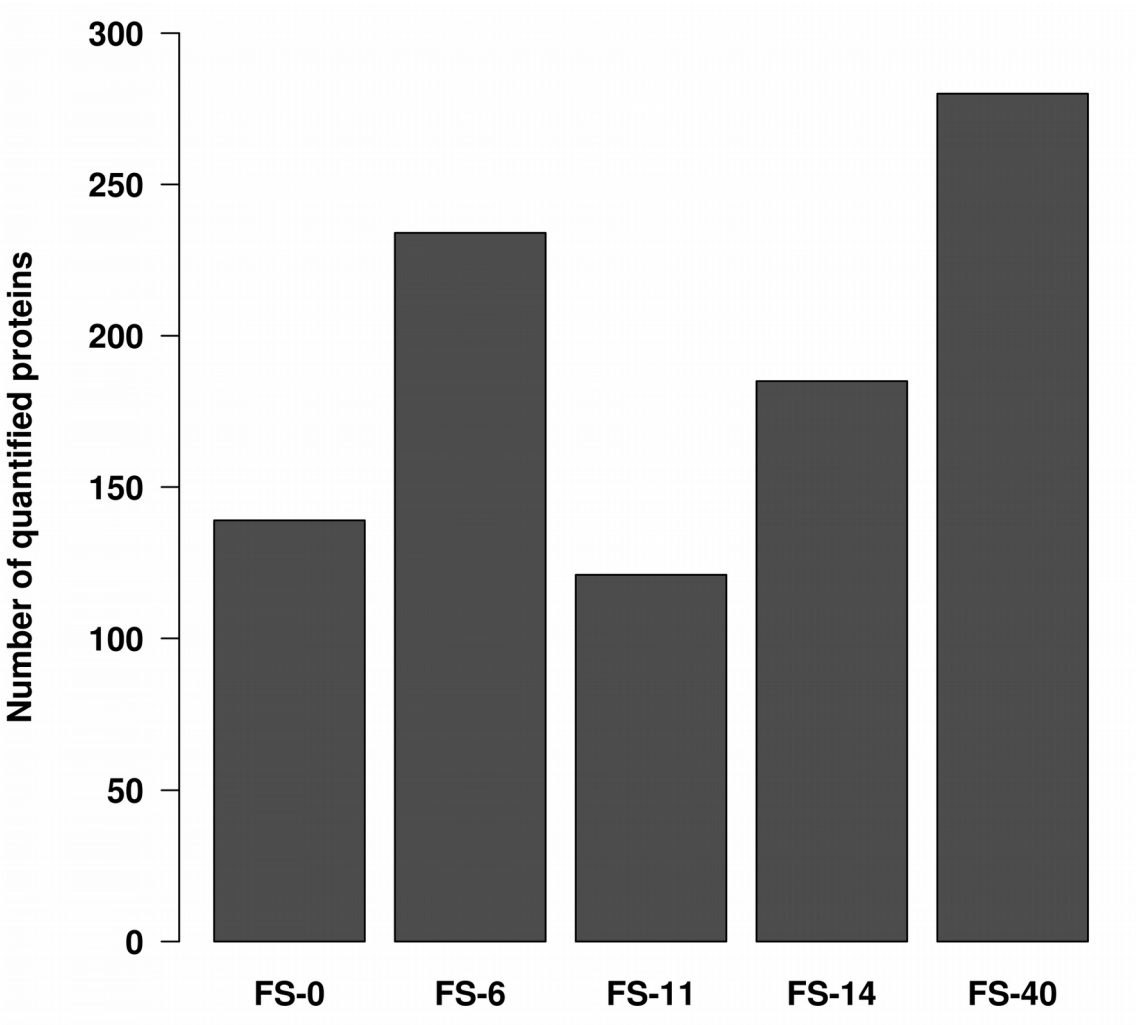
Supplemental figure 6 Relative abundance of functional categories exhibiting a statistically significant change due to AB treatment. These categories were obtained from a regression analysis based on a Poisson model, considering the sampling time during the antibiotic course.



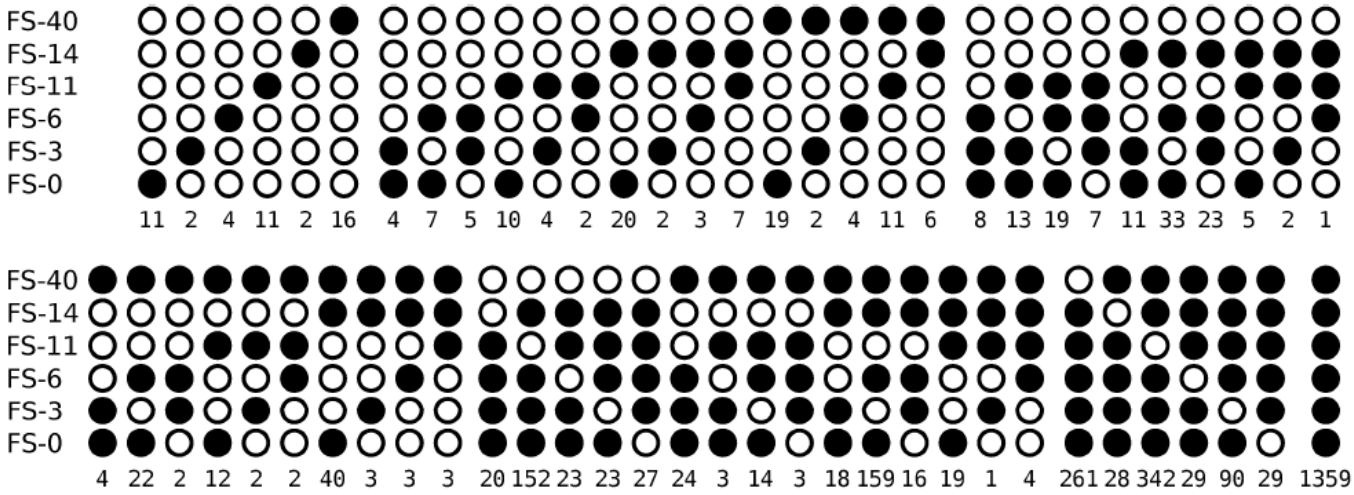
Supplemental figure 7 Correspondence analysis based on the relative proportion of functional categories showing a statistically significant change due to AB treatment.



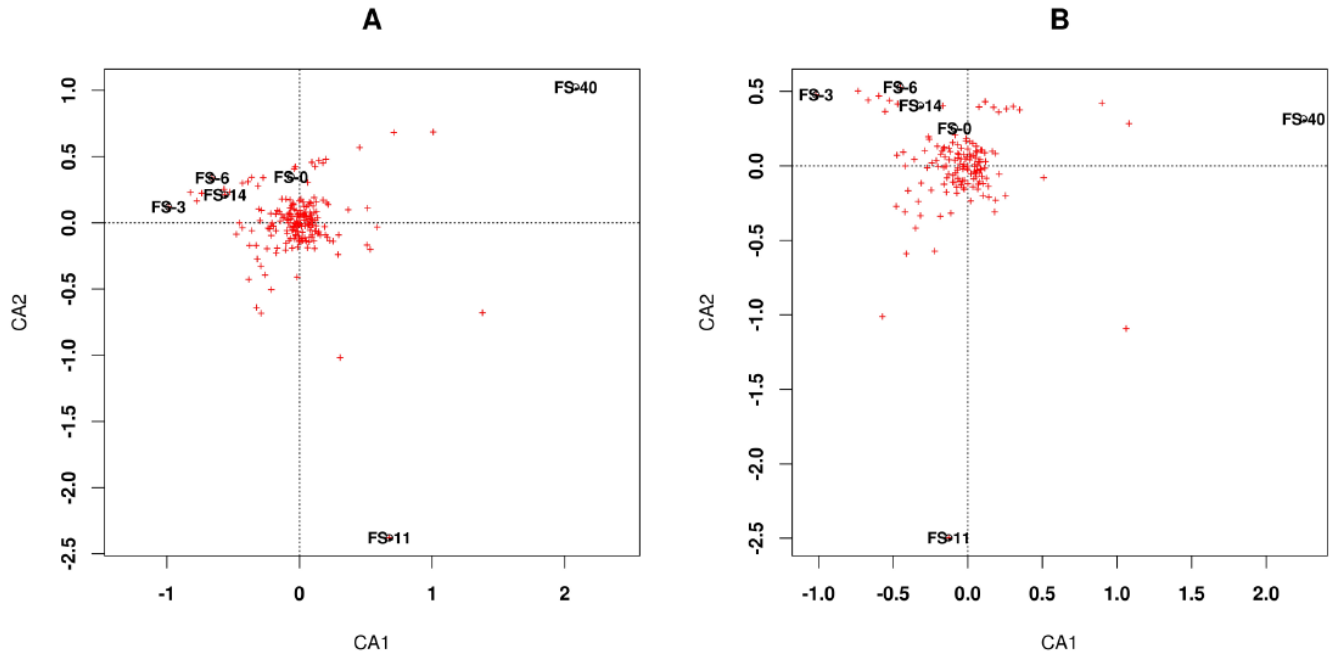
Supplemental figure 8 Distribution of common and distinct mass features identified in the gut communities of the human gut microbiota during and after AB treatment. Clustering was performed with a matrix of the 382 accurate masses representing significant differences from each sample. The number of mass features identified in the corresponding sample(s) is shown at the bottom. The black color indicates the presence of mass features in a given sample, whereas the white color represents the absence of such features. As shown, only 29 of 382 features were identified in all of the gut samples examined in this study, and 8, 9, 6, 14 and 80 were uniquely obtained in the FS-0, FS-6, FS-11, FS-14 and FS-40 samples, respectively



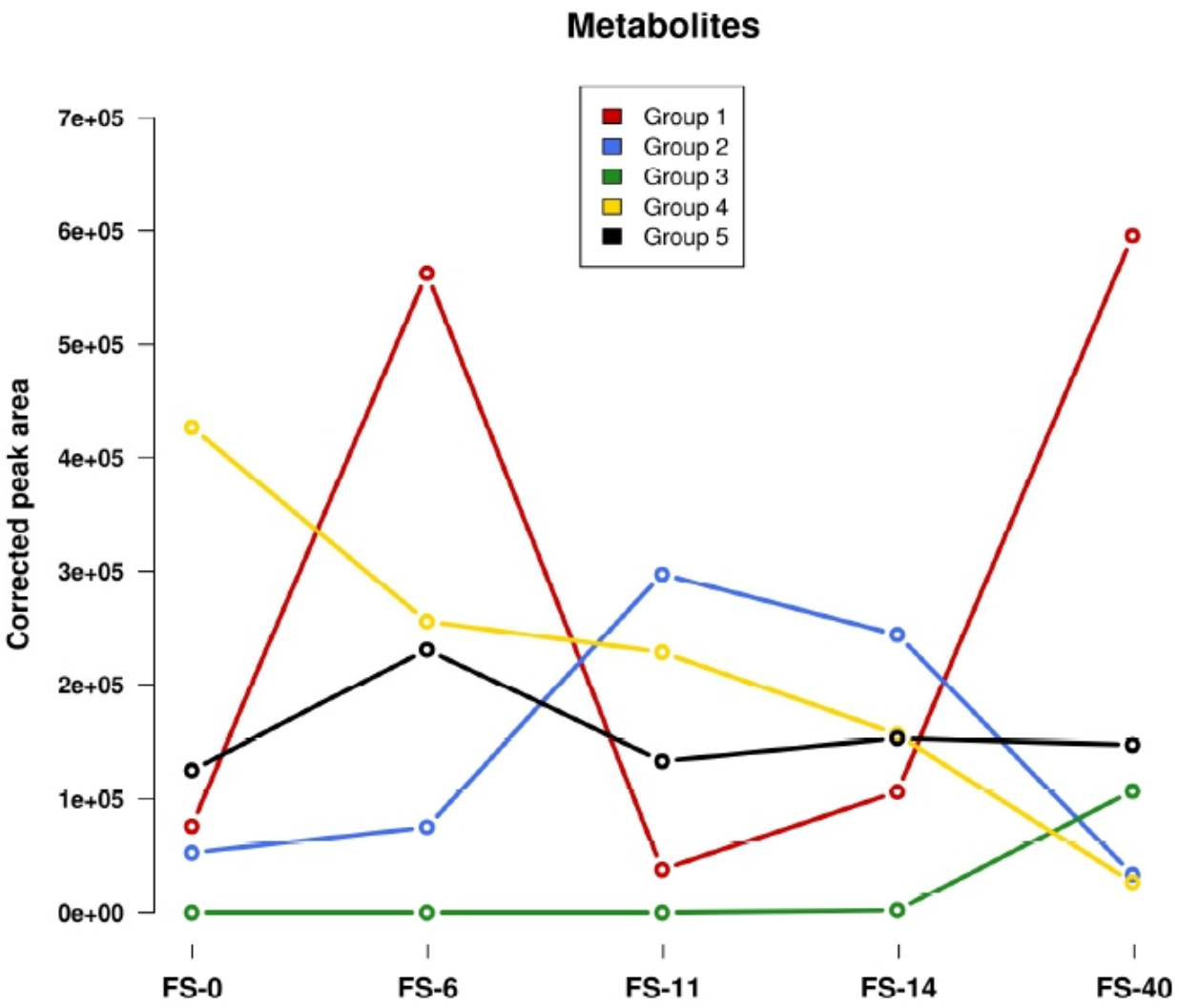
Supplemental figure 9 Number of mass features that passed the filtering and statistical treatments and were found in microbial cells from faecal samples in this study.



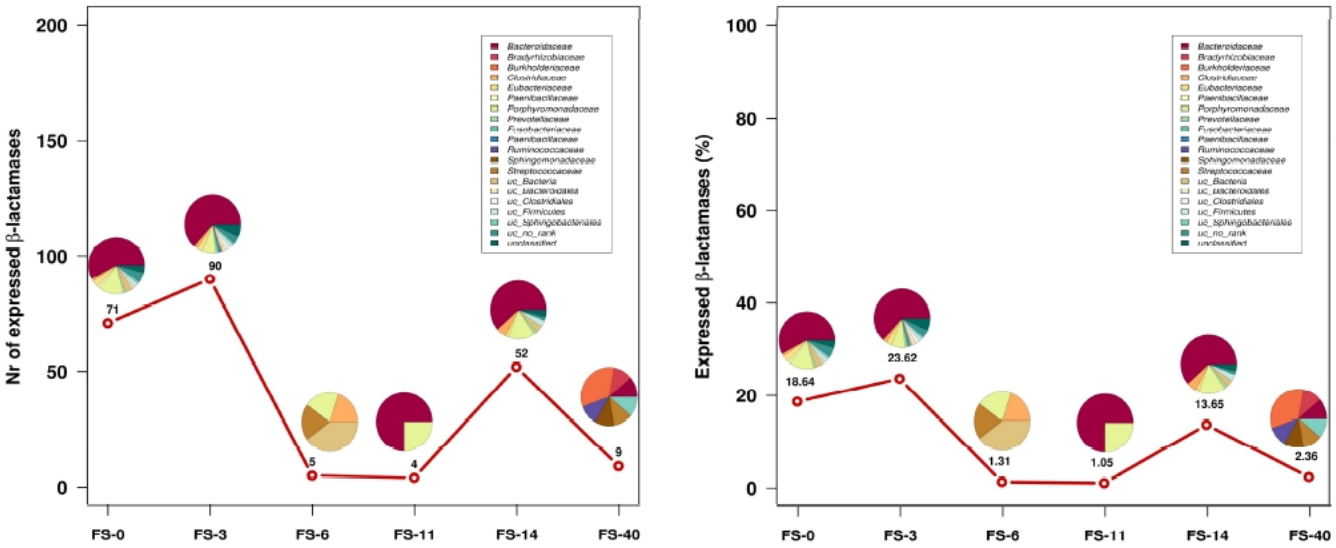
Supplemental figure 10 Distribution of the common and distinct proteins found in the gut communities of the human gut microbiota during AB treatment. Clustering was performed with a matrix of the total protein that has passed the filtering treatment for each sample. The number of proteins identified in the corresponding sample(s) is shown at the bottom. The black color indicates the presence of proteins in a given sample, whereas the white color represents the absence of such proteins. Note: a total of 3,011 proteins (FS-0: 2,802; FS-3: 2,429; FS-6: 2,696; FS-11: 2,044; FS-14; 2,716; and FS-40: 2,289) were unambiguously quantified using GeLC-MS / MS approaches. Only 11 (FS-0), 2 (FS-3), 4 (FS-6), 11 (FS-11), 2 (FS-14) and 16 (FS-40) were community-specific, whereas 1,359 (or 45%) conformed the common set. As shown, 1359 proteins were found to be expressed in all of the gut samples examined in this study.



Supplemental figure 11 Correspondence analysis based on the relative proportion of functional categories exhibiting a statistically significant metaproteome change due to AB treatment. (A) CA for the COG categories where CA1 explains the 33% of the variance and CA2 the 28%. (B) Clustering of the samples based on type and abundance of expressed proteins, applying the Pearson's correlation to calculate the distances.



Supplemental figure 12 Metabolomic-based model of the response of the human gut to antibiotic treatment. Schematic representation of distinct mass feature profiles based on the abundance level in bacterial cells from the faecal samples investigated in this study. Features were grouped based on the metabolite class, and the average corrected intensity values were calculated. Group 1: fatty acids (18), sphingolipids (1), glycerolipids (2), glycerophospholipids – LPA (1), sterol lipids – alkaloid (1); Group 2: fatty acid aldehyde / alcohol (1), sphingolipid (1), glycero(lyso)phospholipids – LysoPE / PA / PC / PE, fatty acid carnitine (2), fatty acid ethanolamide (1); Group 3: fatty acid ethanolamide (1), sterol lipid – D3 / bile acid / cholesterol (4), prostaglandin derivative (2), unsaturated fatty acid (1); Group 4: sphingolipid (1), sterol lipid – D3 / bile acid / cholesterol (1); Group 5: sterol lipid – corticoid (1). The number of metabolites per class is indicated in brackets.



Supplemental figure 13 Number of genes having close sequence similarity to genes that encode beta-lactamases found in metagenomes of microbial cells from faecal samples in this study that were found to be expressed (mRNAs). Functional assignment of predicted genes encoding beta-lactamases was performed via BLASTP analysis against the NCBI-nr database for similar sequences. All hits with an E-value of less than e^{-05} and sequence homology ≥ 50% were considered and manually analyzed. As a result, out of 401,555 sequences (FS-0: 83,622; FS-3: 63,159; FS-6: 58,853; FS-11: 19,267; FS-14: 33,288; FS-40: 143,366), 381 distinct beta-lactamase proteins (FS-0: 55 or 0.065% total open reading frames; FS-3: 69 or 0.109%; FS-6: 52 or 0.088%; FS-11: 11 or 0.057%; FS-14: 27 or 0.081%; FS-40: 167 or 0.116%) were identified. Among them 231 distinct genes were found to be expressed in the faecal microbiota (mRNAs) at different levels before, during and after AB treatment. The total number and relative percentage (referred to the total number of expressed beta-lactamases: 231) of expressed genes coding betalactamases is shown on the left and right, respectively.

3.2

"DIFFERENTIAL EFFECTS OF ANTIBIOTIC THERAPY ON THE STRUCTURE AND FUNCTION OF HUMAN GUT MICROBIOTA"

AUTHORS: Ana Elena Pérez Cobas, Alejandro Artacho, Henrik Knecht, María Loreto Ferrús, Anette Friedrichs, Stephan Ott, Andrés Moya, Amparo Latorre, María José Gosalbes Soler.

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Differential Effects of Antibiotic Therapy on the Structure and Function of Human Gut Microbiota

Ana Elena Pérez-Cobas[1,2], Alejandro Artacho[1], Henrik Knecht[3], María Loreto Ferrús[1], Anette Friedrichs[3,4], Stephan J. Ott[3,4], Andrés Moya[1,2], Amparo Latorre[1,2]*, María José Gosalbes[1,2]*

1 Unidad Mixta de Investigación en Genómica y Salud del Centro Superior de Investigación en Salud Pública e Instituto Cavanilles de Biodiversidad y Biología Evolutiva de la Universitat de València, Valencia, Spain, **2** CIBER en Epidemiología y Salud Pública, Madrid, Spain, **3** Institute for Clinical Molecular Biology at the Christian-Albrechts University, Kiel, Germany, **4** Department for Internal Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

Abstract

The human intestinal microbiota performs many essential functions for the host. Antimicrobial agents, such as antibiotics (AB), are also known to disturb microbial community equilibrium, thereby having an impact on human physiology. While an increasing number of studies investigate the effects of AB usage on changes in human gut microbiota biodiversity, its functional effects are still poorly understood. We performed a follow-up study to explore the effect of ABs with different modes of action on human gut microbiota composition and function. Four individuals were treated with different antibiotics and samples were taken before, during and after the AB course for all of them. Changes in the total and in the active (growing) microbiota as well as the functional changes were addressed by 16S rRNA gene and metagenomic 454-based pyrosequencing approaches. We have found that the class of antibiotic, particularly its antimicrobial effect and mode of action, played an important role in modulating the gut microbiota composition and function. Furthermore, analysis of the resistome suggested that oscillatory dynamics are not only due to antibiotic-target resistance, but also to fluctuations in the surviving bacterial community. Our results indicated that the effect of AB on the human gut microbiota relates to the interaction of several factors, principally the properties of the antimicrobial agent, and the structure, functions and resistance genes of the microbial community.

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* E-mail: maria.jose.gosalbes@uv.es (MJG); amparo.latorre@uv.es (AL)

These authors contributed equally to this work.

Introduction

Throughout evolution mammals have established symbioses with microbial communities, which are located in different organs and tissues of the body such as skin, mucosa, or the gastrointestinal tract. The gut microbiota in humans is a particularly complex ecosystem with few dominant phyla (Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria) but show greater microbial diversity at lower taxonomic levels and a high functional redundancy [1,2]. The gut microbiota seems to be host-specific and rather stable under non- or small perturbations [3] and is involved in a large number of host beneficial functions such as food processing, growth regulation of the intestinal epithelium, development of the immune system, or protection against pathogens [2,4,5]. Because of the essential role of the microbiota in host life, imbalances in the gut microbial community may have an important impact on human health. This is apparent in some intestinal pathologies such as inflammatory bowel diseases or antibiotic-associated diarrhea [6].

Systematic antibiotic (AB) therapy represents a major public health problem because gut microbiota may be transformed into a reservoir of antibiotic resistance genes, promoting the appearance of harmful resistant strains [7,8,9,10]. It also suppresses some protective members of the resident microbiota promoting overgrowth of opportunistic pathogens such as *Clostridium difficile* [11]. Moreover, AB therapy disturbs the gut microbiota and, concomitantly, affects human physiology, for instance carbohydrate metabolism or immunity [7,12].

Antibiotic features such as class, spectrum or pharmacological properties affect the gut microbiota in different ways [13]. In addition, host-associated factors such as diet, life history, genetic or health status, properties of the gut microbial ecosystem itself like resistance and resilience, or even the interplay between the microbiota and its host also have an effect on microbiota composition and function. All these factors can mask changes caused exclusively by antibiotics, representing a real challenge when it comes to understand microbiota responses. Most recent studies into the impact of antibiotics on the microbiota have focused on the emergence of resistant strains, but few have described their influence on the microbial community itself [14,15,16,17,18]. These latter surveys, using 16S rRNA gene sequencing, have shown that short and long-term AB courses affect diversity and biomass of the intestinal microbiota, with microbial composition resilience remaining deficient for long time

after AB-treatment [15,16,17,18]. By contrast, the functional impact of AB on the microbial ecosystem has been addressed less frequently [19].

The use of the meta-"omics" approaches (metagenomics, metatranscriptomics, metaproteomics) has provided deeper insights into microbial communities in different ecosystems [2,20,21,22,23]. A recent integrated analysis has provided a better understanding of the nature of the complex processes underlying the whole human gut microbiota and its responses during beta-lactamic-therapy [12].

In the present work we studied the effect of different antibiotics on the human gut microbiota by a follow-up study comparing microbial communities before, during and after AB therapy in four individuals. We analyzed the changes in composition of the total (16S rRNA gene) and active (16S rRNA transcripts) microbiota throughout treatment. Furthermore, the functional analysis of the total gene content of the community showed, for the first time, how the mode of action and the antimicrobial effect of AB affected the functional potential of the community. Finally, we described the dynamics of resistance genes (i.e. the resistome) throughout the study, paying particular attention to those that become resident after AB-therapy.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of the Medical Faculty of the Christian-Albrechts University Kiel, Germany. Informed written consent was obtained from all patients involved in the study.

Sample collection and AB treatment regimen

Fecal samples were collected from four patients (herein referred to as patient A, B, C and D) at the Department of Internal Medicine of the University Hospital Schleswig-Holstein, Campus Kiel, Germany (UK-SH). Patient A was treated with moxifloxacin (400 mg/day) for 13 days. Moxifloxacin is a fourth-generation synthetic fluoroquinolone antibacterial agent with a bactericidal effect inhibiting cell replication. AB treatment for patient B consisted of a combined therapy with penicillin G and clindamycin on the day of admission, and subsequently with clindamycin alone (3×300 mg/day) for seven days. This semi-synthetic derivative belongs to the lincosamide class exerting a bacteriostatic effect due to the inhibition of protein synthesis. For patient C, AB therapy was initiated with cefazolin (3×2 g/day) for seven days and continued with ampicillin/sulbactam (2×750 mg) for seven more days. Patient D received an amoxicillin (3×1000 mg/day) treatment. The antibiotics used for these two latter two patients belong to the beta-lactam class and have a bactericidal effect inhibiting cell envelope synthesis. Main features of patients and therapy are shown in Table 1.

Fecal samples from patients (named A, B, C and D) were collected on the day of admission, before the antibiotic treatment (day 0), during and after AB therapy. In two cases (A and B), the last sample was taken 3 days after therapy, in the other two cases (B and D) the last sample was provided 7 and 28 days after treatment, respectively (Table1). Patients did not present any intestinal disorder. Samples were collected in sterile tubes and stored at −80°C until further processing.

DNA extraction

Tubes containing fecal samples with sterile PBS (containing, per liter, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2PO4 [pH 7.2]) were centrifuged at 1250 g and 4°C for 2 min to remove fecal waste. DNA was extracted from bacterial pellets using QIAamp® DNA Stool Kit (Quiagen) following the manufacturer's instructions. The product was concentrated by precipitation using 0.1 V of NaCl 3 M and 2 V of ethanol 100% and diluted in 75 µl of nuclease-free water. A standard agarose gel electrophoresis was run to check the integrity of DNA. The total DNA obtained was quantified with Nanodrop-1000 Spectrophotometer (Thermo Scientific) and with the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen).

Amplification of the 16S rRNA gene

For each sample a region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR). The primers used were the universal E8F (5'-AGAGTTTGATCMTGGCTCAG-3') with adaptor A and 530R (5'-CCGCGGCKGCTGGCAC-3') with adaptor B using the sample-specific Multiplex Identifier (MID) for pyrosequencing. The amplified region comprises hyper-variable regions V1, V2 and V3. For each sample a 50 µl PCR mix was prepared containing 5 µl of Buffer Taq (10X) with 20 mM MgCl2, 2 µl of dNTPs (10 mM), 1 µl of each primer (10 mM), 0.4 µl of Taq Fast start polymerase (5 u/ µl), 39.6 µl of nuclease-free water and 1 µl of DNA template. PCR was run under the following conditions: 95° for 2 min followed by 25 cycles of 95° for 30 s, 52° for 1 min and 72° for 1 min and a final extension step at 72° for 10 min. The amplification process was checked by electrophoresis in agarose gel (1.4%). PCR products were purified using NucleoFast® 96 PCR Clean-Up Kit (Macherey-Nagel) and quantified with Nanodrop-1000 Spectrophotometer (Thermo Scientific) and with the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen). The pooled PCR products were directly pyrosequenced.

Total RNA extraction and double-strand cDNA synthesis

Total RNA was extracted from fecal samples using RiboPure™-Bacteria Kit (Ambion). DNase treatment was applied to remove traces of genomic DNA from the eluted RNA using the same kit. The integrity of RNA was checked by electrophoresis in agarose gel (0.8%). The efficiency of the DNase treatment was checked by amplifying each RNA sample by PCR. To retro-transcribe total RNA into single-stranded cDNA the High-Capacity cDNA Reverse Transcription Kit (Ambion) was used. To synthesize double-stranded cDNA, 7.5 µl of *Escherichia coli* ligase buffer (10X), 2 µl of dNTPs (10 mM), 0.2 µl of *E. coli* RNAse H (5 u/µl), 3 µl of *E. coli* DNA pol I (10 u/µl), 0.5 µl of *E. coli* ligase (10 u/µl) and 41.8 µl of nuclease-free water were added to each single-stranded cDNA sample. The mixture was placed in a Thermocycler at 15°C for 2 hours. Then, 2.5 µl of T4 DNA polymerase (3 u/µl) were added and kept at 15°C for 30 min. The metatranscriptome obtained thus was purified by precipitation and quantified using Nanodrop-1000 Spectrophotometer (Thermo Scientific) and the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen). A standard agarose gel electrophoresis was run to check the integrity of double-stranded cDNA.

Pyrosequencing

For each sample, the total DNA (metagenome), double-stranded cDNA and amplicons of the 16S rRNA gene were sequenced with a Roche GS FLX sequencer and Titanium chemistry in the company Life Sequencing (Valencia, Spain) and in the Center for Public Health Research (CSISP-FISABIO) (Valencia, Spain). We obtained an average of 58,928, 41,838 and 4,872 reads per sample, respectively.

Table 1. Main features of the follow-up study.

Patient	Antibiotic	Mode of action/ Antimicrobial effect	Pathology	Sampling date	Samples
A	Moxifloxacin	Cell replication inhibitor/ Bactericidal	Bronchitis, pneumonia	day0-before AB	A_before
				day3-during AB	A3_D
				day6-during AB	A6_D
				day10-during AB	A10_D
				day13-during AB	A13_D
				3 days after AB	A_after
B	Clindamycin	Protein synthesis inhibitor/ Bacteriostatic	Erysipelas	day0-before AB	B_before
				day2-during AB	B2_D
				day5-during AB	B5_D
				day6-during AB	B6_D
				28 days after AB	B_after
C	Cefazolin/ Ampicillin/ Sulbactam	Cell envelop synthesis inhibitor/ Bactericidal	Infection pacemaker	day0-before AB	C_before
				day3-during AB	C3_D
				day6-during AB	C6_D
				day10-during AB	C10_D
				3 days after AB	C_after
D	Amoxicillin	Cell envelop synthesis inhibitor/ Bactericidal	Chronic sinusitis maxillans	day0-before AB	D_before
				day3-during AB	D3_D
				7 days after AB	D_after

AB, antibiotic; D, during the treatment.
doi:10.1371/journal.pone.0080201.t001

Taxonomic assignment of 16S rRNA amplicons

We have used the Ribosomal Database Project (RDP) pyrosequencing pipeline [24] to trim off the MID and primers and to obtain the taxonomic classification. Sequences with a phred quality score less than 20 (Q20) and short length (<250pb) were discarded. We considered only annotations that were obtained with a bootstrap value greater than 0.8, leaving the assignation at the last-well identified level and consecutive levels as unclassified (uc).

Taxonomic assignment of 16S rRNA transcripts

Due to the procedure followed to obtain the metatranscriptome, the vast majority of transcripts belonged to ribosomal genes (16S and 23S). The 16S rRNA reads were obtained from the total cDNA by comparing the total reads against the Small Subunit rRNA Reference Database (SSUrdb) [25] with BLASTN [26] and an e-value of 10-^{16}. All sequences with detected homology were considered as 16S rRNAs and used to evaluate the phylogenetic diversity of the active bacteria. The taxonomic classification was performed in the same way as the amplicons.

Analysis of total and active microbiota

To study the phylogenetic structure of the bacterial community we applied two approaches that involved the 16S rRNA gene. The widely used analysis of 16S rRNA gene amplicons shows the composition of the total microbiota (16S rRNA gene). However, since the growing (active) bacteria contain more ribosomal RNA than latent or starved cells, studying the 16S ribosomal RNA transcripts enabled the active members of the microbiota to be identified (16S rRNA transcripts) [12,22]. We calculated sample diversity of the throughout the treatment for total and active bacteria by applying three parameters: two richness estimators, Chao1 [27] and the abundance-based coverage (ACE) [28], and the Shannon index [29]. These estimators are implemented in package Vegan [30] under R software (http://cran.r-project.org) [31]. The biodiversity index and richness estimators were calculated after sub-sampling with the multiple_rarefactions.py script of QIIME to avoid the bias of the sequencing effort [32]. We used heat maps based on taxonomic composition to study the similarity between samples due to the relative abundance of each taxon using the Vegan library in the R software (http://cran.r-project.org) [30,31]. Canonical correspondence analysis (CCA) was performed to determine the relation between the sample composition and the class and mode of AB-treatment. To statistically assess the effect of such factors on the bacterial composition a multivariate ANOVA based on dissimilarity tests (Adonis) was applied, as implemented in the package Vegan, R software (http://cran.r-project.org) [30,31].

Metagenomics: functional analysis

To eliminate reads that were artifact replicates of pyrosequencing, we used the 454 Replicate Filter Program [33] with the following parameters: sequence identity cutoff = 1; length difference requirement = 0; number of beginning base pairs to check = 10. Unique reads were compared against the human genome using BLASTN [26] with an e-value of 10^{-10} in order to remove human sequences. To identify the sequences encoding the ribosomal gene 16S rRNA we compared the dataset against the Small Subunit rRNA Reference Database (SSUrdb) described in Urich et al. [25] using BLASTN [26] with an e-value of 10^{-16}. Sequences that did not give homology were used to identify the reads corresponding to the ribosomal gene 23S rRNA by BLASTN [26] against the Large Subunit rRNA Reference Database (LSUrdb) described in Urich et al. [25] with an e-value of 10^{-4}. Reads that matched with the LSUrdb were discarded. The remaining reads were compared to the NCBI-nr protein

database using BLASTX [26] to identify the protein-coding genes. Taxonomic assignment was based on the output of BLASTX applying the lowest common ancestor (LCA) algorithm. Fasta files were used to identify the Open Reading Frames (ORFs) by applying the facility of Fraggenscan from the web server of metagenomic analysis (WebMGA) [34]. To annotate the functions of the predicted ORFs, we applied HMMER 3.0 program [35] against TIGRFAM database [36] using default parameters. To identify the genes involved in resistance to antibiotics, we compared the identified ORFs against the Antibiotic Resistance Genes Database by BLASTp [37] with an e-value of 10^{-10}. We used the ShotgunFunctionalizeR package [38] in the R software http://www.R-project.org/ [31] for functional comparison of metagenomes. Specifically, we applied the testGeneCategories.-dircomp function to compare the distribution of functional categories between groups of samples. The test is based on a Poisson model and compares each gene family of a higher functional category to decide if the category is statistically significant among two groups of samples [38].

Data accession number

All sequences have been entered in the European Bioinformatics Institute database, under accession number ERP002192.

Results

Dynamics of total and active microbiota composition throughout therapy

We analyzed total (16S rRNA gene) and active (16S rRNA transcripts) microbiota from the four patients (A, B, C and D) throughout AB treatment. The antibiotics administered to patients A, C and D had a bactericidal antimicrobial effect, whereas in patient B the effect was bacteriostatic. Regarding the mode of action, the antibiotic used in patient A was a cell replication inhibitor, in patient B it was an inhibitor of protein synthesis, whereas patients C and D were treated with a cell envelop synthesis inhibitor (Table 1). Each patient not only presented their own microbiota profile for both total (Figure 1A) and active (Figure 1B) microbiota before treatment, but also there was apparently a rather large variation in bacterial taxa abundance during and after treatment, which we describe succinctly. In patient A, both total and active microbiota showed a high presence of the families Lachnospiraceae (*Coprococcus* and *Roseburia* genera) and Ruminococcaceae (*Faecalibacterium*, *Blautia* and *Subdoligranulum* genera) during AB treatment with fluoroquinolone (Figure 1). However, some genera such as *Faecalibacterium* and *Subdoligranulum* were negatively affected by the AB, while others such as *Blautia*, *Coprococcus*, *Coprobacillus* and *Collinsella* appeared to be resistant in the first stage of treatment. We also found that the bactericidal effect of AB had a negative impact on *Bacteroides* genus (Bacteroidetes phylum) in the first days of treatment, but the trend changed on day 13 with a great increase in its abundance. Treatment with clindamycin of patient B resulted in a high presence of Enterobacteriaceae (*Escherichia*, *Salmonella* genera), as shown in Figure 1B. We also observed an increase in the *Bacteroides* genus after the 5th day of treatment in active microbiota. For patient C, Oscillibacteriaceae and Ruminococcaceae families (Firmicutes phylum) as well as Rikenellaceae and Bacteroidaceae (Bacteroidetes phylum) constituted the most abundant taxa in the total microbiota (Figure 1A). The first important change occurred on day 6 with an increase in *Parabacteroides* (Bacteroidetes phylum), which remained abundant after treatment. However, in the active microbiota we observed a shift towards the Bacteroidetes phylum (*Alistipes* and *Bacteroides* genera) at the two last time points. On the 10th day of treatment, an increase in facultative anaerobic families, Enterobacteriaceae (Proteobacteria) and Enterococcaceae (Firmicutes) was found. Finally, in patient D, the initial microbiota composition consisted mainly of Enteriobacteriaceae (*Escherichia* genus) and Ruminococcaceae (*Faecalibacterium* genus). However, both genera were greatly affected by the antibiotic as there was an increase in resistant bacterial taxa of the *Bacteroides* genus (Bacteroidetes).

After the AB course, patients A, C and D who received a bactericidal antimicrobial agent clustered together in both cases, total (Figure 2A) and active (Figure 2B) microbiota, apart from the patient treated with a bacteriostatic antibiotic (patient B) (Figure 1 and 2). Moreover, we observed that the two patients treated with cell envelope synthesis inhibitors (C and D) grouped together in the case of the active microbiota (Figure 2B).

For all patients, the diversity parameters (Shannon index, Chao1 and ACE estimators) of both total and growing microbiota, showed notable fluctuations with a decrease in the number of bacterial taxa and evenness on the first days of treatment (Figure S1). At the end of the AB course, these three biodiversity parameters increased but they did not reach the initial values observed before AB therapy (Figure S1).

Effect of the class of antibiotic

To evaluate the pattern of variation shown by bacterial taxa or gene abundances and its relationship with two variables (the antimicrobial effect -bactericidal and bacteriostatic- and the mode of action of the antibiotic -protein synthesis inhibitor, cell replication inhibitor and cell envelope synthesis inhibitor-) we applied a CCA at the different levels: 16S rRNA gene, 16S rRNA transcripts, genes and the taxonomy of the identified coding regions (gene taxonomy). The results showed that these two factors (antimicrobial effect and mode of action) accounted for variability in a particular direction and with different strength (Figure 3). Figure 3A shows that the first axis explained 19% of variability, splitting the total microbiota (16S rRNA gene) of the patients that were medicated with bactericidal AB (patients A, C and D) from the one treated with a bacteriostatic AB (patient B). The second axis explained 12% of variability, placing the two groups of samples treated with cell replication inhibitor (patient A) and protein synthesis inhibitor (patient B) antibiotics on one side of the graph; these ABs inhibit both essential and related cellular processes, such as DNA replication and protein synthesis. By contrast, the samples from patient C, treated with a cell envelope synthesis inhibitor AB affecting synthesis of the bacterial cell wall, fell on the other side of the graph. Both variables (antimicrobial effect and mode of action) introduced significant variance in the microbiota composition (Adonis test: $p = 0.02$, $p = 0.04$, respectively).

Regarding active microbiota (Figure 3B), the first and second axes explained 12% and 6% of the total variability, respectively. With respect to the first axis, the samples from patient B (protein synthesis inhibitor antibiotic) situated on the right side of the graph. The latter AB, as occurred for the total microbiota, introduced higher variance. The second axis separated the samples taken from patients C and D, treated with cell envelope synthesis inhibitor AB from the rest. Despite both factors, the antimicrobial effect and mode of action were not significant (Adonis test: $p = 0.18$, $p = 0.069$), the second explained more variability (Adonis test, $p = 0.069$).

The CCA applied at gene level (Figure 3C) showed a distribution similar to that found for the total microbiota (Figure 3A), with the first and the second axes explaining 25% and 6% of the total variance, respectively. The samples are separated chiefly

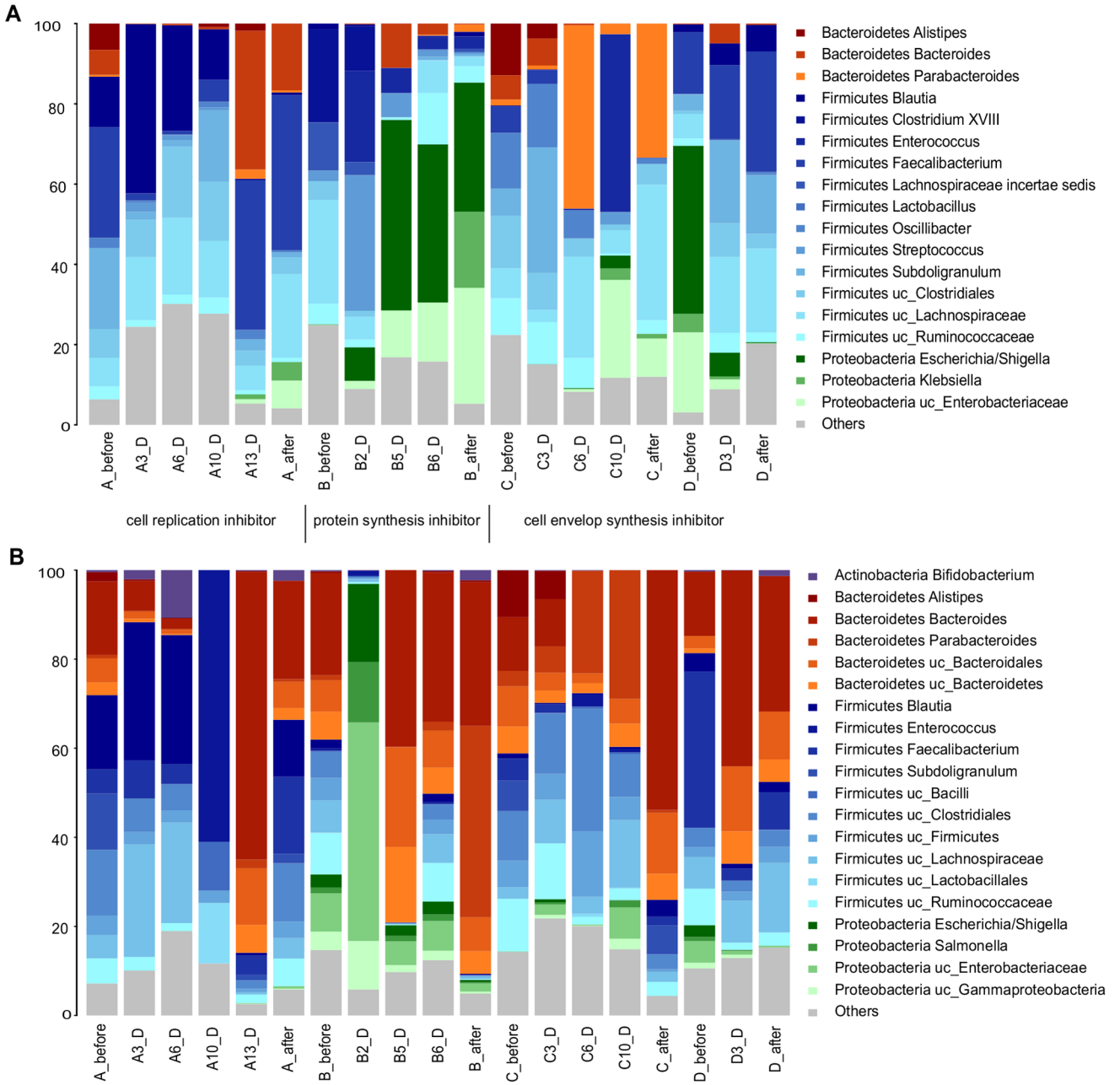

Figure 1. Microbiota composition of patients A, B, C, and D. (A) total microbiota (16S rRNA gene) (B) active microbiota (16S rRNA transcripts). The mode of action for each AB used is indicated.
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by antimicrobial effect and then by mode of action. However, in this case the strength of the different vectors is weaker, probably due to the great functional homogeneity of the gut microbial community (Adonis test: antimicrobial effect $p = 0.27$, mode of action $p = 0.41$).

Finally, we performed a CCA using the taxonomy of the identified coding regions (Figure 3D). The two axes explained 19% and 7% of the total variation of the data. As can be seen, the different classes of antibiotics affected the taxonomy of the identified coding regions in a similar way to the results reported for total microbiota (Figure 3A) and gene analysis (Figure 3C) (Adonis test: antimicrobial effect $p = 0.044$, mode of action $p = 0.049$).

Functional analysis of the metagenomes

The functional annotation of the ORFs was derived using the TIGRFAM database, providing a hierarchical order: main roles, the highest functional level (described in Figure S2), sub-roles, more specific metabolic functions for each one of the main roles and genes [36]. Regarding the main roles, the profiles were fairly homogeneous for all patients and time points. The most abundant categories before, during and after treatment were "Protein synthesis", "Energy metabolism", "Cellular processes" and "Transport and Binding Proteins" with average values of relative abundance of 13.5%, 13.2%, 9.6% and 9.5%, respectively, which highlights the importance of these functions performed by the gut microbiota (Figure S2). However, when considering the sub-roles,

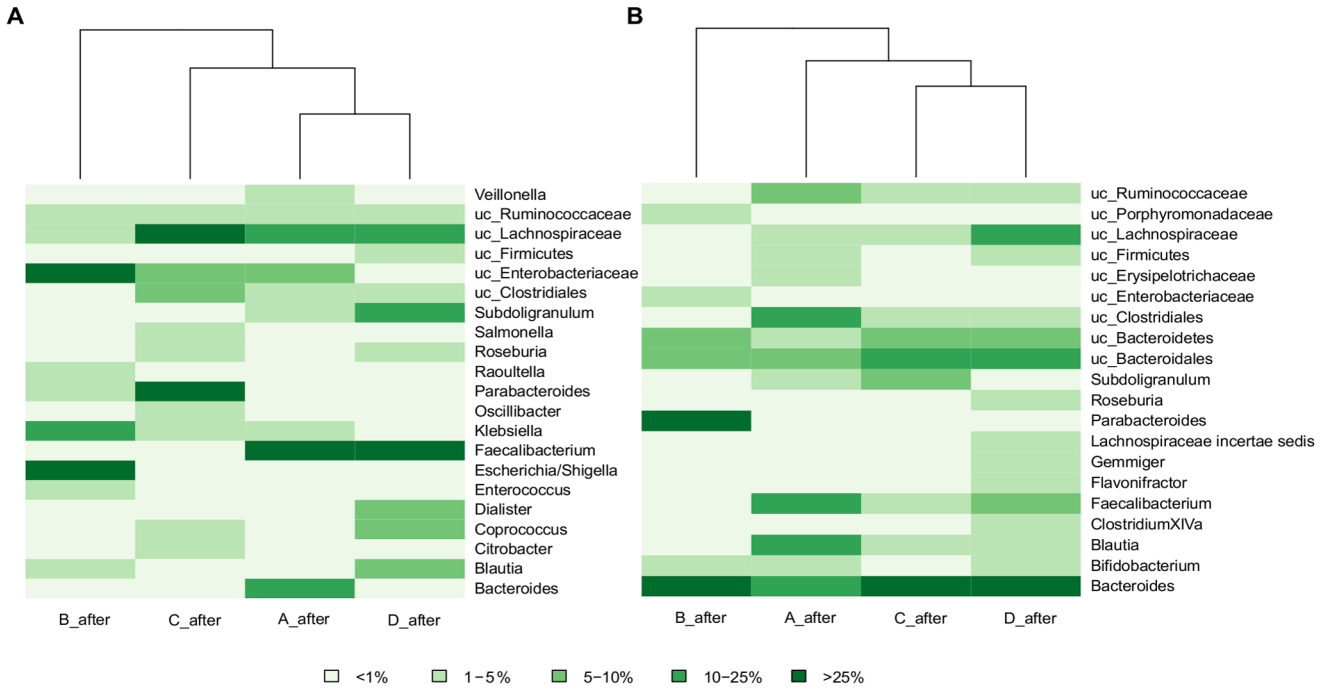

Figure 2. Heat map and clustering based on taxon composition and abundance. (A) total microbiota, (B) active microbiota. Colors in the figure depict the percentage range of sequences assigned to main taxa (abundance >1% in at least one sample).
doi:10.1371/journal.pone.0080201.g002

we detected significant changes in the corresponding profiles for each patient before, during and after treatment. For all patients we detected a total of 53 sub-roles that differed significantly in gene content (Table 2). Only two sub-role functional categories changed significantly in all patients: "Menaquinone and ubiquinone" (within the main function "Biosynthesis of cofactors, prosthetic groups, and carriers") and "Carbohydrates, organic alcohols, and acids" (within the main function of "Transport and binding proteins"). Genes participating in the biosynthesis of menaquinone and ubiquinone were under-represented during the treatment for all patients, except in the case of patient B. Regarding the sub-role of "Carbohydrates, organic alcohols, and acids" the gene functions were overrepresented during the treatment in patients A, B and C and under-represented in patient D. Most of the genes belonging to this functional group were related to the phosphotransferase system (PTS), which is essential for translocating carbohydrates in bacteria [39]. Within this family, we have found genes involved in the transport of various sugars such as mannose, fructose, sorbose, glucitol or glucose. It is noteworthy that the related sub-role "PTS" (within the main role "Signal transduction"), associated to genes participating in regulation, was also over-represented during the treatment in patients A and C and under-represented in patient D.

The changes in the sub-roles "Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides" and "Other" (from the "Cell envelope main role") were significant in patients A, B and D. In general, we detected a lower presence during treatment of genes involved in the synthesis of lipopolysaccharides (LPS). The sub-role "Pathogenesis" (within the main category "Cellular processes") and "Degradation" (within the "Fatty acid and phospholipid metabolism" main category) decreased significantly during treatment for patients A and D and increased in patient B. The fatty acid and phospholipid metabolism genes were involved in fatty acid beta-oxidation. On the contrary, the sub-role "Sporulation and germination" (within "Cellular processes") was more abundant during treatment in patients A and D, with most of the genes being involved in different stages of endospore formation. Finally, we found that the sub-role "TCA cycle" (within the main category "Energy metabolism") and "Amino acids, peptides and amines" (within "Transport and binding proteins) underwent an increase in the number of genes encoding different enzymes of the citric acid cycle during antibiotic treatment for patient A and B and a decrease for D. Regarding the transport of amino acids, peptides and amines, we found the presence of genes encoding ABC transporters for amino acids and urea.

Analysis of the resistome

We performed a search of the resistome by identifying AB resistance genes in the 19 metagenomes analyzed by comparing the predicted ORFs against the Antibiotic Resistance Genes Database [37]. We identified the resistance genes that represented 0.2%, 0.8%, 0.22%, and 0.5% of the total determinants found for patients A, B, C and D respectively. We found that while patients A, B, and C showed an increase in resistance genes after treatment, patient D presented the lowest relative abundance of these determinants, decreasing from 0.81% to 0.14% (Figure 4A). Figure 4B shows the profiles of resistance genes that varied during the treatment for patient A, B and C, administered with antibiotics belonging to different classes: fluoroquinolone, lincosamide and beta-lactams, respectively (Table 1). Overall, we observed that the resistance induced by each antimicrobial was associated with other resistance determinants. Also, we found that its profiles matched fairly well with the oscillatory dynamics of the surviving bacterial community. Patient A showed an increase in the relative abundance of the total resistance genes at the end of the treatment, raising values from 0.18% before treatment to 0.28% after the AB course. Fluoroquinolone resistance, multidrug

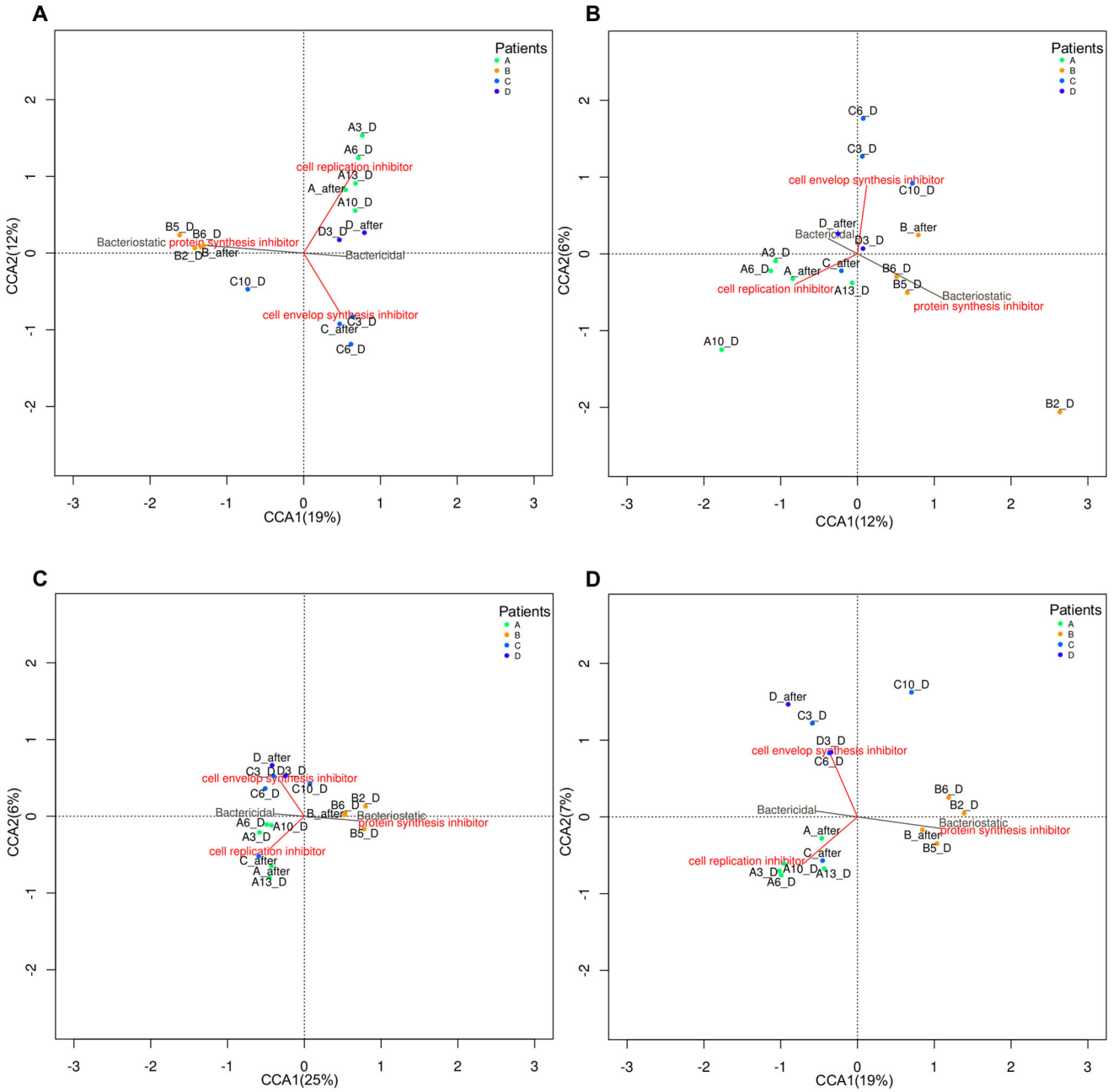

Figure 3. Canonical Correspondence Analysis (CCA) of the patients A, B, C and D in the follow-up study. (A) total microbiota, (B) active microbiota, (C) genes and (D) gene taxonomy. The antimicrobial effect is represented as a vector with two levels (bactericidal and bacteriostatic). The mode of AB action is represented as a vector with three levels (cell envelop synthesis inhibitor, cell replication inhibitor and protein synthesis inhibitor).
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resistance efflux pump, appeared on day 10, when the microbiota composition was dominated by the Firmicutes phylum, with high abundance of members of the genus *Enterococcus*, described as resistant to this type of antimicrobial [40]. This patient presented high relative abundance of *tetq* gene, which confers tetracycline resistance before and after the treatment, being *Bacteroides* genus one of the most abundant taxa. Bacitracin profile showed a maximum on day 6, with *baca* gene being associated to *Streptococcus* and Clostridiales taxa (Figure 1 and 2). Patient B showed a strong increase in the relative abundance of resistance genes during treatment, increasing from 0.29% up to 0.89%. In fact all the genes increased in abundance after AB treatment except those involved in bacitracin resistance. The most remarkable increase was found in a group of genes coding for multidrug resistance efflux pump, which confer resistance against clindamycin and related antimicrobials (aminoglycoside, glycylcycline, beta-lactam, macrolide, and acriflavine). Patient C also showed an increase in the total relative abundance of resistance genes at the last time point and after treatment, from 0.16% to 0.36%. The genes that code for multidrug resistance efflux pumps are the most abundant on the 10th day of treatment. However, beta-lactamase genes increased throughout the AB course and reached the maximum at

Table 2. Functional profiles.

		Patient			
Main Role	**Sub-Role**	**A**	**B**	**C**	**D**
Amino acid biosynthesis	Glutamate family	↑ 2.06E-004	NS	NS	NS
	Histidine family	↑ 0.02	NS	NS	NS
	Serine family	↑ 4.36E-004	NS	NS	↑ 0.01
Biosynthesis of cofactors*	Biotin	NS	↑ 0.02	NS	↓ 2.33E-004
	Glutathione and analogs	NS	↑ 2.95E-003	NS	↓ 0.04
	Menaquinone and ubiquinone	↓ 0.02	↑ 2.19E-005	↓ 0.04	↓ 4.68E-003
	Molybdopterin	NS	NS	NS	↓ 4.35E-003
	Pantothenate and coenzyme A	↑ 0.01	NS	NS	NS
	Other	↑ 1.33E-004	NS	NS	NS
Cell envelope	Biosynthesis and degradation of surface**	↓ 0.05	↑ 3.01E-005	NS	↓ 0.02
	Other	↑ 0.01	↓ 0.01	NS	↑ 0.02
	Surface structures	NS	NS	NS	↑ 0.04
Cellular processes	Biosynthesis of natural products	NS	NS	NS	↓ 0.05
	Cell division	NS	↓ 0.01	NS	↑ 3.07E-004
	Chemotaxis and motility	NS	NS	NS	↑ 0.01
	Detoxification	NS	NS	NS	↓ 0.04
	DNA transformation	↓ 2.32E-003	NS	↓ 0.01	NS
	Pathogenesis	↓ 9.43E-004	↑ 1.47E-005	NS	↓ 2.22E-009
	Sporulation and germination	↑ 0.03	↓ 7.07E-017	NS	↑ 2.65E-017
	Toxin production and resistance	NS	↓ 2.85E-004	NS	NS
Central intermediary metabolism	Amino sugars	NS	NS	NS	↑ 0.03
	Nitrogen metabolism	NS	↑ 0.02	NS	NS
DNA metabolism	Chromosome-associated proteins	NS	NS	↓ 0.01	NS
	Restriction/modification	↑ 0.02	NS	NS	NS
Energy metabolism	Aerobic	↓ 0.01	NS	NS	↓ 0.01
	Amino acids and amines	NS	↑ 9.26E-007	NS	↓ 2.22E-009
	Anaerobic	NS	NS	NS	↓ 4.80E-005
	Biosynthesis and degradation of polysaccharides	↓ 1.58E-003	NS	NS	NS
	Chemoautotrophy	NS	↓ 0.01	NS	NS
	Electron transport	NS	↑ 0.01	NS	NS
	Entner-Doudoroff	NS	↑ 2.85E-004	NS	↓ 0.01
	Fermentation	NS	NS	NS	↑ 0.01
	Pentose phosphate pathway	NS	↑ 0.01	NS	↓ 0.03
	Sugars	NS	↑ 2.47E-005	NS	↓ 0.04
	TCA cycle	↑ 0.05	↑ 0.02	NS	↓ 4.59E-003
Fatty acid and phospholipid metabolism	Degradation	↓ 0.05	↑ 0.04	NS	↓ 0.03
Protein fate	Protein and peptide secretion and trafficking	NS	NS	NS	↓ 0.02
	Protein folding and stabilization	NS	↓ 0.01	NS	NS
Protein synthesis	Other	↓ 0.03	↓ 4.57E-005	NS	NS
	Ribosomal proteins: synthesis and modification	NS	↓ 2.85E-004	NS	↑ 4.80E-005
	tRNA and rRNA base modification	NS	NS	↑ 2.04E-003	NS
Regulatory functions	Other	NS	NS	↑ 0.01	NS
Signal transduction	PTS	↑ 1.40E-005	NS	↑ 3.59E-009	↓ 7.09E-009
	Two-component systems	NS	NS	NS	↓ 1.52E-003
Transcription	DNA-dependent RNA polymerase	NS	↓ 4.49E-003	NS	↑ 0.03
Transport and binding proteins	Amino acids, peptides and amines	↑ 4.36E-004	↑ 2.85E-004	NS	↓ 4.32E-005
	Anions	↓ 4.36E-004	NS	NS	NS
	Carbohydrates, organic alcohols, and acids	↑ 4.36E-004	↑ 3.62E-006	↑ 1.46E-004	↓ 3.76E-033

Table 2. Cont.

		Patient			
Main Role	**Sub-Role**	**A**	**B**	**C**	**D**
	Cations and iron carrying compounds	↑ 3.03E-003	NS	NS	↓ 0.04
	Nucleosides, purines and pyrimidines	NS	↑ 2.61E-003	NS	↓ 1.52E-003
	Other	NS	↑ 1.33E-003	NS	↓ 5.56E-004
	Porins	NS	↑ 0.01	NS	NS
Unknown function	Enzymes of unknown specificity	↓ 0.04	NS	NS	↓ 4.97E-005

Main roles and sub-roles that change significantly during treatment and their associated p-values (p-value < 0.05). The upward arrow indicates those categories that were more abundant during treatment and the downward arrow those that were less abundant. NS, not significant.
*Biosynthesis of cofactors, prosthetic groups, and carriers.
**Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides.
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the end of treatment. Patient C also presented a high abundance of tetracycline resistance genes before treatment (*teto*, *tetq* and *tetw*) associated to different taxa (*Blautia*, *Bacteroides*, *Clostridium*, *and Ruminococcus*), which have been described as resistant to this antibiotic [41,42,43] but underwent a dramatic decrease on day 10 associated with a major presence of the Proteobacteria phylum.

Discussion

Dynamics of the gut microbiota structure over the AB course

The human gut microbiota consists of a highly complex community whose members establish close relationships with the host. ABs have strong direct and indirect effects on the human gut microbiota and consequently on the functions they perform, affecting the ecosystem maintenance and therefore host physiology [7,13]. The microorganisms that carry certain genetic determinants have an advantage under AB pressure, allowing them to survive and grow. It is well known that the human gut microbiota presents a high inter-individual variability and that its composition depends on factors such as genetics, age, diet, health status and AB-therapy, among others.

In our study, each patient presented their own initial microbiota and thus there was an individual response to AB treatment with fluctuations in the bacterial diversity and composition for both total and active gut microbiota. These results highlight the importance of the initial microbial structure in shaping the changes in microbiota during the AB course. The individual character of the response and incomplete recovery of initial microbiota after AB treatment has previously been described by Dethlefsen and coworkers [18] in a follow-up study of three patients that received two courses of ciprofloxacin. However, we also observed that the selection of resistant microorganisms led to a similar microbiota composition after analogous antibiotic treatment. Thus, AB seems to have a major impact on the structure of the final bacterial community.

The gut microbiota has been described as an ecosystem that is relatively resistant to perturbations [16]. However we observed that a particular assembly of microorganisms can confer greater resistance to a disturbance than others in terms of presence and abundance of taxa, which could be related with the specific effect of the AB. In patient A, there was a decrease in *Faecalibacterium* and *Bacteroides* genera during the AB course, with AB-resistant strains appearing at the end of treatment. However, on the first days of treatment, other butyrate-producing taxa (*Roseburia* and *Lachnospiraceae incertae sedis)* and H2-consuming bacteria (*Blautia*, *Collinsella* and *Bifidobacterium*) were present as active microbiota, obtaining energy sources for the colonocytes of the host. A similar behavioral pattern of these members of the intestinal microbiota has also been reported by the above mentioned group of Dethlefsen et al. [15,18] when they used ciprofloxacin, as in our patient A, an AB belonging to the cell replication inhibitor group. In the case of patient B, since clindamycin affected anaerobic bacteria, there were marked decreases in *Bacteroides* and *Blautia* genera in the active microbiota just after AB administration (Figure 2B). However, three days later, high abundances of *Bacteroides* were detected in the active bacterial community, suggesting these bacteria acquired resistance. The presence of clindamycin-resistant *Bacteroides* in gut microbiota has also been described in other studies [9,44]. Moreover, we observed that the decrease in anaerobic bacteria is compensated for by an important increase in members of the family Enterobacteriaceae. Patients C and D were treated with ABs which have a similar mode of action as both are of the β-lactam class. As stated, the initial microbiota composition was very different between both subjects, showing a differential response to ABs. However, the active microbiota changed throughout AB treatment with both patients acquiring a similar composition by the end of it (Figure 2B). Patient C received a combination of two ABs, Cefazolin and Ampicillin/Sulbactam, which cover a broad spectrum of microorganisms and showed a significant increase in *Parabacteroides* and *Bacteroides* genera. Interestingly, resistance genes against ampicillin and cephalosporin in these two taxa have been described previously [43]. On day 10 of treatment, an increase in the Enterobacteriaceae family occurred and some of its genera, such as *Escherichia* or *Klebsiella* are considered as opportunistic pathogens [45], suggesting that AB use creates opportunistic infections by these harmful microorganisms. Patient D was treated with amoxicillin, described as active against some Proteobacteria such as *Escherichia* or *Klebsiella*. During treatment, these genera were almost eliminated, whereas *Bacteroides*, *Blautia* and *Faecalibaterium* taxa proved less susceptible to treatment, as occurred in patient C. It is worth pointing out that this bacterial profile, with *Bacteroides*, *Blautia* and *Faecalibaterium* after the AB stress, has been found in the case of bactericidal agents but not when a bacteriostatic antimicrobial was used. Furthermore, a previous study [12] found a similar pattern of active bacteria after beta-lactam treatment.

Antimicrobial effect and mode of action of ABs on the gut microbiota

It has been stated that external variables such as ABs shift the microbial composition [46]. In our study, the class of AB

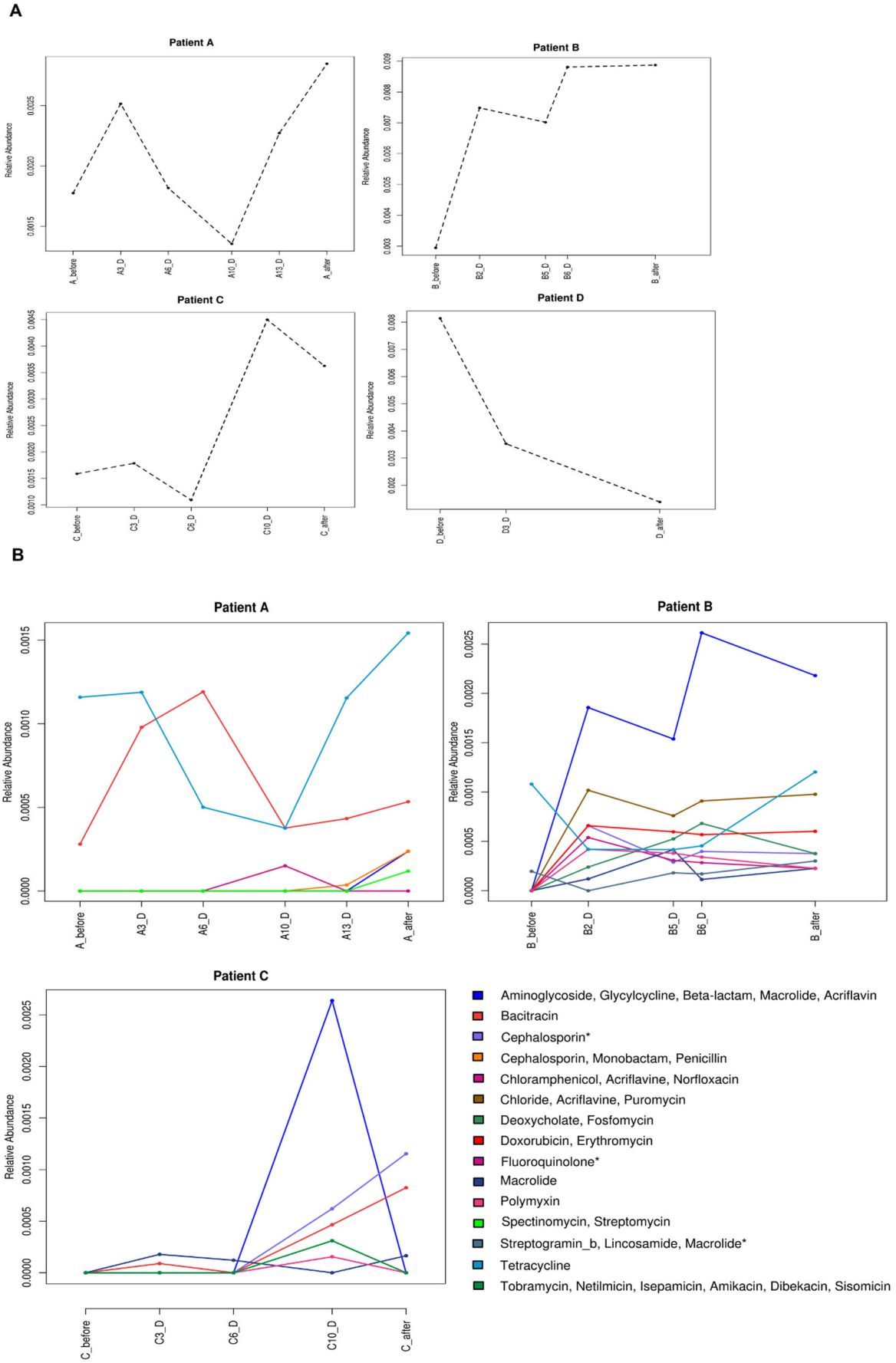
A
Patient A
Relative Abundance
A_before
A3_D
A6_D
A10_D
A13_D
A_after
Patient B
B_before
B2_D
B5_D
B6_D
B_after
Patient C
C_before
C3_D
C6_D
C10_D
C_after
Patient D
D_before
D2_D
D_after
B
Patient A
Patient B
Patient C
Aminoglycoside, Glycylcycline, Beta-lactam, Macrolide, Acriflavin
Bacitracin
Cephalosporin*
Cephalosporin, Monobactam, Penicillin
Chloramphenicol, Acriflavine, Norfloxacin
Chloride, Acriflavine, Puromycin
Deoxycholate, Fosfomycin
Doxorubicin, Erythromycin
Fluoroquinolone*
Macrolide
Polymyxin
Spectinomycin, Streptomycin
Streptogramin_b, Lincosamide, Macrolide*
Tetracycline
Tobramycin, Netilmicin, Isepamicin, Amikacin, Dibekacin, Sisomicin

Figure 4. Resistance gene profiles. (A) The dashed lines represent the relative abundance of the total number of resistance genes for patients A, B, C, and D. (B) Relative abundance of the resistance genes throughout AB treatment for patients A, B, and C. The symbol "*" highlights the resistance gene profiles which coincide with the antibiotic administered to patients C, A and B, respectively.
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significantly shaped the microbiota on the basis of the antimicrobial effect (bactericidal or bacteriostatic) and the mode of action. In addition, we have found that specific mechanisms of action affect some organisms more than others, leading the bacterial community towards an alternative temporary equilibrium state. Clindamycin (protein synthesis inhibitor) introduced higher variance in microbiota composition than the other agents, giving way to a different bacterial community structure. This was probably due to the bacteriostatic nature of clindamycin when compared to the bactericidal effect of the other AB treatments. Interestingly, in the case of the active microbiota representing the surviving community, the bacterial composition was affected by the mode of action rather than the antimicrobial effect clearly distinguishing the three modes of action (Figure 3B). At a functional level, the microbial community profile was driven by the antimicrobial effect rather than by the mode of action. However, the strength of the AB class, considered as an external factor exerted at gene level was less intense, resulting in major uniformity.

AB impact on bacterial metabolic functions

High homogeneity was observed in the main roles for all the patients. This uniformity at a functional level has been also shown in both DNA and RNA-based surveys [22,47,48,49,50] since the microbiota is characterized by high functional redundancy. When we considered sub-roles, 51% with significant variation corresponded to inter-individual variability, representing the specific-subject response to AB course. The over-representation of genes involved in sugar transport in most of the patients suggested that this functional category could play an important role under stress conditions, as is the case of AB treatment. The phosphotransferase system (PTS), in addition to its main role in sugar transport, which is an essential function in itself, is involved in different regulatory processes such as stress response in bacteria and, hence, it could confer some extra advantages in presence of ABs [51]. Then, an efficient system of importing sugars could facilitate the energetic metabolism and, therefore, it could counteract the negative effect of ABs on the bacterial growth. Pérez-Cobas and coworkers [12] showed an increase in proteins belonging to the glycolysis pathway and pyruvate metabolism, as well as higher expression of genes related to energy metabolism/sugars category during beta-lactam treatment.

As we mentioned previously, the bacteriostatic effect drives the bacterial community to a characteristic composition, which is also reflected at a functional level. In patient B, most of the functional categories over-represented during treatment could be related with the increase in Enterobacteriaceae members. In this regard, we found an increase in the number of genes involved in lipopolysaccharide synthesis, which is the main component of the outer membrane for most Gram-negative bacteria. This barrier plays an important role in nutrient uptake and also confers resistance against ABs [52]. Likewise, the genes of secretion systems typical of Gram negative bacteria pathogenesis showed an increase only in patient B. However, patients A, C, and D, who received bactericidal treatment, presented a high abundance of Gram positive Ruminococcaceae and Lachnospiraceae families, and an over-representation of genes involved in endospore formation, a resistance mechanism typical of Gram positive bacteria. Another category that presented differences between bactericidal and bacteriostatic ABs was catabolism of fatty acids and phospholipids, to produce acetyl-CoA through the beta-oxidation process. As clindamycin inhibits mainly the anaerobic bacteria, the genes belonging to this sub-role were more abundant in patient B whose bacterial composition proved rich in Enterobacteriaceae family members.

Changes in the resistome

It has previously been pointed out that the AB usage is the most influential agent in the spread and stabilization of resistance genes in the gut environment [13]. One of the multidrug resistant genes that increased in patients A and B was a multidrug resistance efflux pump, which confers resistance against aminoglycoside, glycylcycline, beta-lactam, macrolide and acriflavine antibiotics. Since these ABs have different properties such as spectrum or mode of action, the transmission of these genes to a pathogen could hinder clinical treatment in the event of infection. These resistance genes have been described in some Proteobacteria genera such as *Escherichia* or *Klebsiella*, thereby supporting the increase in the abundance of these genera during treatment, principally in patient B. Patients A, B and C reached higher values of gene resistance abundances after AB treatment, with patient B, who was treated with clindamycin, attaining maximum values. It has been reported that besides the strong effect on the microbial composition, clindamycin also promotes increased AB resistance, which can persist in the microbial population for a long time [53]. In contrast, patient D showed a decrease in the relative abundance of resistance genes in the bacterial community. In fact, we found different dynamics in patient D as compared to the other three patients. This sample presented an initial composition with prevalence of the Enterobacteriaceae family, which has been described as a considerable source of resistance genes [54] and hence, the data indicated that these taxa were strongly affected by the ABs. Thus, the final resistome in the human gut after AB therapy would be determined by the resistance genes carried by the surviving bacteria and by the class of AB administered.

Conclusions

In this study, using high-throughput methodology, we have provided new insights into the complex antibiotic resistance scenario, related to the different modes of action of antibiotics and the consequences for the gut microbiota composition and function during antibiotic therapy. We have shown that specific properties of ABs such as antimicrobial effects or mode of action, are powerful forces for the selection of intestinal microbiota, and are partially responsible for the shifts in bacterial composition during AB therapy. The resulting structure of the microbial community showed its specific metabolic capabilities giving a different functional profile. Additionally, we have shown that the AB also modified the resistome composition, increasing the abundance of resistance genes in the gut environment, which is also important in shaping the post-treatment composition of the microbiota. However, further research into a larger group of subjects would be necessary to establish a quantitative evaluation of changes in gut microbiota.

Supporting Information

Figure S1 Evolution of diversity parameters along the treatment for patient A, B, C, and D. (A) Shannon Index. (B) Richness estimators: N, Chao1 and ACE. N is the number of observed taxa.
(TIFF)

Figure S2 Abundance of the main functional roles for all the samples.
(TIFF)

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Author Contributions

Conceived and designed the experiments: MJG AL AM. Performed the experiments: AEPC MLF. Analyzed the data: AEPC AA MJG AL. Contributed reagents/materials/analysis tools: HK AF SJO AEPC AL AM MJG. Wrote the paper: MJG AEPC AM AL. Discussion of data: AEPC HK AF MJG AM AL.

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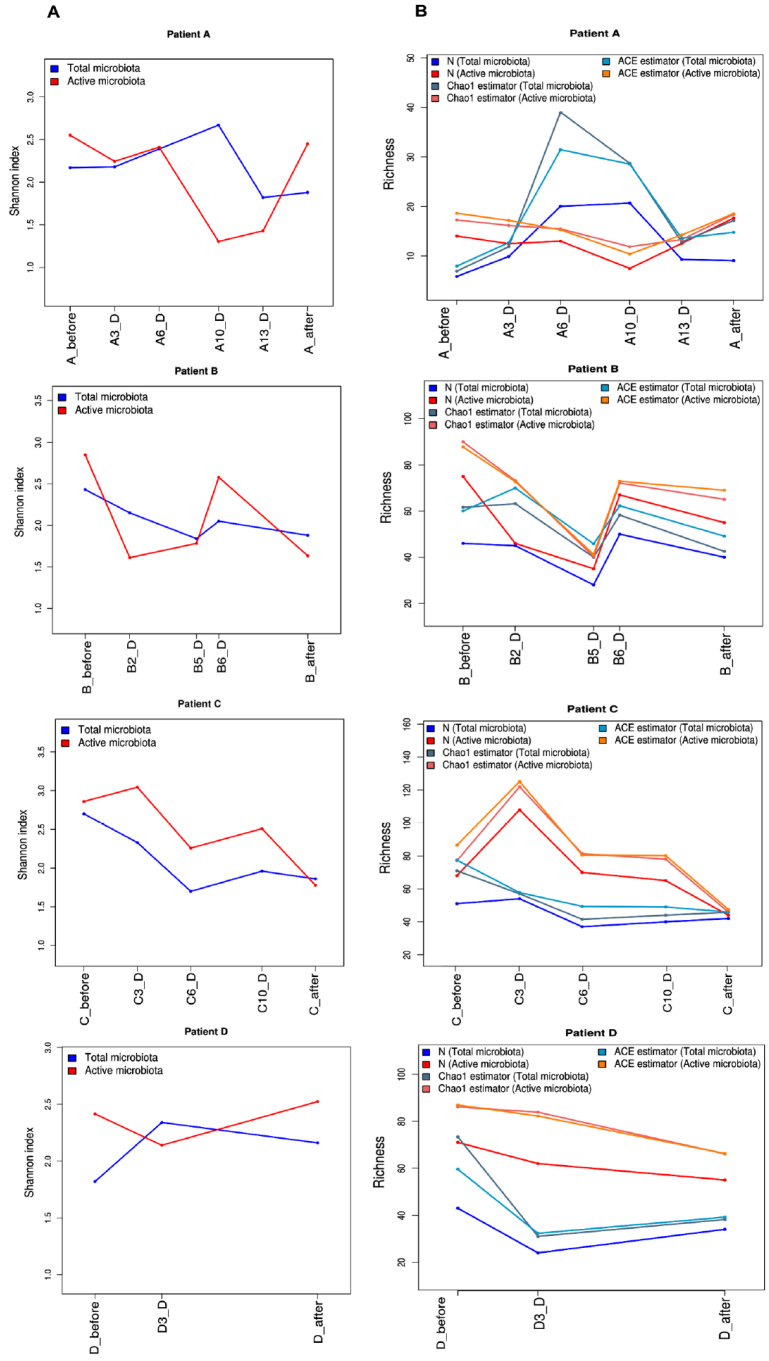
SUPPORTING INFORMATION



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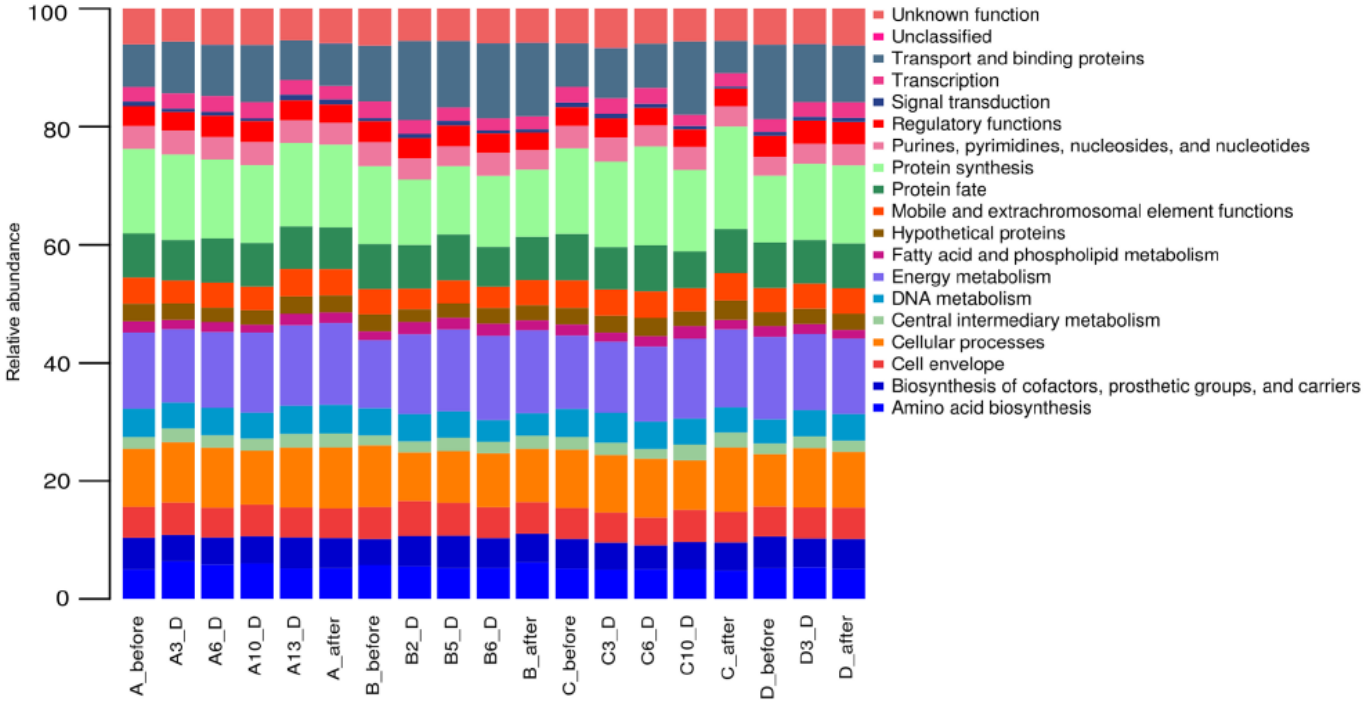

Figure S2. Abundance of the main functional roles for all the samples.

3.3

"STRUCTURAL AND FUNCTIONAL CHANGES IN THE GUT MICROBIOTA ASSOCIATED TO *Clostridium difficile* INFECTION"

AUTHORS: Ana Elena Pérez Cobas, Alejandro Artacho, Stephan Ott, Andrés Moya, María Jose Gosalbes Soler, Amparo Latorre.

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Structural and functional changes in the gut microbiota associated to *Clostridium difficile* infection

Ana E. Pérez-Cobas[1,2], Alejandro Artacho[1], Stephan J. Ott[3,4], Andrés Moya[1,2], María J. Gosalbes[1,2†] and Amparo Latorre[1,2*†]

[1] *Unidad Mixta de Investigación en Genómica y Salud de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO) y el Instituto Cavanilles de Biodiversidad y Biología Evolutiva de la Universitat de València, València, Spain*
[2] *CIBER en Epidemiología y Salud Pública, Madrid, Spain*
[3] *Institute for Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany*
[4] *Department for Internal Medicine, University Hospital Schleswig-Holstein, Kiel, Germany*

Edited by:
Monica Medina, Pennsylvania State University, USA

Reviewed by:
Sébastien Duperron, Université Pierre et Marie Curie, France
Elisabeth Margaretha Bik, Stanford University School of Medicine, USA

****Correspondence:***
Amparo Latorre, Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universitat de València, C/ Catedrático José Beltrán 2, 46980 Paterna (València), PO Box 46071, València, Spain
e-mail: amparo.latorre@uv.es

† These authors have contributed equally to this work.

Antibiotic therapy is a causative agent of severe disturbances in microbial communities. In healthy individuals, the gut microbiota prevents infection by harmful microorganisms through direct inhibition (releasing antimicrobial compounds), competition, or stimulation of the host's immune defenses. However, widespread antibiotic use has resulted in short- and long-term shifts in the gut microbiota structure, leading to a loss in colonization resistance in some cases. Consequently, some patients develop *Clostridium difficile* infection (CDI) after taking an antibiotic (AB) and, at present, this opportunistic pathogen is one of the main causes of antibiotic-associated diarrhea in hospitalized patients. Here, we analyze the composition and functional differences in the gut microbiota of *C. difficile* infected (CDI) vs. non-infected patients, both patient groups having been treated with AB therapy. To do so we used 16S rRNA gene and metagenomic 454-based pyrosequencing approaches. Samples were taken before, during and after AB treatment and were checked for the presence of the pathogen. We performed different analyses and comparisons between infected (CD+) vs. non-infected (CD−) samples, allowing proposing putative candidate taxa and functions that might protect against *C. difficile* colonization. Most of these potentially protective taxa belonged to the Firmicutes phylum, mainly to the order Clostridiales, while some candidate protective functions were related to aromatic amino acid biosynthesis and stress response mechanisms. We also found that CDI patients showed, in general, lower diversity and richness than non-infected, as well as an overrepresentation of members of the families Bacteroidaceae, Enterococcaceae, Lactobacillaceae and Clostridium clusters XI and XIVa. Regarding metabolic functions, we detected higher abundance of genes involved in the transport and binding of carbohydrates, ions, and others compounds as a response to an antibiotic environment.

Keywords: Gut microbiota, bacterial composition, metabolic functions, *C. difficile* infection, colonization resistance

INTRODUCTION

The human intestinal microbiota is involved in many host functions, such as food processing, regulating intestinal epithelium growth, immune system development, synthesis of essential vitamins, or protection against pathogens (Hooper et al., 2002; Guarner and Malagelada, 2003; Hattori and Taylor, 2009; Leser and Mølbak, 2009; Montalto et al., 2009). Because of its role in human health, imbalances in the gut microbiota have been associated to pathologies such as inflammatory bowel disease, diabetes, obesity, or Crohn's disease (Kang et al., 2010; Sekirov et al., 2010; Morgan et al., 2012; Shanahan, 2013). Antibiotic (AB) therapy has been crucial to treat bacterial infections for over half a century, but it strongly disturbs the gut commensal bacteria and, consequently, the beneficial functions they perform (Jernberg et al., 2010; Willing et al., 2011; Pérez-Cobas et al., 2013a). In fact, AB usage has been associated to short and long-term changes in the intestinal microbiota, reducing colonization resistance to opportunistic pathogens such as *Clostridium difficile* (Vollaard and Clasener, 1994; Bartlett, 2002; Jernberg et al., 2010; Reeves et al., 2011; Britton and Young, 2012). *C. difficile* is an anaerobic, spore-forming, Gram-positive toxin-producing bacterium, which is the most common cause of nosocomial diarrhea, and broad spectrum ABs constitute one of the primary risk factors for infection by this pathogen (Hookman and Barkin, 2009; Cohen et al., 2010). Under normal conditions, the human gut microbiota is able to prevent pathogen invasion through general mechanisms such as direct inhibition (by releasing inhibitory compounds, bacteriocins), nutrient depletion (consuming growth-limiting nutrients) or stimulating host immune defenses (reviewed in Stecher and Hardt, 2011). The exact mechanism by which the microbiota protects against *C. difficile* infection (CDI), preventing its growth and virulence, is still unknown. In this regard, direct antagonism

was found *in vitro* since *C. difficile* is a target of bacteriocin produced by an intestinal strain of *Bacillus thuringensis* (Britton and Young, 2012). Since the gut microbiota participates actively in the fermentation of diet carbohydrates, amino acid and lipid metabolism and protein digestion, Theriot et al. used a metabolic model of CDI in mice and found that ABs affect all these functions, leading to a disturbed microbiota functional state that favors *C. difficile* germination and growth (Theriot et al., 2014). Moreover, gut microorganisms participate in bile acid transformation, which otherwise stimulate *C. difficile* spore germination and growth (Britton and Young, 2012). Thus, the loss of key taxa which play these roles can trigger a structural and functional imbalance, allowing colonization by this opportunistic pathogen.

In recent years, high-throughput molecular techniques, such as 16S rRNA gene sequence analyses (taxonomic composition of microbial communities), metagenomics (genetic potential of microbial communities) and other meta-"omics" (metatranscriptomics, metaproteomics, metabolomics) have extended our knowledge of intestinal microbiota diversity and functions (Gill et al., 2006; Kurokawa et al., 2007; Zoetendal et al., 2008; Tap et al., 2009; Gosalbes et al., 2011; Pérez-Cobas et al., 2013a,b). Some of these approaches have recently been used to address the effects of ABs in the gut ecosystem (Dethlefsen et al., 2008; Antonopoulos et al., 2009; Dethlefsen and Relman, 2010; Jakobsson et al., 2010; Antunes et al., 2011; Pérez-Cobas et al., 2013b) showing that ABs considerably alter the gut microbial ecology and the host-microbiota interactions (Pérez-Cobas et al., 2013a). The response of the microbiota to ABs is related to properties of the agent, such as the antimicrobial effect, mode of action, dosage and duration of treatment, or route of administration (Jernberg et al., 2010; Pérez-Cobas et al., 2013b). In addition, biological factors of the host-microbial ecosystem itself such as taxonomic and functional composition, resistance gene reservoir, or host immune homeostasis also contribute to the microbial community shifts associated to AB therapy (Jernberg et al., 2010; Willing et al., 2011; Relman, 2012). To date, few studies have aimed to ascertain whether specific changes in the microbiota composition due to AB therapy lead to CDI. Past surveys have shown that diversity of the intestinal microbiota is significantly reduced in patients prior and/or during CDI, as well as important structural changes associated to infection (Antharam et al., 2013; Vincent et al., 2013).

The main goal of the present follow-up study is to gain insights into the development of CDI and its relation to an altered human gut microbiota. We have used 16S rRNA gene and metagenomic approaches to characterize the structure and functions of the intestinal microbiota before, during and after broad spectrum AB therapy in patients who developed CDI. In two previous studies we explored the effect of broad spectrum ABs on human gut microbiota composition and function in patients that did not develop CDI at any time (Pérez-Cobas et al., 2013a,b). Comparative analyses of these two groups of patients identified bacterial taxa and metabolic functions associated to an infection status, as well as specific taxa and functions that could protect against the *C. difficile*, and thus contribute to colonization resistance of the human gut microbiota.

MATERIALS AND METHODS

SAMPLE COLLECTION

Three patients under AB therapy at the Department of Internal Medicine of the University Hospital Schleswig-Holstein, Kiel, Germany were recruited for the study due to the fact that they were positive for *C. difficile* at some time points of the treatment. The patients were older than 65 years, no antibiotic therapy was administered to them in the previous 6 months to their hospital admission. The diagnosis at the entrance to the hospital were ischaemic colitis, sigmoid diverticulitis and infection of unknown origin for patients referred as F, G, and H, respectively. The patients stayed in the hospital during the AB therapy. Written, informed consent was obtained from all the subjects.

Fecal samples were collected, before, during and after AB treatment, from the three patients in sterile tubes and stored at −80°C until processing all sample together. Fecal samples were screened by multiplex PCR for *C. difficile* toxin genes, *tcdA* and *tcdB*, and triose phosphate isomerase gene (*tpi*), considering *C. difficile* positive those samples that resulted positive for the three examined genes (referred as CD+, whereas CD− is used for the rest of samples). Patients F and H were found positive after 16 and 35 days after AB treatment, respectively, whereas patient G was found positive on entering hospital (**Table 1**). The three patients presented diarrhea during AB theraphy.

In two previous studies we evaluated the effect of broad-spectrum antibiotics on five patients (A, B, C, D, E) through similar approaches of those presented in this work (16S rRNA gene and metagenomics) as part of the same research survey (Pérez-Cobas et al., 2013a,b) that was approved by the Ethical Committee of the University Hospital Schleswig-Holstein. None of these patients developed CDI (they were negative for the multiplex PCR for *C. difficile tcdA*, *tcdB*, and *tpi* genes), or presented diarrhea. The main features and therapy of all patients (A, B, C, D, E, F, G and H) are shown in **Table 1**. Only the time-points used in this study are shown for the patients A, B, C, D, and E (all CD− samples) of the previous studies.

DNA EXTRACTION AND SEQUENCING PROCESS

The fecal samples were resuspended in sterile PBS [containing, per liter, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 (pH 7.2)] and centrifuged at 1250 g and 4°C for 2 min to remove fecal debris. The supernatants were centrifuged at maximum speed at 4°C for 5 min to pellet the cells. DNA was extracted with the QIAamp® DNA Stool Kit (Quiagen) following the manufacturer's instructions. Total DNA integrity was checked by running a standard agarose gel electrophoresis and the concentration was quantified with the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen). For each sample, except of F_after from which there was no enough amount of DNA, the total DNA (metagenome) was directly pyrosequenced with a Roche GS FLX sequencer and Titanium chemistry in the Center for Public Health Research (FISABIO-Salud Pública) (Valencia, Spain). Thus, a total of 12 metagenomes were analyzed. We obtained a mean of 78,976 reads per sample with an average length of 374 bp.

Table 1 | Description of the patients involved in the study.

Patient	Antibiotic	Sampling date	Samples code	Study	CD (+/−)
A	Moxifloxacin	Day0—before AB	A_before	Pérez-Cobas et al., 2013b	−
		Day3—during AB	A3_D		−
		Day6—during AB	A6_D		−
		Day10—during AB	A10_D		−
		Day13—during AB	A13_D		−
B	Clindamycin	Day0—before AB	B_before	Pérez-Cobas et al., 2013b	−
		Day2—during AB	B2_D		−
		Day5—during AB	B5_D		−
		Day6—during AB	B6_D		−
C	Cefazolin/Ampicillin/Sulbactam	Day0—before AB	C_before	Pérez-Cobas et al., 2013b	−
		Day3—during AB	C3_D		−
		Day6—during AB	C6_D		−
		Day10—during AB	C10_D		−
D	Amoxicillin	Day0—before AB	D_before	Pérez-Cobas et al., 2013b	−
		Day3—during AB	D3_D		−
E	Ampicillin/Sulbactam/Cefazolin	Day0—before AB	E_before	Pérez-Cobas et al., 2013a	−
		Day3—during AB	E3_D		−
		Day6—during AB	E6_D		−
		Day11—during AB	E11_D		−
		Day14—during AB	E14_D		−
F	Amoxicillin/Ciprofloxacin/Clarithromycin	Day0—before AB	F_before	Present study	−
		Day16—during AB	F16_D		+
		3 days after AB	F_after		+
G	Ciprofloxacin	Day0—before AB	G_before	Present study	+
		Day4—during AB	G4_D		+
		4 days after AB	G_after		+
H	Vancomycin/Ampicillin/Sulbactam	Day0—before AB	H_before	Present study	−
		Day7—during AB	H7_D		−
		Day14—during AB	H14_D		−
		Day20—during AB	H20_D		−
		Day35—during AB	H35_D		+
		Day38—during AB	H38_D		+
		26 days after AB	H_after		−

CD (+/−), positive and negative detection for C. difficile. AB, antibiotic.

16S rRNA GENE AMPLIFICATION

A region of the 16S rRNA gene (V1, V2, and V3) was amplified by polymerase chain reaction (PCR) for each sample. The primers were the universal E8F (5′-AGAGTTTGATCMTGGCTCAG-3′) with adaptor A and 530R (5′-CCGCGGCKGCTGGCAC-3′) with adaptor B using the sample-specific Multiplex Identifier (MID) for pyrosequencing according to 454 standard protocols. For each sample a 50 μl PCR mix was prepared, containing 5 μl of Buffer Taq (10X) with 20 mM MgCl2, 2 μl of dNTPs (10 mM), 1 μl of each primer (10 mM), 0.4 μl of Taq Fast start polymerase (5 u/μl), 39.6 μl of nuclease-free water and 1 μl of DNA template. PCR conditions were: 95°C for 2 min followed by 25 cycles of 95°C for 30 s, 52°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 10 min. The amplification products were checked by electrophoresis in agarose gel (1.4%). PCR products were purified using NucleoFast® 96 PCR Clean-Up Kit (Macherey-Nagel) and quantified with the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen). The pooled PCR products were directly pyrosequenced in the same way as the total DNA (described above). We obtained an average of 5394 reads per sample.

ANALYSIS OF THE 16S rRNA GENE READS

We used the Ribosomal Database Project (RDP) pipeline (Cole et al., 2009) to trim off the MID and primers and also to filter sequences by quality. Sequences with a phred quality score

below 20 (Q20) and short length (<250 bp) were discarded. The denoising of the sequences was performed with the usearch program in the QIIME pipeline (Caporaso et al., 2010). Then, the pyrosequencing chimeras were discarded using the uchime filtering also in the QIIME pipeline. After, OTUs were calculated at 97% of sequence similarity by clustering with the usearch program in the QIIME pipeline. The taxonomic assignment of the amplicons was based on the database of RDP. We included only annotations obtained with a confidence level (bootstrap cut-off) greater than 0.8, leaving the assignment at the last-well identified level and the consecutive levels as unclassified (uc).

BIODIVERSITY AND ECOLOGICAL ANALYSIS

To analyze the microbial community structure at OTU level (97%) we calculated two diversity parameters: number of OTUs and Shannon index (Shannon, 1948) and two richness estimators: Chao1 (Chao, 1984) and abundance-based coverage (ACE) (Chao et al., 2000). These estimators are implemented in Vegan package (Oksanen et al., 2011) under R software (http://cran.r-project.org) (R Development Core Team, 2011). To statistically compare the mean ranks of the biodiversity measures between groups, we used the Wilcoxon signed-rank test implemented in the R software.

We also performed a clustering based on OTU composition to study the similarity between samples using the pvclust library (Suzuki and Shimodaira, 2006) in the R software. This analysis assesses the uncertainty in hierarchical clusters using bootstrap resampling techniques. We used the approximately unbiased (AU) p-value with 10,000 bootstraps to estimate the probability of each cluster. This AU p-value indicates how strong the cluster is supported by data.

FUNCTIONAL CLASSIFICATION OF METAGENOMES

We used the 454 Replicate Filter Program (Gómez-Alvarez et al., 2009) to eliminate artifact replicate reads of pyrosequencing following the parameters: sequence identity cutoff = 1; length difference requirement = 0; number of beginning base pairs to check = 10. Reads were compared against the human genome database using BLASTN (Altschul et al., 1990) with an e-value of 10^{-10} to eliminate possible contamination with human sequences. To identify the sequences encoding the ribosomal 16S rRNA and 23S rRNA genes we compared the dataset against the Small Subunit rRNA Reference Database (SSUrdb) and the Large Subunit rRNA Reference Database (LSUrdb) described in Urich et al. (2008) using BLASTN with an e-value of 10^{-16} and 10^{-4} respectively. After removing the ribosomal genes, the remaining reads were compared to the NCBI-nr protein database using BLASTX (Altschul et al., 1990) to identify the protein-coding genes, and then we performed an Open Reading Frames (ORFs) search with the Fraggenscan program from the metagenomic analysis web server (WebMGA) (Wu et al., 2011). The predicted ORFs were functionally annotated by HMMER 3.0 (Durbin et al., 1998) against the TIGRFAM database (Haft et al., 2003) using default parameters.

STATISTICAL ANALYSIS

Canonical correspondence (CCA) and detrended correspondence (DCA) analyses were performed to explore the relationship between different groups of samples and with *C. difficile* infection as a variable that could explain the variability pattern. To statistically assess the effect of that variable in explaining the bacterial composition differences, a multivariate ANOVA based on dissimilarity tests (Adonis) was applied, implemented in the Vegan package under the R software. These approaches were applied to two different levels: the taxonomy based on the 16S rRNA gene, and the biological functions based on the TIGRFAMs annotations. We used the ShotgunFunctionalizeR package (Kristiansson et al., 2009) in the R software to statistically compare samples at diversity and functional levels. The differences in composition between samples were addressed comparing groups of multiple samples with the function "testGeneFamilies.dircomp." On the other hand, we applied the "testGeneCategories.dircomp" function to compare the distribution of functional categories between groups of samples. It compares each gene family from a higher functional category to decide whether the global category is statistically significant among two groups of samples. All tests were based on Poisson models.

SEARCHING FOR PUTATIVE CANDIDATE TAXA AND METABOLIC FUNCTIONS TO PROTECT AGAINST CDI

We also used the "testGeneCategories.dircomp" test to identify taxa and metabolic functions that could play a protective role against *C. difficile* colonization. Specifically, we performed three comparisons between groups of samples to identify taxa and functions that were significantly over-represented in CD− compared to CD+ samples. The taxa and functions resulting from the different comparisons were intersected to define the candidate protective group. For this purpose, we performed the following comparisons:

Comparison 1.Since patients F and H were negative to the pathogen before treatment but positive during therapy, we compared the CD− samples before AB (F_before and H_before) against the CD positive samples (CD+) during AB (F_16D, H35_D and H38_D) (**Table 1**). We aimed to identify taxa and functions that significantly decreased (p-value < 0.1) due to treatment, presumably allowing *C. difficile* overgrowth.

Comparison 2. Since patients A, B, C, D and E did not develop CDI, we performed a comparison of the samples before AB treatment against their samples during treatment (**Table 1**). We aimed to identify taxa and functions that significantly increased (p-value < 0.1) due to therapy or that changed less drastically than those in Comparison 1, since their presence could play a role in preventing *C. difficile* infection.

Comparison 3. Since patient H was negative for the pathogen 26 days after AB, we carried out a comparison of the CD+ samples of patient H (H35_D and H38_D) against the CD− sample taken after AB (H_after) (**Table 1**). We aimed to identify taxa and functions whose significant increase (p-value < 0.05) could be incompatible with pathogen overgrowth as this was not detected.

Finally, we intersected all these results to obtain a group of candidate taxa and functions that could participate in *C. difficile* colonization resistance.

CO-OCCURRENCE BAYESIAN NETWORKS OF CANDIDATES (TAXA AND METABOLIC FUNCTIONS) IN CD− SAMPLES

To find positive correlations between candidate protective taxa or functions found in the previous analyses and other taxa and functions obtained for samples from patients A, B, C, D and E during AB treatment (all CD− samples), we performed Bayesian networks based on their relative abundance. The Bayesian networks were inferred using the bnlearn package (Scutari, 2010) in the R software. The optimal network inference was constrained so that only those interactions exhibiting a Spearman correlation *p*-value below 0.01 were included in the network. Correlations and *p*-values were computed using the Spearman method implemented in R software.

DATA ACCESSION NUMBER

All sequences have been entered in the European Bioinformatics Institute database, under accession numbers: ERP002192 (patients A, B, C, and D), ERP001506 (patient E) and PRJEB5771 (patients F, G, and H).

RESULTS

MICROBIAL DIVERSITY AND BACTERIAL COMPOSITION IN PATIENTS DEVELOPING CDI

Analysis of the gut microbiota of the three CDI patients (F, G, and H) showed large variations in bacterial composition during therapy (**Figure 1**).

Before AB treatment, the bacterial composition of patient F was dominated by the *Akkermansia* genus (30.6%) belonging to the family Verrucomicrobiaceae. Other bacterial families were also abundant such as Ruminococcaceae (20.8%), Oscillibacteriaceae (*Oscillibacter*, 11.7%) and Bacteroidaceae (*Bacteroides*, 14.8%). When *C. difficile* was detected, at day 16 of AB treatment, all these taxa were almost absent in the community except *Bacteroides*, which had increased to 41.9%, becoming a predominant genus of the gut ecosystem. The Clostridium cluster XlVa increased dramatically (from 0.7% before AB course to 46.8% at day 16), being the most abundant group at this time point. After treatment, the abundance of the main taxa of the microbial community changed again, the predominant being Enterococcaceae *(Enterococcus*, 48.3%), Streptococcaceae (*Streptococcus*, 43.2%), Staphylococcaceae (*Staphylococcus*, 4.1%) and Clostridium cluster XI (3.5%).

Patient G was found positive to *C. difficile* detection before, during, and after AB treatment, showing the most similar bacterial composition at the three time points, though there are some remarkable differences. The initial composition (G_before) consisted mostly of Bacteroidaceae (*Bacteroides*, 36.7%) and Ruminococcaceae (*Faecalibacterium*, 29.6%). During AB (G4_D), although *Bacteroides* decreased in abundance to 25%, it remained the most abundant genus, while *Faecalibacterium* (5.9%) decreased radically. However, *Enterococcus* increased during AB (from 1.3 to 14.9%). After therapy (G_after), Clostridium cluster XI became the predominant group (62.4%) whereas *Streptococcus* genus decreased progressively at each time point (3, 2.2, and 0.2%, respectively).

Patient H had a very unusual gut microbiota before AB treatment, being dominated (85.7%) by Enterobacteriaceae family, mainly *Escherichia* genus, but its abundance decreased dramatically reaching the lowest values at days 35 and 38 of the broad-spectrum AB treatment (4.2 and 2.8%, respectively), when *C. difficile* was detected. During days 7 and 14 of AB treatment the genus *Bacteroides* showed the higher abundance values (20.4 and 34.8%); however this taxon decreased on day 20, becoming undetectable by days 35 and 38. *Streptococcus* genus increased slightly in the two CD+ samples (1.5 and 4.5%, respectively). The most striking shift occurred in the Lactobacillaceae family (*Lactobacillus* genus), whose frequency increased from less than 1% at the beginning of treatment to 83.3 and 70% on days 35 and 38 of the AB course, and was reduced to 15.5% after AB. We performed a statistical comparison to evaluate the differences in bacterial composition between the samples immediately prior to *C. difficile* detection (H14_D, H20_D) and in the CD+ samples (H35_D, H38_D). (Table S2). The main significantly overrepresented taxa were *Lactobacillus*, *Streptococcus*, *Proteus*, *Sutterella* and the uc_Lactobacillaceae, while the Clostridium cluster XlVa, *Enterococcus*, *Bacteroides*, *Escherichia*, *Klebsiella*, and *Roseburia* were the least abundant taxonomic groups.

The three individuals exhibited great fluctuations in the number of observed OTUs, as well as in the diversity parameters analyzed (Table S1). The diversity (based on Shannon, Chao 1 and ACE estimators) of patient F was reduced in the CD+ samples, being minimal after the therapy. The microbial diversity of patient G also reached the lowest values after treatment. The decreased diversity after the course in these two patients could be due to both, the AB and CDI effects. However, the patient H, which was recovered of the infection after the therapy, presented the lowest diversity parameters before the AB that could be due to the massive presence of members of the Enterobacteriaceae family detected in this sample and also during CDI (**Figure 1**).

Finally, we performed a cluster analysis to find similarities in microbiota composition between samples at OTU level (97%) (**Figure 2**). The three samples corresponding to patient G (G_before, G4_D and G_after) were clustered with F_after, being all CD+ samples. This cluster was closer to the others two samples of patient F (F_before and F16_D). Patient H samples formed two clear groups. One of the clusters included the prior infection samples (before AB and 7, 14 and 20 days during AB) whereas the CD+ samples (days 35 and 38), which are the most similar samples, grouped in a second cluster with the sample after treatment (H_after). The clustering shows that both the individual and the *C. difficile* presence contributed to explain the similarity pattern of the samples.

DIFFERENCES IN MICROBIAL STRUCTURE BETWEEN *C. DIFFICILE*-INFECTED AND NON-INFECTED PATIENTS

In previous studies we analyzed changes in bacterial composition in AB-treated patients that did not develop *C. difficile* infection (A, B, C, D and E), and thus all samples were CD−. To search for differences in microbiota composition possibly related to infection, we compared the 15 time points during the AB therapy of these CD− patients with samples from patients that were positive for *C. difficile* detection (CD+) (F16_D, F_after, G_before, G4_D, G_after, H35_D and H38_D) (**Table 1**).

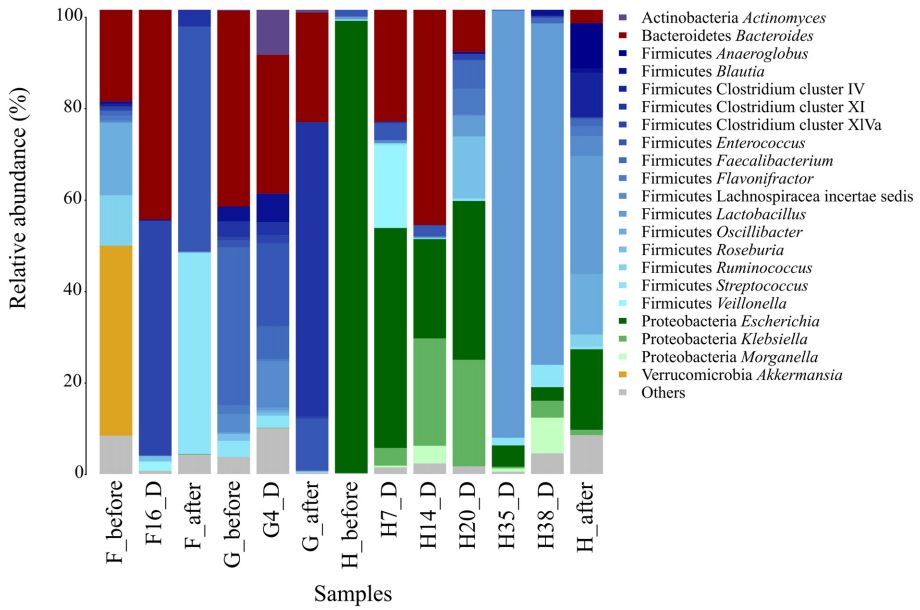

FIGURE 1 | Fecal microbiota composition in CDI patients (F, G, and H). The composition of each sample is based on the RDP taxonomic assignment of the 16S rDNA sequences. The phylum and the genus level are shown for the most abundant bacterial groups (>5%).

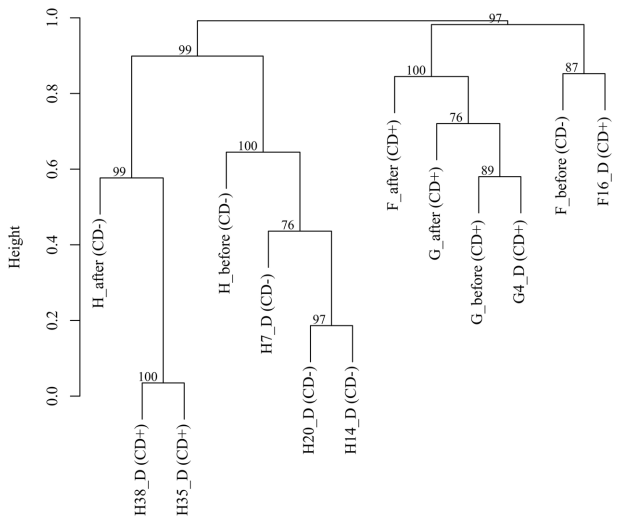

FIGURE 2 | Cluster analysis based on OTUs (97%) of CDI patients (F, G, and H). The approximate unbiased (AU) *p*-values are shown.

First, we compared the Shannon index distributions between CD+ and CD− samples (Figure S1). We found a lower diversity for CD+, with an average of 3.1 ± 1.0 compared to CD− samples with 3.9 ± 0.8, respectively. The richness estimator, Chao1, showed great variations for both groups; even so, the means were also lower in the CD+ populations with values of 210 ± 132 vs. 287 ± 157 in CD− individuals. The Wilcoxon signed-rank test was performed to compare the diversity measures between both groups, and was not significant for the Shannon index ($p = 0.14$) and the Chao1 estimator ($p = 0.33$). The gut microbiota of CD+ samples seems to be more heterogeneous and less rich than the CD− samples corresponding to patients that did not develop CDI, but a larger number of samples would be required to support this observation.

Second, we performed a detrended correspondence analysis (DCA) to explore the variations in bacterial composition between the same CD+ and CD− samples tested above (**Figure 3A**). The two axes explained 26. 7% of the total variance, and there was large variability in the microbiota of both groups. Despite this variability, two clusters can be distinguished with minimal overlapping. We applied the Adonis test to evaluate whether developing *C. difficile* infection is a factor that influences the microbiota structure. The factor proved to be significant with a *p*-value of 0.005.Thus, although the CD+ samples do not form a well-defined cluster, they share some features in their microbiota composition that differ from CD− samples.

Finally, we performed a statistical test to find those taxa that explained the differences in composition between CD− and CD+ groups. In CD+ samples there was significant over-representation of the genera *Lactobacillus*, *Bacteroides*, *Enterococcus*, *Faecalibacterium*, the family Lachnospiraceae incertae sedis, and the Clostridium clusters XIVa and XI, the latter included *C. difficile*. However, commensal members of the intestinal community, such as *Roseburia*, *Coprococcus*, *Blautia*, *or Subdoligranulum* genera and the families Erysipelotrichaceae and Ruminococcaceae were underrepresented (**Table 2**).

CANDIDATE TAXA INVOLVED IN *C. DIFFICILE* COLONIZATION RESISTANCE

In order to obtain a subset of candidate bacteria that could be involved in *C. difficile* colonization resistance, we performed statistical comparisons between different groups of

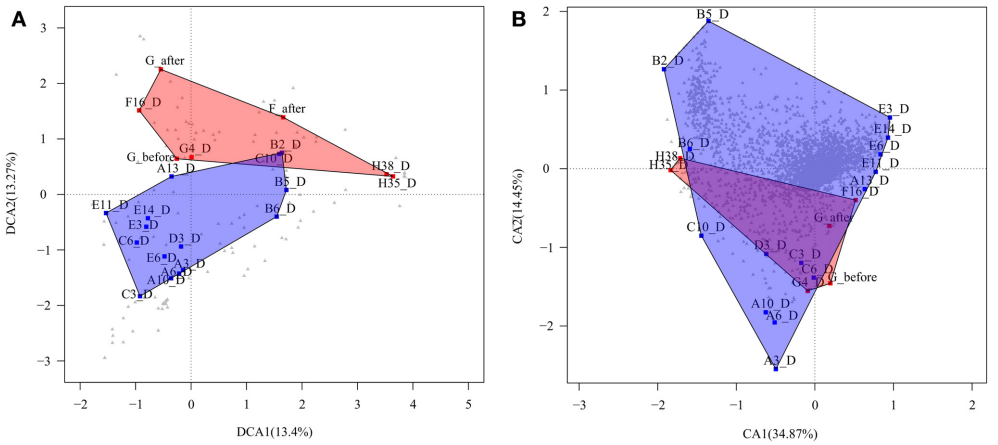

FIGURE 3 | Correspondence analyses. (A) Detrended correspondence analysis (DCA) based on taxa abundance and composition of CD+ samples of patients (F, G, and H) (red square) and CD− samples of patients (A, B, C, D, and E), during treatment (blue square). Gray triangle indicates taxa distribution. **(B)** Correspondence analysis (CA) based on the functional profile (TIGRFAMs) of the same samples. Gray triangle indicates functions.

Table 2 | Differential taxa abundance between CD− (during time points of A–E patients) and CD+ (F16_D, F_after, G_before, G4_D, G_after, H35_D and H38_D) samples.

Taxa	Abundance in CD+ samples	*P*-value
Clostridium cluster XIVa	Increase	0
Clostridium cluster XI	Increase	0
Lactobacillus	Increase	0
Bacteroides	Increase	0
Lachnospiracea incertae sedis	Increase	5.65E-137
Faecalibacterium	Increase	2.30E-121
Enterococcus	Increase	7.06E-120
uc_Lachnospiraceae	Decrease	0
Blautia	Decrease	0
uc_Ruminococcaceae	Decrease	0
uc_Enterobacteriaceae	Decrease	0
Roseburia	Decrease	0
Parabacteroides	Decrease	0
Subdoligranulum	Decrease	0
Oscillibacter	Decrease	0
Coprococcus	Decrease	2.01E-84
Alistipes	Decrease	1.78E-46
uc_Erysipelotrichaceae	Decrease	8.51E-54
Butyricicoccus	Decrease	1.18E-48
Lactococcus	Decrease	1.62E-46
Streptococcus	Decrease	0.01

samples (see Materials and Methods for the three specific comparisons). The three comparative analyses gave a number of statistically significant taxa (**Table 3**), and intersection of the results of the three analysis indicated which taxa may participate in colonization resistance to *C. difficile*. We found that the major number of taxa belonged to the order Clostridiales (Firmicutes), specifically to the families Ruminococcaceae (*Ruminococcus*, *Subdoligranulum*, and *Gemmiger*), Oscillibacteraceae (*Oscillibacter*) and Eubacteriaceae (*Anaerovorax*). We also found unclassified Ruminococcaceae and Erysipelotrichaceae belonging to the Clostridiales and Erysipelotrichales orders, respectively, as well as other Clostridia and Clostridiales members. Finally, the genus *Escherichia* from the family Enterobacteriaceae, Proteobacteria phylum, was also detected.

Once the candidate protective taxa had been detected, we performed a Bayesian network (see Materials and Methods) to find other related members of the bacterial community and hence also putatively involved in pathogen colonization resistance (**Figure 4**). *Gemmiger*, *Subdoligranulum* and uc_Erysipelotrichaceae did not show any significant correlation and thus they are not represented in the figure. It is worth noting that most taxa showing a positive and significant correlation with the candidates were phylogenetically related to them, mainly belonging to the Clostridiales order, such as *Roseburia* and *Coprococcus* (Lachnospiraceae family) and *Anaerotroncus* (Ruminococcaceae family).

FUNCTIONAL CHANGES IN PATIENTS DEVELOPING CDI

In the present work, we performed the functional annotation of the 12 metagenomes sequenced (metagenome of sample F_after could not be analyzed, see Materials and Methods) by comparison against the TIGRFAM database, obtaining the following hierarchical classification: main roles (the highest functional level), sub roles (more specific metabolic functions for each one of the main roles) and genes (metabolic functions) for all the reads. Figure S2 shows great homogeneity in the main role distribution of different samples for all three patients (F, G, and H). On average, the most abundant main role categories were: energy metabolism (12.3% ± 2.1), protein synthesis (12% ± 2), transport and binding proteins (8.6 ± 2.5%) and cell processes (8.6 ± 1.3%). Similar main role distribution was described for patients A, B, C, and D in our previous study (Pérez-Cobas et al., 2013b), which is expected due to the importance of these household functions for the survival and growth of gut bacteria.

Table 3 | Significant taxa and associated *p*-value resulting from the three comparative analyses to find protective candidate taxa.

Taxa	Comparison 1(a)	Comparison 2(b)	Comparison 3(c)
Akkermansia	0	1.66E-4	NS
Anaerotruncus	4.62E-6	NS	NS
Anaerovorax	1.12E-70	1.43E-3	3.41E-3
Clostridium cluster IV	6.11E-14	NS	1.27E-32
Clostridium cluster XIVb	0.02	NS	NS
Clostridium cluster XVIII	2.16E-18	6.44E-73	NS
Enterococcus	2.63E-7	2.62E-258	NS
Erysipelotrichaceae incertae sedis	1.46E-6	1.23E-6	NS
Escherichia	0	6.48E-22	6.01E-12
Faecalibacterium	6.71E-5	NS	NS
Gemmiger	0.07	0.05	3.41E-3
Holdemania	0.07	1.52E-11	NS
Oscillibacter	0	1.33E-15	8.23E-42
Pseudoflavonifractor	5.42E-16	NS	NS
Pyramidobacter	0.04	NS	NS
Ruminococcus	4.85E-228	1.19E-6	5.25E-10
Subdoligranulum	1.03E-19	2.76E-8	3.41E-3
uc_Clostridia	1.62E-3	4.27E-5	3.46E-5
uc_Clostridiales	2.35E-11	0.06	1.57E-18
Clostridiales incertae sedis XIII	1.62E-3	0.04	NS
uc_Enterobacteriaceae	3.18E-6	1.73E-17	NS
uc_Erysipelotrichaceae	0.02	0.07	3.46E-5
uc_Ruminococcaceae	0	7.85E-117	4.44E-129
Anaeroglobus	NS	NS	1.47E-33
Bacteroides	NS	NS	5.85E-11
Dialister	NS	NS	5.25E-10
Selenomonas	NS	NS	4.73E-9
uc_Betaproteobacteria	NS	NS	0.04
uc_Lachnospiraceae	NS	NS	1.50E-9

In bold are the candidate taxa that were significant in the three comparisons.
(a) (F_before and H_before) vs. (F_16D, H35_D, and H38_D).
(b) A, B, C, D, and E samples before vs. during AB treatment.
(c) (H35_D and H38_D) vs. (H_after).

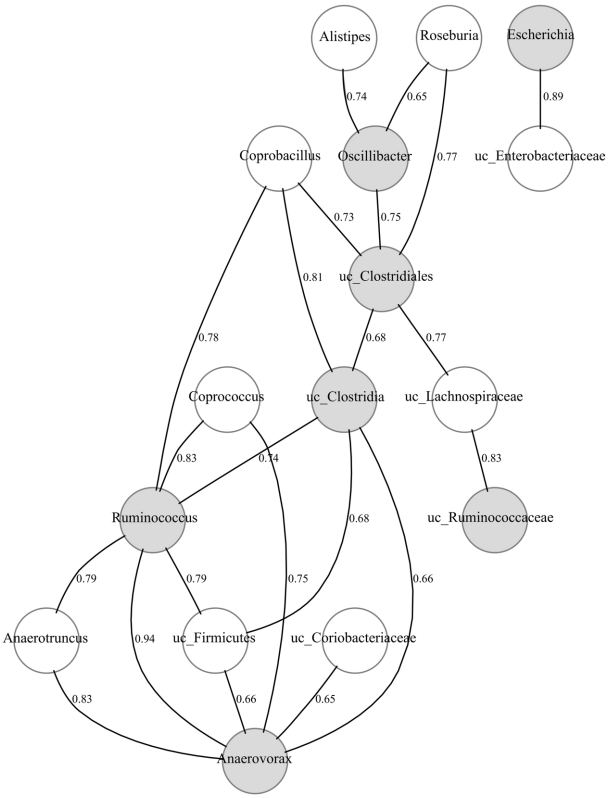

FIGURE 4 | Bayesian network of microbial composition in CD− samples of patients (A–E), during treatment, and the candidate protective taxa. The significant positive correlations (p-value < 0.01) between the candidate protective taxa and other members of the bacterial community are shown. The correlation coefficients are indicated. Gray and white nodes represent candidate and correlated taxa, respectively. Candidate taxa with no-correlations are not included.

In patient F, there were 51 significantly different sub roles between samples corresponding to before and during AB treatment (F_before vs. F16_D) (Table S3). The most significant over-represented categories in AB treated samples were: DNA metabolism/chromosome-associated proteins; cellular processes/DNA transformation; cell envelope/biosynthesis and degradation of surface polysaccharides and lipopolysaccharides; and energy metabolism/ pentose phosphate pathway. The under-represented categories were: protein synthesis/tRNA aminoacylation; transport and binding proteins/amino acids; peptides and amines; and cell envelope/surface structures.

Three different comparisons were made for patient H: (i) before vs. during treatment but before *C. difficile* detection (H_ before vs. H7_D, H14_D and H20_D); (ii) before vs. CD+ samples (H_before vs. H35_D and H38_D) and (iii) CD− prior to CDI vs. CD+ samples (H14_D and H20_D vs. H35_D and H38_D). In total, we found 37 significant sub roles increased or decreased in CD− samples. (i) Those that increased during AB but before CDI were mainly involved in "cell processes/DNA transformation" and "protein synthesis/translation factors," whereas we observed a significant decrease in "amino acid biosynthesis/folic acid"; "mobile and extrachromosomal elements function/plasmid functions"; "signal transduction/PTS" and "transport and binding proteins/carbohydrates, organic alcohol and acids." (ii). A similar functional profile was found when we compared before vs. CD+ samples. (iii) Finally, when we specifically compared the two samples previous to infection with the two CD+ samples we found 54 significant sub-roles. The most significant over-represented in the CD+ samples were: "signal transduction/PTS"; "transport and binding proteins/carbohydrates, organic alcohols, and acids"; "transport and binding proteins/amino acids, peptides and amines" and "cell envelope/biosynthesis and degradation of mureinsacculus and peptidoglycan" (Table S3).

Patient G proved to be infected by the pathogen throughout the study. Thus, we compared the sub roles distribution, before vs. during treatment, to find those functions that

could be AB-related. The comparison showed that only two categories changed during AB: "amino acid biosynthesis/serine family" decreased ($p = 0$) while "cell envelope/other" increased ($p = 0.04$).

DIFFERENCES IN THE FUNCTIONAL PROFILE BETWEEN *C. DIFFICILE*-INFECTED AND NON-INFECTED PATIENTS

To compare the whole functional composition of CD+ sample of patients F, G, and H with CD− samples during treatment of patients A, B, C, D, and E, we applied a correspondence analysis based on TIGRFAM functions abundance, with both axes explaining a total of 49.3% of sample variance. The analysis did not show a clear differential functional pattern between the CD+ and CD− groups given the CD+ samples seem to be a subset of the CD− group (**Figure 3B**). We also used the Adonis test to evaluate the significance of ABs in structuring the functional profile of the microbial community in a different way for the two groups (CD+ and CD−). The factor was not significant at the hierarchical level sub roles and metabolic functions, the *p*-values being 0.63 and 0.73, respectively. To find specific sub roles that could be associated to CD+ samples, we compared the functional profile of the same previously tested samples (**Table 4**), finding significant enrichment in "transport and binding proteins," mainly for "carbohydrates, organic alcohols and acids," and "signal transduction" by the phosphotransferase system (PTS). However, "mobile and extrachromosomal element functions" and "aromatic amino acid family biosynthesis" were significantly underrepresented.

CANDIDATE FUNCTIONS INVOLVED IN *C. DIFFICILE* COLONIZATION RESISTANCE

Just as in the 16S rRNA gene survey, we performed three comparative analyses to find (in the intersection) those metabolic functions that may play a role in colonization resistance. **Table 5** shows the roles, sub roles, and functions that may be protective. Those with a clearly assigned role are involved in "aromatic amino acid biosynthesis (chorismate mutase)," "endospore formation (stage IV sporulation protein B and anti-sigma F factor)," "metabolism of amino groups (agmatine diminase)," and "stress response mechanisms (rrf2 family protein, redox-active disulfide protein 2 and glutamate decarboxylase)." Doubled CXXCH domain belongs to a protein of unknown function but it is postulated to be part of c-type cytochromes that participate in electron transfer. UDP-N-acetylglucosamine 4,6-dehydratase participates in the biosynthesis of pseudaminic acid. No sub-roles were assigned to indolepyruvate ferredoxin oxidoreductase and RNA polymerase sigma-70 factor.

We also performed a Bayesian network to find significant and positive associations between the candidate protective functions and other functions that may be important in pathogen infection resistance. **Figure 5** shows the functional network according to hierarchical categories. In a general overview, most of the candidate functions were connected with several different sub roles, and correlations between candidates were also found. The most frequently connected function was the doubled CXXCH domain (26 correlations), and chorismate mutase (25 correlations). Additionally, these two candidate functions shared some connections whose nodes are involved in different roles, the majority

Table 4 | Comparisons of sub-roles distribution between CD− (during time points of A–E patients) and CD+ (F16_D, F_after, G_before, G4_D, G_after, H35_D and H38_D) samples.

Main role	Sub-role	*P*-value
Amino acid biosynthesis	Aromatic amino acid family	↓1.5E-29
Biosynthesis of cofactors, prosthetic groups, and carriers	Pantothenate and coenzyme A	↓8.2E-7
	Riboflavin, FMN, and FAD	↑1.2E-2
Cellular processes	DNA transformation	↓1.0E-3
	Sporulation and germination	↓1.2E-2
	Toxin production and resistance	↓4.9E-3
Central intermediary metabolism	Amino sugars	↑2.0E-6
	One-carbon metabolism	↑2.3E-2
	Other	↑3.9E-6
	Phosphorus compounds	↓6.0E-4
	Sulfur metabolism	↓8.6E-3
DNA metabolism	Chromosome-associated proteins	↑6.1E-8
	Degradation of DNA	↓1.4E-2
	DNA replication, recombination, and repair	↑1.1E-2
	Restriction/modification	↓1.0E-3
Energy metabolism	Electron transport	↓1.7E-3
	Fermentation	↑3.0E-4
	Glycolysis/gluconeogenesis	↑1.6E-4
	Pentose phosphate pathway	↑1.5E-2
	Sugars	↑2.3E-2
Hypothetical proteins	Conserved	↓9.4E-7
Mobile and extrachromosomal element functions	Other	↓3.4E-17
Protein synthesis	Other	↑4.7E-2
	tRNA and rRNA base modification	↑8.2E-3
Purines, pyrimidines, nucleosides, and nucleotides	2′-Deoxyribonucleotide metabolism	↓1.4E-2
	Purine ribonucleotide biosynthesis	↑3.6E-7
	Salvage of nucleosides and nucleotides	↑3.0E-4
Regulatory functions	Protein interactions	↓3.6E-5
Signal transduction	PTS	↑1.5E-20
Transcription	Degradation of RNA	↑1.5E-3
	DNA-dependent RNA polymerase	↑1.5E-2
	RNA processing	↑8.2E-3

(Continued)

Table 4 | Continued

Main role	Sub-role	*P*-value
Transport and binding proteins	Anions	↑9.5E-4
	Carbohydrates, organic alcohols, and acids	↑6.3E-18
	Cations and iron carrying compounds	↑1.2E-5
	Unknown substrate	↑3.6E-5
Unknown function	General	↑2.0E-6

Arrows indicate the sub-roles significantly over-represented (upward) and under-represented (downward) in the CD+ samples.

Table 5 | Candidate functions involved in *C. difficile* colonization resistance.

Main role	Sub-role	Function
Amino acid biosynthesis	Aromatic amino acid family	Chorismate mutase
Cellular processes	Sporulation and germination	Stage IV sporulation protein B
Regulatory functions	Protein interactions	Anti-sigma F factor
Central intermediary metabolism	Polyamine biosynthesis	Agmatine deiminase
Unknown function	General	rrf2 family protein
		Redox-active disulfide protein 2
NA	NA	Glutamate decarboxylase
		Doubled CXXCH domain
		Indolepyruvate ferredoxin oxidoreductase
		RNA polymerase sigma-70 factor*
		UDP-N-acetylglucosamine 4,6-dehydratase

**RNA polymerase sigma-70 factor, Bacteroides expansion family 1.*

being related to energy metabolism, protein synthesis and fate, as well as amino acid biosynthesis. The UDP-N-acetylglucosamine 4,6-dehydratase showed 21 correlations, mainly with cell envelope, protein fate and transport system roles. Also, this function was connected to another important candidate: glutamate decarboxylase, with which it shares some correlations. The redox-active disulfide protein 2 and glutamate decarboxylase presented 17 correlations each. The former, which is correlated to the two candidates known as chorismate mutase and indolepyruvate ferredoxin oxidoreductase, showed associations with energy metabolism and protein synthesis, while glutamate decarboxylase is correlated to protein fate, regulatory and transport functions.

DISCUSSION

In this study, we have analyzed changes in the bacterial composition and functional profile of the gut microbiota of two patients (F and H) that were positive for *C. difficile* (CD+ samples) after AB treatment and one patient (G) that despite not having taken AB was already CD+ when entered to the hospital. Patients F and H had an unusual microbiota at the start of the study (before AB treatment), enriched in *Akkermansia* genus (30.6%) and highly abundant in *Escherichia* genus (85.7%), respectively. We also compared the gut microbiota of those three patients with five individuals from two previous studies (Pérez-Cobas et al., 2013a,b), who were also treated with AB but did not develop CDI. All the patients fit the same inclusion criteria. Despite the heterogeneity of the samples and only 15 time points are overall compared, we consider that the results obtained with the different analyses performed, provide new insights into the effect of CDI on the structure and metabolic functions of the human gut microbiota. Furthermore, we identified members of the bacterial community and metabolic functions that are differentially present in the CD− samples compared to the CD+ samples and thus could be involved in resistance to *C. difficile* colonization.

The gut microbiota of the three CDI patients showed large variations in bacterial composition and diversity throughout the therapy, confirming that antibiotics disturb the ecological equilibrium of microbial communities. Previous studies showed great fluctuations and low diversity of the human gut microbiota under the effects of a wide variety of ABs, although patients did not develop CDI (Dethlefsen et al., 2008; Dethlefsen and Relman, 2010; Pérez-Cobas et al., 2013a,b). In addition to the influence of AB on the microbiota structure, this survey found that CDI contribute to decreasing bacterial diversity since the infected samples showed, in general, lowest biodiversity index values and richness estimators than non-infected samples. In this respect, a mouse colitis model-based study has suggested that intestinal inflammation during colonization by some pathogens, including *C. difficile*, affect microbiota equilibrium (reviewed in Stecher and Hardt, 2011), contributing to reduced microbial diversity.

Similarly, significant alterations in the abundance of some taxa (mainly from the Firmicutes phylum) and a decrease in microbial diversity and species richness were found in individuals with CDI (Antharam et al., 2013).

We have found that the microbiota of the infected samples (CD+) share some common features, being depleted in commensal genera such as *Ruminococcus*, *Roseburia*, *Subdoligranulum*, *Blautia* or *Coprococcus* and enriched in *Lactobacillus*, *Enterococcus*, Clostridium clusters XlVa and XI. The latter being the phylogenetic cluster which contains the *C. difficile* species (Collins et al., 1994). Although the relative abundance of cluster XI was variable between the infected samples, its presence is higher in CD+ than in CD− samples, probably due to the high abundance of *C. difficile*. The higher abundance of Clostridium cluster XIVa could be a consequence of the microbiota imbalance, since members of this group have been characterized as opportunists (Lozupone et al., 2012). This may also be the case of *Enterococcus*, which is a common opportunistic pathogen that becomes dominant when the normal gut microbiota is disturbed by ABs (Donskey, 2004;

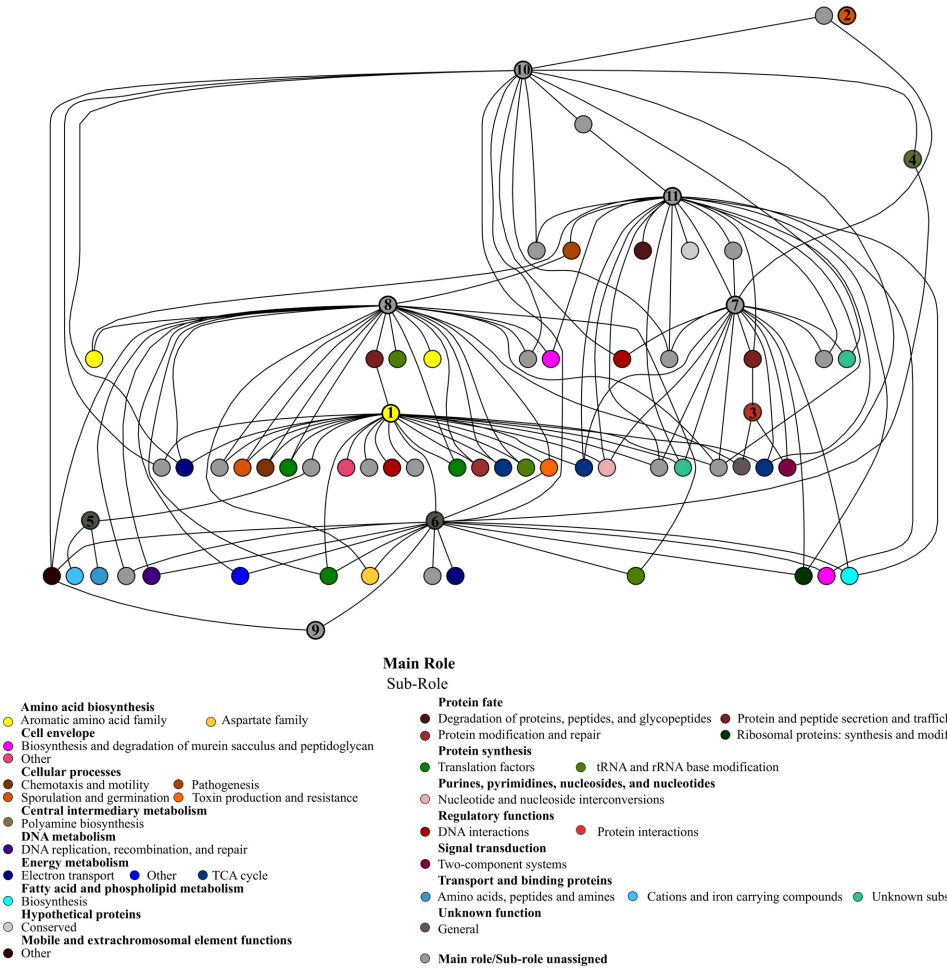

FIGURE 5 | Bayesian network of microbiota potential functions in CD− samples of patients (A–E), during treatment, and the protective candidate taxa. The significant positive correlations (p-value < 0.01) between the candidate protective functions and other functions of the gut ecosystem are shown. Each node represents a specific function with the corresponding subrole color. The candidate protective function nodes are indicated by numbers: (1) Chorismate mutase; (2) Stage IV sporulation protein B; (3) Anti-sigma F factor; (4) Agmatine deiminase; (5) rrf2 family protein; (6) Redox-active disulfide protein 2; (7) Glutamate decarboxylase; (8) Doubled CXXCH domain; (9) Indolepyruvate ferredoxin oxidoreductase; (10) RNA polymerase sigma-70 factor, Bacteroides expansion family 1; (11) UDP-N-acetylglucosamine 4,6-dehydratase.

Ubeda et al., 2010). *Enterococcus* was also over-represented in samples of reduced biodiversity in other CDI studies (Antharam et al., 2013; Vincent et al., 2013). The higher abundance of *Lactobacillus* in the CD+ samples is also interesting. For example, a murine model-based study found that Lactobacillaceae was dominant in CDI samples (Rea et al., 2011) as did a study of CDI in humans (Antharam et al., 2013). Although *Lactobacillus* has been described as an intestinal probiotic genus, different studies show that only specific strains (e.g., *L. delbrueckii*) can inhibit *C. difficile* growth (Naaber et al., 2004; Banerjee et al., 2009). Further research would be needed to clarify the role of *Lactobacillus* strains in gut colonization by *C. difficile*.

The three comparisons performed enabled us to identify taxa that were significantly over-represented in CD− samples, due to AB therapy, in individuals that either did not develop CDI (comparison 2) or recover from CDI (comparison 3), but decreased in those CD+ samples (Comparison 1). Thus, *Anaerovorax*, *Escherichia*, *Gemmiger*, *Oscillibacter*, *Ruminococcus*, *Subdoligranulum*, uc_Clostridia, uc_Clostridiales, uc_Erysipelotrichaceae, and uc_Ruminococcaceae were found as candidates for protecting against *C. difficile* colonization. Bayesian correlation networks are a powerful tool to search and study ecological or metabolic associations in microbial communities (Durbán et al., 2012), and thus we used them to look for other taxa associated to the above, which may be also indirectly involved in resistance by ecologically interacting with the candidates. Most of the taxa in the network belonged to Clostridia: *Ruminococcus*, *Subdoligranulum*, *Oscillibacter*, *Anaerovorax*, *Roseburia*, *Coprococcus*, *Anaerotroncus*, *Gemminger* and other unclassified members of Lachnospiraceae and Ruminococcaceae families. It has been proposed that competition of normal gut microbiota members with their related pathogens for limiting resources or sites, called "niche exclusion," could be a colonization resistance mechanism (reviewed in Britton and Young,

2012). Thus, this niche hypothesis could explain the role of these related taxa belonging to Clostridiales in protecting against CDI. In this regard, some studies in mice have shown that Clostridia members, such as Lachnospiraceae, are *C. difficile* antagonists and restore the microbiota when fed to infected mice (Itoh et al., 1987; Reeves et al., 2011, 2012; Lawley et al., 2012). Another study in hamsters showed that non-toxigenic *C. difficile* were able to prevent the toxigenic pathogen (Sambol et al., 2002; Merrigan et al., 2003), suggesting a more efficient utilization of limiting nutrients (niche exclusion) as the protection mechanism. In human studies, members of the Ruminococaceae and Lachnospiraceae families were significantly depleted in CDI patients (Antharam et al., 2013).

Some of the Clostridia members found to be associated to the main protective candidate taxa, such as *Roseburia* or *Coprococcus*, are active anaerobic short-chain fatty acids (SCFA) producers (Barcenilla et al., 2000; Pryde et al., 2002). This could be other mechanism through they are candidates to protect against CDI, since SCFA are reported to inhibit *C. difficile* growth and also to decrease the production of toxin *in vitro* (May et al., 1994). Moreover, it has been postulated that the anaerobic fraction of the microbiota is essential for gut ecosystem stability in healthy individuals, because the butyrate and other SCFAs they produce have anti-inflammatory effects and stimulate the immune system and, thus, this imbalance increases the risk of *C. difficile* overgrowth (Bartlett, 2002; Roy et al., 2006; Jernberg et al., 2010). However, a recent study in mice found that SCFA production was no correlated with lower levels of *C. difficile* colonization (Reeves et al., 2012). In addition, these authors found that the microbiota composition of CDI mice was partially restored when they used only one isolate of the Lachnospiraceae family for inoculation. Nevertheless, total restoration was obtained when total fecal content was transferred from a wild-type mouse. These results agree with our findings because we have found several putative candidate protective taxa, indicating that more than one bacterial group is involved in pathogen protection. Hence, further research should test *in vivo* the colonization resistance capacity of the specific ensemble we have proposed.

In a previous study, we showed that the metabolic profiles of AB-associated shifts in human gut microbiota were less dramatic than those in bacterial composition, principally when considering main roles. This is due to functional redundancy of the human gut microbiota, and the fact it has a very general set of functions (Pérez-Cobas et al., 2013b). We have also found great homogeneity in distribution of the main role in all the samples. However, differences appear when considering more inclusive functional levels (sub-roles and functions). In this study, patients showed different functional responses (sub-roles) to ABs, in agreement with our previous study where a great inter-individual variability was found in AB-treated patients. Although no significant differences between both groups of AB-treated patients (CDI and non-infected) as a whole were detected, a specific functional profile was found. Thus, the transport, metabolism, and regulation of sugars such as mannose, fructose, lactose, glucitol, or mannitol were over-represented functions in CDI samples, the major sugar transport system being the phosphotransferase system (PTS). In a previous work, we found that AB increases PTS in metagenomes, since it seems to give advantage to bacteria carrying them under stress conditions (Deutscher et al., 2006; Pérez-Cobas et al., 2013b). The higher presence of these functions in CD+ samples compared to CD− is noteworthy, even when both were treated with ABs, because it could be related with the infection, as shown in a metabolomic study in mice that developed CDI (Theriot et al., 2014). The same authors found an increase in carbohydrates like mannose, fructose, lactose, glucitol, or mannitol after AB treatment, and they postulated that these increases favored *C. difficile* germination and growth. Related to this finding, a transcriptomic study revealed that sugars released by an altered microbiota are exploited by enteric pathogens such as *Salmonella enterica* and *C. difficile* (Ng et al., 2013). Thus, *C. difficile* and other opportunistic bacteria can efficiently catabolize the excess of carbohydrates generated by the disrupted microbiota and, in the absence of competitors, increase colonization rates.

Using the same three comparisons, we also found metabolic functions that may play a role in *C. difficile* colonization resistance (**Table 5**). Overall, there was a higher abundance of functions related to aromatic amino acid biosynthesis, being chorismate mutase the central node of the network, since it was strongly connected to other important functions like energy metabolism or protein fate. The chorismate mutase, which participates in tyrosine, phenylalanine and tryptophan biosynthesis, could be involved in colonization resistance through stimulation of the immune system, since the tryptophan metabolite participates in immune system equilibrium and inflammation regulation (Zelante et al., 2013). Future research should be conducted to discover the mechanism by which aromatic amino acid synthesis could protect against colonization by *C. difficile*. Also, some energy metabolism pathways seem important, such as TCA cycle, electron transport, or fatty acid biosynthesis. A great number of different transporter families, regulator genes, and genes involved in responses to osmotic or acid stress were also highlighted in the network, possibly playing a role in colonization resistance.

Another possible protective pathway was peptide catabolism via tryptophan metabolism. Low abundance of protein digestion markers was associated to susceptibility to CDI in the mouse gut (Theriot et al., 2014). Regarding host immune response, we found polyamine biosynthesis (putrescine or cadaverine) by decarboxylation of amino acids to be another potential protective pathway. A previous study reported that these metabolites interact with the gut microbiota, stimulating the immune system and playing a role in intestinal maturation (Gómez-Gallego et al., 2012). In this regard, Jung et al. (2003) found that glutamate decarboxylase activity, related to polyamines, was also a protective determinant, playing a role in protection against acid stress. It is also relevant that this enzyme is connected to other functions in the network, such as protein fate, transcription regulation, or transport systems, thus reinforcing its protective role. Moreover, other protective gene-products regulate metabolic pathways that are important for several cellular physiology processes, like osmotic stress resistance and responses to environmental changes (Wouters et al., 2010; Shepard et al., 2011).

In summary, we found specific fecal microbiota in CDI patients as it was enriched in *Lactobacillus*, *Enterococcus*,

Clostridium clusters XIVa and XI but depleted in SCFA-producing bacteria. The latter bacterial group could be involved in *C. difficile* colonization resistance. A group of metabolic processes related to the metabolism of proteins, amino acids and responses to stress would seem to participate in avoiding pathogen invasion in the human gut ecosystem. Further research into these pathways should be undertaken to unravel the mechanism by which they participate in colonization resistance to *C. difficile*. A larger cohort of patients with similar sampling would be needed to deeper define the CDI microbiota at taxonomic and functional level.

AUTHOR CONTRIBUTIONS

Andrés Moya, María J. Gosalbes, and Amparo Latorre conceived the work. Ana E. Pérez-Cobas performed all the analyses. María J. Gosalbes and Alejandro Artacho help with some of the analyses. The manuscript was written by Ana E. Pérez-Cobas, María J. Gosalbes, and Amparo Latorre. Andrés Moya and Stephan J. Ott revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00335/abstract

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SUPPLEMENTARY TABLES

Supplementary table 1 Biodiversity measures for C. difficile positive patients F, G and H based on OTUs (97%).

Sample	CD(+/-)	N	Shannon	Chao1	SE (Chao 1)	ACE	SE (ACE)
F_before	CD-	211	2.83	255.2	16.99	255.54	7.99
F16_D	CD+	114	2.48	172.58	30.18	158.46	6.34
F_after	CD+	31	2.38	49	49.09	44.63	3.12
G_before	CD+	272	4.31	311.84	15.39	309.99	8.63
G4_D	CD+	357	4.77	433.78	22.06	428.53	10.11
G_after	CD+	158	2.45	236.55	26.97	263.6	9.98
H_before	CD-	45	2.44	52.2	9.02	51.68	3.51
H7_D	CD-	79	3.39	95.87	10.32	107.61	5.68
H14_D	CD-	102	3.58	123	14.34	125	5.5
H20_D	CD-	187	3.72	216.4	11.51	229.16	7.52
H35_D	CD+	53	2.25	87	36.98	72.82	4.45
H38_D	CD+	114	2.85	177.91	33.8	155.8	6.43
H_after	CD-	112	4.09	140.96	13.41	147.94	6.32

N: Number of OTUs; SE (Standard Error); ACE: Abundance coverage estimator; NaN: not a number

Supplementary table 2 Differential taxa abundance between CD+ (H35_D, H38_D) and CD- samples (H14_D, H20_D) in patient H.

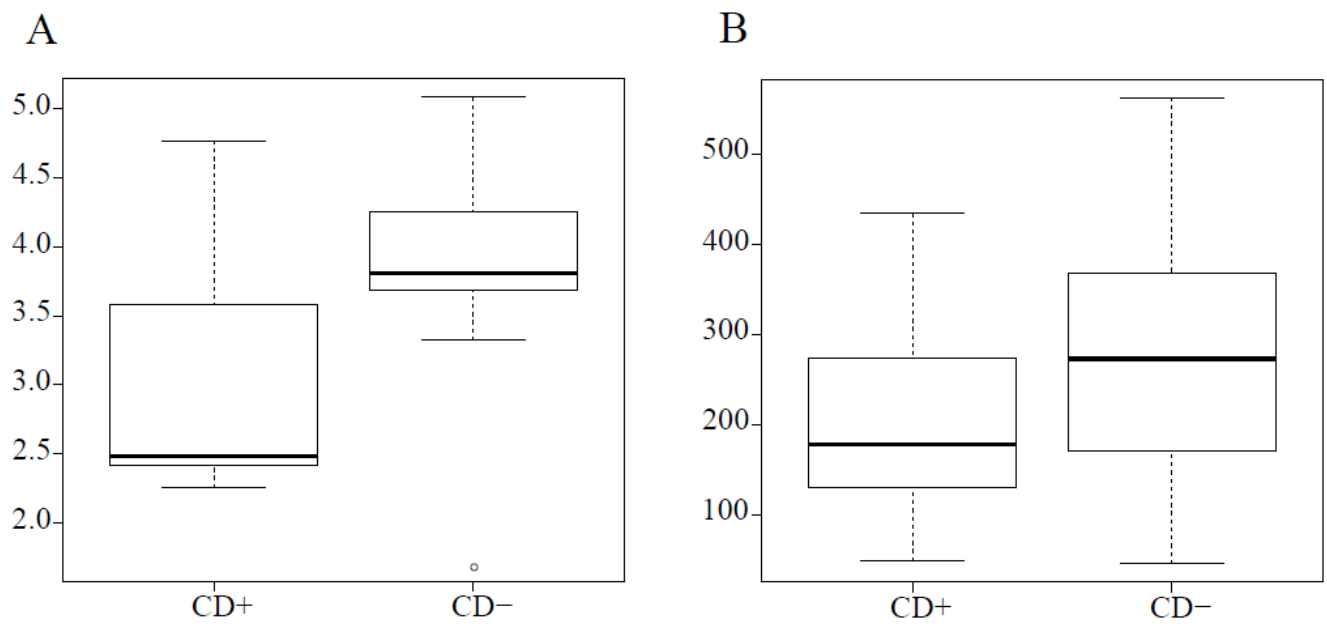
Bacterial taxa	Abundance in CD+ samples	P-value
Lactobacillus	increase	0
Streptococcus	increase	1.49E-47
uc_Lactobacillaceae	increase	9.24E-10
Proteus	increase	3.83E-4
Sutterella	increase	3.74E-3
Bacteroides	decrease	7.33E-103
Escherichia	decrease	6.73E-165
Klebsiella	decrease	1.18E-145
Enterococcus	decrease	6.43E-10
Raoultella	decrease	3.72E-3
Clostridium cluster XIVa	decrease	1.31E-2

Supplementary Table 3. Comparisons of the sub-role abundance and p-value associated. Patient F: (B / D+) before and during therapy (F_before *vs* F16_D). Patient H: (B / D-) before *vs* during the treatment but prior *C. difficile* detection (H_before *vs* H7_D, H14_D and H_20_D); (B / D+) before *vs* CD+ samples (H_before *vs* H35_D and H38_D); (D- / D+) during AB (H14_D and H20_D *vs* H35_D and H38_D). Arrows show sub-roles more (upward) and less (downward) abundant. NS, not significant.

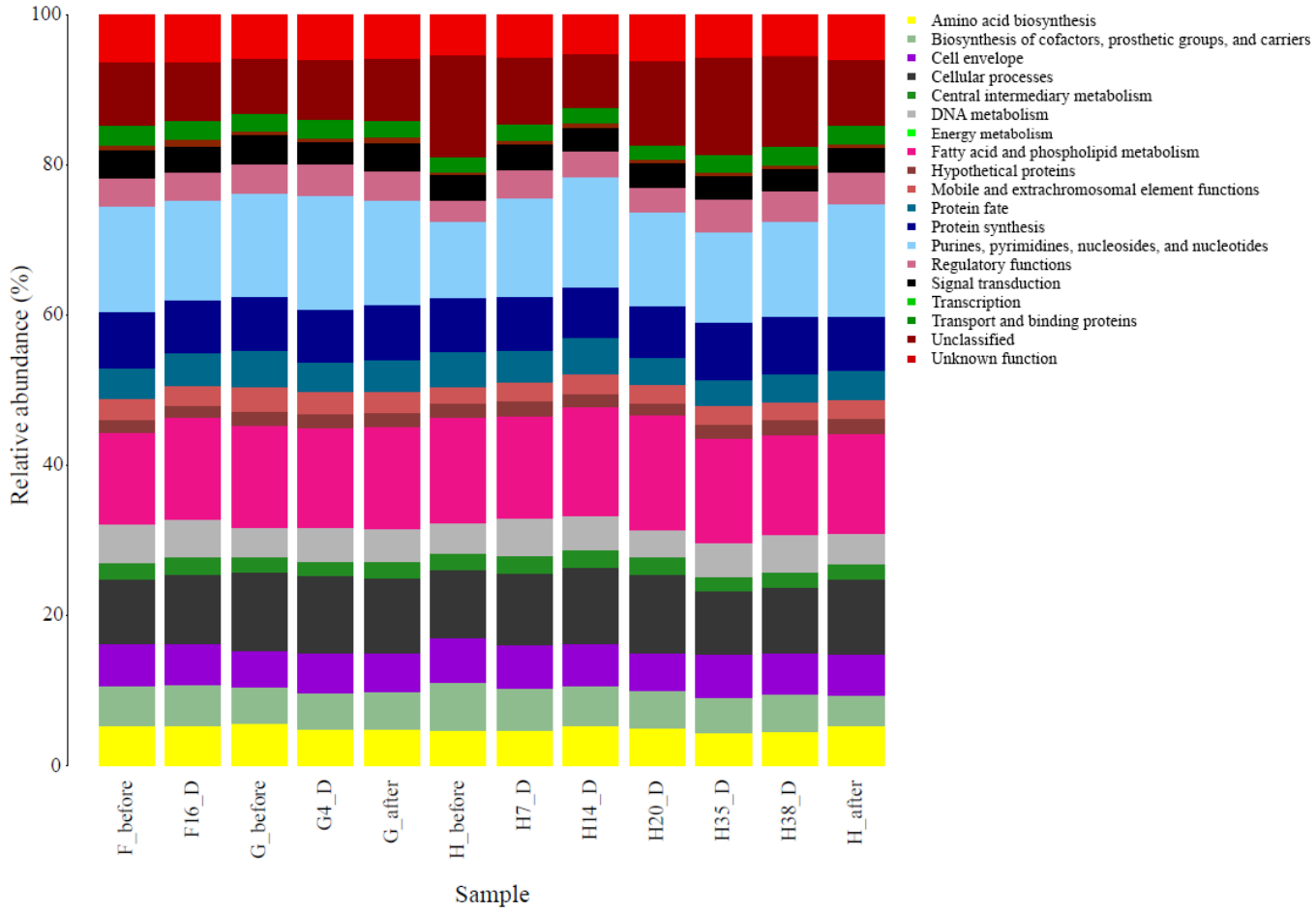
		Patient F	Patient H		
Main Role	Sub-Role	B/D+	B/D-	B/D+	D-/D+
Amino acid biosynthesis	Aromatic amino acid family	NS	↑0.02	NS	↓3E-04
	Aspartate family	NS	↑0.02	↑2E-03	NS
	Glutamate family	↓0.01	↓0.02	NS	↑5E-05
	Histidine family	NS	NS	↓2E-03	↓2E-04
	Pyruvate family	NS	↓0.04	↓2E-04	NS
	Serine family	NS	NS	NS	↓0.02
Biosynthesis of cofactors, prosthetic groups, and carriers	Biotin	↑4E-03	NS	↓3E-03	↓3E-05
	Chlorophyll and bacteriochlorphyll	↓0.04	NS	NS	NS
	Folic acid	NS	↓9E-014	↓2E-26	NS
	Heme, porphyrin, and cobalamin	NS	NS	NS	NS
	Menaquinone and ubiquinone	↓0.04	↓0.04	NS	↑0.01
	Molybdopterin	NS	NS	NS	↑0.03
	Other	NS	NS	↓4E-03	↓5E-03
	Pyridine nucleotides	NS	NS	NS	↑0.03
	Pyridoxine	↑4E-03	NS	NS	↓0.01
	Riboflavin, FMN, and FAD	↑0.02	NS	NS	NS
Cell envelope	Murein sacculus and peptidoglycan*	↓2E-06	↓0.03	↑4E-03	↑8E-012
	Surface polysaccharides**	↑7E-019	NS	NS	NS
	Other	↓0.04	NS	NS	NS
	Surface structures	↓2E-011	↓2E-07	↓8E-12	NS
Cellular processes	Adaptations to atypical conditions	↑2E-06	NS	NS	NS
	Biosynthesis of natural products	↓4E-03	NS	NS	↑0.02
	Cell division	↓1E-04	NS	NS	NS
	Chemotaxis and motility	NS	↓0.02	↓3E-02	NS
	Detoxification	↑0.01	NS	NS	NS
	DNA transformation	↑6E-019	↑5E-08	NS	↓2E-07
	Pathogenesis	↓0.01	↓0.01	↓7E-03	NS
	Sporulation and germination	↓0.01	↑0.01	NS	↓5E-03
	Toxin production and resistance	↓0.02	↑2E-04	NS	↓2E-06
Central intermediary metabolism	Amino sugars	NS	↓2E-06	↓4E-05	NS
	Nitrogen fixation	NS	↑4E-03	NS	↓5E-03
	Nitrogen metabolism	NS	NS	↓2E-03	NS
	One-carbon metabolism	NS	NS	↑1E-02	NS
	Other	NS	NS	↑2E-02	↑5E-03
	Phosphorus compounds	↓2E-04	NS	↑5E-02	↑3E-03
	Polyamine biosynthesis	NS	↑0.01	NS	↓3E-04
	Sulfur metabolism	NS	NS	↓4E-04	↓5E-03
DNA metabolism	Chromosome-associated proteins	↑9E-037	↑2E-05	NS	↓2E-07
	Degradation of DNA	↓3E-03	NS	NS	NS
	DNA replication, recombination and repair	↓5E-03	NS	↑2E-03	↑4E-05
	Restriction/modification	NS	NS	↓1E-02	NS
Energy metabolism	Aerobic	NS	NS	NS	↑0.01
	Amino acids and amines	↑2E-05	NS	NS	↓0.01
	ATP-proton motive force interconversion	↑3E-03	NS	↑1E-02	↑0.02
	Biosynthesis/degradation of polysaccharides	↓2E-08	↓0.01	NS	↑2E-06
	Electron transport	↑0.03	NS	↓3E-05	↓3E-010
	Entner-Doudoroff	NS	↓0.01	NS	NS
	Fermentation	↑1E-03	↓0.02	↑2E-02	↑1E-07
	Glycolysis/gluconeogenesis	NS	NS	↑2E-03	↑0.05
	Other	NS	NS	NS	↓0.01
	Pentose phosphate pathway	↑8E-011	↓0.01	NS	NS

	Photosynthesis	↑0.01	↑2E-04	NS	↓0.01
	Pyruvate dehydrogenase	↓2E-05	NS	NS	NS
	Sugars	↓0.01	↓0.01	NS	↑0.01
	TCA cycle	NS	NS	↓2E-03	↓2E-03
Fatty acid+	Biosynthesis	↓0.02	NS	NS	NS
	Degradation	NS	NS	NS	↑0.05
Hypothetical proteins	Conserved	↓0.04	NS	NS	NS
	Domain	↓0.04	NS	NS	NS
Mobile and extrachromosomal element functions	Other	NS	NS	↓3E-05	↓2E-011
	Plasmid functions	NS	↓6E-010	↓4E-25	↓6E-04
	Prophage functions	NS	↑0.05	NS	NS
	Transposon functions	NS	NS	↑3E-02	NS
Protein fate	Proteins, peptides, and glycopeptides***	NS	NS	↑1E-03	↑2E-03
	Protein and peptide secretion and trafficking	↓2E-03	NS	NS	NS
	Protein folding and stabilization	↓2E-04	NS	↑3E-02	↑1E-05
	Protein modification and repair	↓0.04	NS	NS	NS
Protein synthesis	Other	NS	NS	↑8E-03	NS
	Ribosomal proteins: synthesis/modification	NS	↑3E-09	↑4E-06	NS
	Translation factors	NS	↑4E-08	↑1E-03	↓0.03
	tRNA aminoacylation	↓2E-014	NS	↑1E-03	↑5E-07
	tRNA and rRNA base modification	NS	↑0.02	NS	↓9E-06
Purines, pyrimidines, nucleosides, and nucleotides	2'-Deoxyribonucleotide metabolism	NS	NS	↑8E-03	NS
	Purine ribonucleotide biosynthesis	NS	NS	↑4E-05	↑3E-03
	Pyrimidine ribonucleotide biosynthesis	↓0.02	NS	↑2E-03	NS
	Salvage of nucleosides and nucleotides	↑0.02	NS	NS	↑0.02
Regulatory functions	DNA interactions	NS	↓0.04	↓2E-03	NS
	Protein interactions	NS	NS	↓2E-03	NS
	Other	↓3E-06	NS	↑3E-03	↑0.01
	Small molecule interactions	↑0.01	NS	NS	NS
Signal transduction	PTS	NS	↓1E-016	NS	↑5E-016
	Two-component systems	↑0	↑4E-05	NS	↓1E-05
Transcription	DNA-dependent RNA polymerase	NS	↑0.01	↑2E-14	↑3E-07
	RNA processing	↑0.02	↓0.05	NS	NS
	Transcription factors	↓0.04	NS	NS	NS
Transport and binding proteins	Amino acids, peptides and amines	↓3E-012	↓1E-07	NS	↑3E-012
	Anions	↓9E-09	↓7E-08	NS	↑3E-08
	Carbohydrates, organic alcohols and acids	NS	↓10E-035	↓3E-08	↑1E-012
	Cations and iron carrying compounds	NS	↓1E-03	NS	↑0.01
	Nucleosides, purines and pyrimidines	↑3E-03	↓0.02	NS	↑0.03
	Other	NS	↓2E-06	NS	↑4E-08
	Unknown substrate	↑0.03	NS	↑1E-02	NS
Unknown function	Enzymes of unknown specificity	↓2E-010	NS	NS	↑0.04
	General	↑0.04	NS	NS	NS

+Fatty acid and phospholipid metabolism.
*Biosynthesis and degradation of murein sacculus and peptidoglycan.
**Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides.
***Degradation of proteins, peptides, and glycopeptides.



Supplementary Figure 1 Diversity analyses in CD+ (n=7) and CD- (n=15) samples. (A)Shannon index and (B) Chao 1 estimator of CD+ (F_16D, F_after, G_before, G4_D, G_after, H_35D and H_38D) and CD- (patients A, B, C, D and E during AB) samples. Thickest line indicates the median.



Supplementary Figure 2 Relative abundance of the main functional roles of samples from patients F, G and H.

4

GENERAL DISCUSSION

4. GENERAL DISCUSSION

Nowadays, some aspects of the modern lifestyle as global mobility, Western diet, sanitation or medical therapies alter human-microbe interactions that are the result of millions years of co-evolution. Some of the most influencing factors are an improved hygiene and sanitation, as well as medical therapies, principally the antibiotic administration (Dethlefsen et al. 2007). Antibiotic administration disrupt the normal gut microbiota, affecting the human-microbiota relationships and therefore the human health. Among others consequences, antibiotic-induced perturbation leads to reduce the colonization resistance of the bacterial community, allowing the overgrowth of opportunistic pathogens as *C. difficile*. Moreover, the potential spread and stabilization of antibiotic resistance genes from gut bacteria to pathogens represents a major problem in public health (Cotter et al. 2012, Jernberg et al. 2007, 2010; Macfarlane 2014, Salyers et al. 2004, Sommer et al. 2009).

In this thesis, we focused on the consequences of antibiotic therapy on the structure, functions and stability of the human gut microbiota, as well as on its role in the infection by the intestinal pathogen *C. difficile*. All the patients of the study were part of the same European survey (ERA-NET project on *C. difficile*) and were recruited for the project at the Department of Internal Medicine of the University Hospital Schleswig-Holstein in Kiel, Germany. We used faecal samples of this set of patients to perform an in-depth follow-up analysis of antibiotic effects and CDI on the human intestinal microbiota by analysing different molecular markers of the microbial community diversity and activity (DNA, RNA, proteins and metabolites).

The general tools we applied consisted on 16S rRNA-based approaches, metagenomics and metatranscriptomics in conjunction with pyrosequencing (Roche GS FLX sequencer and Titanium chemistry), as well as metaproteomics and meta-metabolomics (these last two, in collaboration with the group of Manuel Ferrer at the Institute of Catalysis, Madrid, Spain). We characterized the changes associated to different antibiotic therapies on the composition and diversity of the total and active fraction of the human faecal microbiota, by the pyrosequencing of the 16S rDNA (total microbiota) and 16S rRNA (active microbiota) molecules, respectively. Shifts on the potential functions of the intestinal microbiome related to antibiotic administration were evaluated through pyrosequencing the total DNA of the faecal microbial community (metagenomes). Moreover, the dynamics of the functions of the metabolically active faecal community under antibiotic stress was addressed by analysing the total mRNA (metatranscriptomes), proteins (metaproteomes) and metabolites (meta-metabolomes) in one patient.

Overall, we were able, for the first time, to obtain a general picture of the responses of the human gut microbiota to antibiotic therapy and CDI, integrating the information of different biological levels. Moreover, we identified bacteria and functions probably involved in colonization resistance against *C. difficile*.

In Chapter 3.1 we performed a follow-up study of the gut microbiota of a hospitalized patient along a beta-lactam antibiotic treatment. This study constitutes a proof of concept, since it was the first report about antibiotic-associated changes of the gut microbiota based on the application of multiple "omics" approaches. This multi-analysis included the characterization of the diversity of the total and active microbiota (16S rRNA based approaches), the gene content (metagenomics), the expressed genes (metatranscriptomics), the proteins (metaproteomics) and the metabolites (meta-metabolomics) of every sample before, during and after the antibiotic course. This work by combining different “omics” provided an overview of the functional status of the gut ecosystem under antibiotic stress.

In Chapter 3.2 we performed a follow-up study of the gut microbiota of four individuals under different antibiotic therapies. Antibiotics administered to the patients presented bactericidal and bacteriostatic antimicrobial effects. Besides, the antimicrobial agents presented different modes of actions that we grouped in three: cell replication inhibitor, protein synthesis inhibitor and cell envelope synthesis inhibitor. In this work, through 16S rRNA-based approaches and metagenomics, we evaluated the effect of inherent properties of the antibiotics (the antimicrobial effect and mode of action) on the modelling of the total and active gut microbiota and its genetic potential. We also analysed the functional changes and possible responses of the microbiota associated to antibiotic stress, including the resistance gene reservoir evolution along antimicrobial therapies.

In Chapter 3.3 we carried out a follow-up study of three individuals under antibiotic therapy, similar to the previous

works, but in this case the three patients developed CDI. We also used 16S rRNA gene and metagenomic approaches to characterize shifts on the structure and functions of the gut microbiota due to antibiotic treatment and CDI. In addition, we performed a comparative analysis of this group of patients with the patients from the two previous studies (Chapters 3.1 and 3.2) that, as stated before, were also treated with broad spectrum antibiotics but that did not develop CDI. Specifically, we performed three different statistical comparisons among the different samples of the three studies that allowed identifying bacterial taxa and metabolic functions related with the infection process, as well as others that could be candidates to protect against *C. difficile*. Moreover, we used Bayesian networks to identify other taxa and functions possibly involved in colonization resistance based on their putative association (positive correlation) with the protective candidates.

Various studies about antibiotic effects on the gut microbiota in animals and humans, including our own work, described large variations in the relative abundance of bacterial taxa, a reduced diversity on the total gut microbiota during antimicrobial therapies, as well as an almost return to the original composition several weeks after treatment cessation, although with some unrecoverable minority taxa (Antonopoulos et al. 2009, Dethlefsen & Relman 2011, Dethlefsen et al. 2008, Jernberg et al. 2007, Macfarlane 2014, Pérez-Cobas et al. 2013a,b). Similar to the non-infected patients, the microbial diversity and the composition of the patients infected by *C. difficile* exhibited strong fluctuations as a consequence of the antibiotics, but also due to the infection when the minimum values were reached (Pérez-Cobas et al. 2014). Moreover, our works were the first studies to address the effects of antibiotics on the active fraction of the gut microbiota. Interestingly, we found that the active fraction during treatment showed less variations (although significant) than the total community, since it contained the resistant bacteria that continue growing and take longer to be affected by the antibiotic (Pérez-Cobas et al. 2013a,b).

In our studies about the effect of broad spectrum antibiotics on the human faecal microbiota, we observed that the oscillatory dynamic in the microbial composition was in correspondence to the metabolism of the administered antibiotics, and the abundance of the resistant microbial populations. For instance, during the first days of a beta-lactam treatment there was a dominance of Firmicutes species (Gram-positive), then, a collapse of diversity occurred due to the continuing therapy leading to a shift toward Bacteroidetes species (Gram-negative) that are considered as naturally resistant to beta-lactams and that also remained more active during the antibiotic course (Pérez-Cobas et al. 2013a). In general, the use of these broad-spectrum antibiotics resulted in an increase in the relative abundance of Gram-negative bacteria as Bacteroidaceae or Enterobacteriaceae families in the microbiota of the patients (Pérez-Cobas et al. 2013a,b). A similar increase in Gram-negative members has been recently described in a cohort of 21 patients treated for a week with broad spectrum antibiotics, specifically beta-lactams and fluoroquinolones (Panda et al. 2014).

In agreement with the high inter-individual variability previously described for the human gut microbiota, we found that each patient exhibited a specific initial microbial assembly, and in consequence an individual response to therapy for both groups, the infected and non-infected patients (Ahmed et al. 2007, Caporaso et al. 2011, Eckburg et al. 2005, Green et al. 2006). We also showed that the initial microbiota composition strongly influenced the shifts on the intestinal microbiota during antibiotic therapy (Pérez-Cobas et al. 2013a,b, 2014). However, the selection of resistant microorganisms after similar antibiotic treatment, led to microbial assemblies which share some features, regardless of the initial composition. Specifically, properties of the antibiotics as the antimicrobial effect and mode of action influenced the gut microbial composition in a significant manner. For instance, the clindamycin, which presents a bacteriostatic antimicrobial effect, led the microbiota of a treated patient to a microbial composition that largely differs from that of the other patients that were treated with bactericidal antibiotics. In addition, the effect of the mode of action was reflected on the gut microbiota composition, mainly of the active fraction, since it contained those bacteria able to resist the treatment (Pérez-Cobas et al. 2013b). A significant influence of antibiotic mode of action on the modelling of the human gut microbiota was recently described in a cohort of elderly subjects under antibiotic therapy (O'Sullivan et al. 2013).

The pattern of similarities between the samples of the infected patients showed an influence of the CDI, but also, as expected a high inter-individual variability. The comparison of the microbiota structure of infected and non-infected patients showed a lower (although not significant) diversity in the *C. difficile* positive samples. However, it was found a significant effect due to the infection by the pathogen in terms of taxa presence and abundance, grouping the infected samples together, despite the large

overall variability aforementioned. The infected samples were characterized by an over-abundance of different genera as *Lactobacillus*, *Bacteroides* or *Enterococcus* and a low representation of commensal members as *Roseburia*, *Coprococcus*, *Blautia* among others (most of them from Firmicutes phyla).

On the other hand, the metagenomic analysis revealed that the variations caused by the antibiotics and the strength of the signal due to the antimicrobial effect and mode of action were lower in the gene content than in the microbial structure (Pérez-Cobas et al. 2013a,b, 2014). This result could be due to the high functional redundancy of the human gut microbiome previously reported (Lozupone et al. 2012, Qin et al. 2010, The Human Microbiome Project Consortium 2012 Turnbaugh et al. 2010).

However, the analysis of the metagenomes during the antibiotic courses showed individual responses of the microbiota also at functional level, for infected and non-infected patients (Pérez-Cobas et al. 2013a,b, 2014). The functional changes associated to antibiotics depended on the strategies of the surviving bacteria and its dynamics along the course. For instance, a patient treated with clindamycin, which had the most different taxonomic composition along the treatment respect to the others, also showed a clearly distinct functional profile, according to the strategies of the resistant bacteria, in this case opportunistic pathogens of Enterobacteriaceae family (*Escherichia*, *Klebsiella* and *Salmonella*). In fact, the gut microbiota of this patient exhibited a higher abundance of genes related to pathogenicity and others involved in the synthesis of lipopolysaccharides that constitute the main component of the outer membrane for Gram-negative bacteria, as it is the case of Enterobacteriaceae family (Pérez-Cobas et al. 2013b). The outer membrane participates in nutrient uptake and confers resistance against antibiotics, taking advantage this bacterial group under adverse conditions (Doerrler 2006). A mechanism of antibiotic response that seems to be quite general is related to sugar transport, since a higher presence of genes from the phosphotransferase system (PTS) was described for most of the patients during the treatments (Pérez-Cobas et al. 2013b). The PTS is the main sugar translocation system for bacteria and also participates in different stress responses (Deutscher et al. 2006). Similarly, enrichment in genes involved in sugar metabolism was described on the gut phage community of antibiotic-treated mice as well as on the gut microbiota of treated pigs (Looft et al. 2012, Modi et al. 2013). In addition, in one of our collaborations, it was found that antibiotic-treated and obese individuals showed higher and less balanced sugar anabolic capability respect to healthy and lean subjects (Hernández et al. 2013). Hence, the beneficial effect of this function may be due to the role of sugar transporters in counteract osmotic stress, as well as to their participation in the energy metabolism of the benefited bacteria, giving to them an advantage for survival and colonization on the gut, especially under unstable conditions. Interestingly, the functional comparison between the metagenomes of infected and non-infected patients revealed an over-abundance of carbohydrate transport genes in the infected samples, showing an association of sugar metabolism and pathogen infection, a point that has been previously described in mice studies (Ng et al. 2013, Theriot et al. 2014). The antibiotic-induced alterations in the sugar availability and metabolism of the gut microbiota could explain the growing of some opportunistic pathogens as *C. difficile* that has been described as able of metabolizing carbohydrates liberated by an antibiotic-disturbed microbiota (Ng et al. 2013). In support to the role of carbohydrates in CDI, a different metabolic study described an increase in sugar alcohols (as sorbitol, mannitol) in the gut metabolome of animals susceptible to infection (Theriot et al. 2014).

The analysis of the resistome during antimicrobial treatments supported the claim that antibiotic usage increases the abundance of resistance genes in microbial communities. Notably, after antibiotic therapy we found not only an increase on the abundance of resistance genes against the administered antibiotic but also against others, as a side effect. Even more, it was found a higher relative abundance on multidrug resistance genes, an issue that is concerning in hospital environments. It was also reported a cross-resistance increase, (including multidrug-resistance genes) after antibiotic therapy in the phage community of the mice gut and also in the swine intestinal microbiota (Looft et al. 2012, Modi et al. 2013). The final gut resistome after antibiotic therapy is a complex process principally determined by the initial microbial composition (the initial resistance genes reservoir), the type of administered antibiotic and the dynamic of the surviving bacteria and the resistance genes they carried, playing an important role the cross-resistance genes (Pérez-Cobas et al. 2013b).

We also studied the antibiotic-associated changes in the gene expression, protein production and metabolic activity of the gut microbial community through metatranscriptomics, metaproteomics and meta-metabolomics approaches, respectively (Pérez-Cobas et al. 2013a).

The metatranscriptomic analysis of a beta-lactam treated patient showed that at the first days of treatment, the gut microbial community responded up-regulating genes involved in avoiding the antimicrobial effects (as expressing beta-lactamases) and down-regulating genes of the CRISPR/Cas system, which could favour the acquisition of resistance genes, while during the last days of treatment the up-regulated genes were related to renewal, maintenance and repair of essential molecules. Besides this, many of the genes participating in antibiotic responses returned to a basal level after cessation of antibiotic treatment, but the expression of genes of energetic metabolism, which was affected by the previous antibiotic administration, was restored after therapy. It is important to highlight that after the suppression of the most abundant bacteria during antibiotic therapies, specific bacterial groups, including minor community members, significantly contribute to the gene expression and in consequence to the overall metabolism of the community (Pérez-Cobas et al. 2013a).

From the meta-metabolomic analysis it is worth to mention that not only bacterial, but also some metabolites produced by the host and further processed by bacteria (derivatives of bile acids, cholesterol, hormones), decreased during antibiotic treatment and increased after it, showing how the antibiotics can alter the interplay between the liver/pancreas and colonic bacterial enzymes, possibly affecting host physiological processes (Pérez-Cobas et al. 2013a). A similar result was reported for a mouse-based study were antibiotics administration strongly disrupted the metabolic homeostasis of the gut, affecting negatively the hormone, bile acid and cholesterol metabolism (Antunes et al. 2011). Also, during treatment, some attenuated bacterial pathways as glycolysis, tricarboxylic TCA cycle, glutamate metabolism or iron uptake, led to a lower amount of iron, sugars, branched amino acids, short organic acids and pyruvate in the gut environment. Similarly, a study of rats treated with beta-lactam antibiotics showed a lower abundance of amino acids (mainly tryptophan), carbohydrates, SCFAs, and TCA cycle metabolites (Zheng et al. 2011).

The metaproteomic approach, showed a notable decrease on protein production as a consequence of treatment. Important metabolic pathways like glycolysis, tricarboxylic acid cycle, glutamate metabolism, metal uptake or vitamin synthesis were affected, suggesting that antibiotics negatively influence the overall metabolic status of the gut ecosystem (Pérez-Cobas et al. 2013a). A related metaproteomic study (in collaboration with our group) also showed that beta-lactam therapies alter the digestion of dietary sugars by the intestinal microbiota, which has detrimental consequences for the metabolic gut status (Hernández et al. 2013). Similar to the gene expression profiles during the course, an enrichment of proteins involved in antibiotic responses like antimicrobial peptide transporters or multidrug efflux pumps was found and the proteins expression seemed to recover after therapy.

As we previously mentioned, antibiotics disrupt the human gut microbiota favouring infection by *C. difficile*, which is the leading cause of antibiotic-associated diarrhea and a current problem in developed countries, since its incidence and severity have increased during the last years. Besides this, the emergence of multidrug resistance strains reduced the efficiency of the standard treatment, and consequently an increase in the probability of relapses (Karadsheh & Sule 2013, Ley 2014). Taur and collaborators suggested that using antibiotics to cure a condition caused by antibiotics is a conceptually incorrect strategy and that it could be the reason for the high recurrence rates for CDI. In fact, the common treatments for relapse cases consist on prolonged or pulsed antibiotic courses with low success (Kelly & LaMont 2008, Taur & Pamer 2014).

Since a healthy gut microbiota is able to maintain the pathogen "out of play" through its colonization resistance capacity, restoration of the gut microbiota seems to be the most promising approach to face CDI, especially for recurrent cases. In this sense it has been proposed a therapy that consists in transferring faeces from a healthy donor to a patient, a process that is known as faecal microbiota transplantation. The cure rates of faecal transplantation on recurrent CDI patients have been by far higher than those of the patients treated with conventional antibiotic treatments (Borody et al. 2004, Petrof et al. 2013, Taur & Pamer 2014, van Nood et al. 2013). However, the microbial ecosystem of faeces is complex, and many biological processes which occur there remain unknown. This fact introduces some concerns about faecal microbial transplantation, such as a possible introduction of pathogens, or alterations of microbiota-host interactions that could trigger some microbiota-related diseases as obesity or metabolic syndrome (Borody & Khoruts 2012, Kassam et al. 2013, Ley 2014, Taur & Pamer 2014). In this regard, an alternative may be to identify the consortia of bacteria involved in pathogen protection, so that, they could be administered to patients in an easier and safety way such as probiotics (Ley 2014, Taur & Pamer 2014). Some recent studies, including our own work have focused on the identification of bacteria involved in resistance to CDI and its role in colonization

resistance against this pathogen for both, mice and humans (Antharam et al. 2013, Lawley et al. 2012, Pérez-Cobas et al. 2014, Petrof et al. 2013, Reeves et al. 2012, Shahinas et al. 2012). Some of the taxa we proposed as candidates to protect against CDI have also been found in many of these studies, regardless of the methods and models used, suggesting that we are about to obtain an optimal set of bacteria that can prevent the pathogen colonization. Members of the Clostridiales, principally species from the families Ruminococcaceae and Lachnospiraceae have been the common factor of the conducted studies (Antharam et al. 2013, Pérez-Cobas et al. 2014, Reeves et al., 2011, 2012; Vincent et al. 2013). For instance, a recent study showed that 33 strains of a healthy donor were able to cure two patients with recurrent CDI, being 11 strains from the Lachnospiraceae family (Petrof et al. 2013). A mouse study showed that a strain of Lachnospiraceae was able to partially restore the gut microbiota after CDI, but the total restoration was only possible when the cecal microbial community of a healthy donor was transferred to the infected animals (Reeves et al. 2012). Others genera belonging to these families, such as *Ruminococcus, Subdoligranulum* (Ruminococcaceae) and *Roseburia*, *Coprococcus* (Lachnospiraceae) could be involved in colonization resistance since they have been also depleted in CDI samples from other human studies (Antharam et al. 2013, Pérez-Cobas et al. 2014, Vincent et al. 2013). Interestingly, unclassified OTUs which belong to these families have also appeared as possible participants of the colonization resistance response, indicating that species with a possible key role in protection could remain undescribed (Antharam et al. 2013, Pérez-Cobas et al. 2014, Reeves et al. 2012). Moreover, we propose to be tested as protectors against *C. difficile* colonization also bacteria from other phylum as *Alistipes* (Bacteroidetes), *Escherichia* (Proteobacteria) or Coriobacteriaceae (Actinobacteria) (Pérez-Cobas et al. 2014).

Most of the bacteria candidates to be protectors have been described as producers of SCFAs, which are the main source of energy of the gut epithelium and that also have anti-inflammatory effects (Lawley & Walker 2013). In addition, SCFAs stimulate defence barriers by increasing antimicrobial peptide levels and mucin production, as well as participate in the generation of a mucosal regulatory T cell subset as part of immune response (Smith et al. 2013, Wong et al. 2006). Thus, a proposed mechanism of colonization resistance for this group of bacteria is through the production of SCFAs and the positive stimulation of the gut epithelial and immune system functions (Lawley & Walker 2013). In this sense, a study that compared three groups of individuals, healthy, *C. difficile* negative with nosocomial diarrhea and *C. difficile* positive with nosocomial diarrhea, found a lower microbial diversity and abundance of SCFAs producers on the individuals with diarrhea, regardless of the infection status (Antharam et al. 2013). Hence, the depletion of these organisms leads to an epithelial dysfunction and diarrhea, reinforcing the role of these microbial products in a correct functioning of the gut. In contrast, some studies found that SCFAs administration to infected mice did not reduce the CDI (Reeves et al. 2012, Su et al. 1987). Therefore, it is still unknown if the SCFAs play a leading or secondary role in avoiding the CDI, but it is clear that they contribute positively to prevent and fight pathogen infection through maintaining and restoring the gut epithelium equilibrium and stimulating human defences.

A different mechanism by which the microbiota could participate in colonization resistance to CDI is through its role in bile acids transformation. Some *in vitro* studies have shown that germination of *C. difficile* spores can be stimulated by primary bile acids produced by the liver and secreted in the small intestine (Sorg & Sonenshein 2008, Wilson 1983). In addition, recently it has been identified a bile acid receptor of *C. difficile* required for germination and colonization, supporting the role of bile metabolism in CDI (Francis et al. 2013).

Moreover, a fraction of the gut microbiota is involved in the transformation of primary bile acids to secondary bile acids (Britton & Young 2014, Ridlon et al. 2006, Sorg & Sonenshein 2008). Specifically, the action of the microbial enzyme 7-dehydroxylase allows to transform the cholate (primary bile acid) into deoxycholate (secondary bile acid). In contrast to the primary bile acids, the secondary ones, as deoxycholate, strongly inhibit the vegetative growth of the pathogen and reduce the ability of *C. difficile* colonization (Britton & Young 2012, Howerton et al. 2013, Sorg & Sonenshein 2010, Taur & Pamer 2014). Administration of antibiotics alters the bile acid equilibrium leading to higher levels of primary bile acids (cholate) and as a consequence a higher rate of *C. difficile* germination and overgrow (Antunes et al. 2011, Giel et al. 2010, Pérez-Cobas et al. 2013a). In our meta-metabolomic survey, we also found an imbalance in the levels of bile acid derivatives of the gut microbiota as consequence of beta-lactam antibiotics administration. Thus, the antimicrobial therapy probably altered the microbial pool involved in bile acid metabolism (Pérez-Cobas et al. 2013a). Additionally, Theriot and colleagues used a mouse model to

investigate CDI and found that the gut metabolome of animals susceptible to infection was characterized for relative increase in primary bile acids and reduced level of secondary ones (Theriot et al. 2014). In this manner, Britton and collaborators suggested that bacteria harbouring the enzyme responsible of primary bile acid transformation (7-dehydroxylase), could be potential probiotics against *C. difficile* colonization and infection (Britton & Young 2012). Interestingly, the dehydrolase activity in the gut has been described mainly for members of Clostridia as *Clostridium* and *Eubacterium* genera (Begley et al. 2006, Ridlon et al. 2010). Since many of our identified candidate bacteria to protect against CDI belonged to Clostridia, it is a possibility that their role in colonization resistance could be related to the metabolism of bile acids (Pérez-Cobas et al. 2014). However, it is so far unknown, which gut microbial members contribute mostly to this function *in vivo*. Further studies should focus on testing the activity of the different bacterial species regarding bile acids transformation.

Since the gut microbiota forms a complex metabolic network where the by-product of a microorganism is the substrate of other, competition for niches and nutrients has been proposed as a resistance mechanism against the establishment of pathogenic bacteria (Britton & Young 2012, 2014; Lawley & Walker 2013). For instance, mucosal-associated bacteria act as an extra barrier to pathogen penetration across the epithelium, since the invaders must compete with the microbiota for adhesion receptors (niche competition) (Juge 2012). Moreover, studies with pathogenic *E. coli* strains showed that this bacteria compete with commensal *E.coli* strains for nutrients (i.e. sugars) and that commensal members are able to prevent the growth of the pathogenic strains in the mouse intestine (Leatham et al. 2009, Maltby et al. 2013). In the case of *C. difficile*, some studies pointed to the competition for available resources, mainly carbohydratees, as a colonization resistance mechanism.

In a preliminary study based on continuous flow-cultures it was found that components of the mucin (sialic acids, N-acetylglucosamine, N-acetylneuraminic acid) are required to promote the expansion of intestinal bacteria able to supress *Clostridium* (Wilson & Perini 1988). Recently, a transcriptomic study showed that *C. difficile*, is able to use sialic acids as a carbon source to reach high densities and colonize the mice intestine (Ng et al. 2013). The authors postulated that when the normal gut microbiota is reduced by antibiotics, sialic acids are liberated for enough bacteria with sialidases but as there are less sialic acids consumers (competitors) and *C. difficile* has a great chance for colonization. In this model, colonization resistance by the gut microbiota works through competition for carbohydrates such as sialic acids, which are a source of energy, carbon and nitrogen for many bacteria (Vimr et al. 2004). In our work, we found an over-representation of sugar transporters in the metagenomes of CDI samples, indicating that an alteration of the carbohydrates metabolism of a disrupted microbiota could favour the *C. difficile* expansion (Pérez-Cobas et al. 2014). Additionally, we found a candidate protector gene involved in the metabolism of N-acetylglucosamine to synthesize pseudaminic acid (sialic-acid-like sugar). The presence of this compound may promote the growth of bacterial groups that are better consumers than *C. difficile*, and therefore they are able of control the pathogen growth. Taking into account that the candidate bacteria that we proposed are phylogenetically related to *Clostridium*, they may share niche and compete for similar resources. This idea is supported by a study where a non-toxigenic *C. difficile* strain was able to prevent the establishment of a toxigenic *C. difficile* since it was more successful at competing for limiting resources (Merrigan et al. 2003, Sambol et al. 2002).

A direct mechanism of colonization resistance by the gut microbiota against CDI, could be the production of antimicrobials that inhibit the pathogen growth. Different antimicrobials produced by the gut microbiota (bacteriocins, toxins) are capable of killing pathogens including *C. difficile* (Britton & Young 2012, Lawley & Walker 2013). An intestinal strain of *Bacillus thuringensis* produces a bacteriocin named Thuricin CD that has narrow activity against spore-forming Gram-positive bacteria including *C. difficile* (Rea et al. 2010). Although the activity of this bacteriocin has been only tested in *in vitro*, this capacity could represent a possible defence mechanism of the gut microbiota. In our CDI study we did not found neither members of *Bacillus* genus nor specific antimicrobials as candidate genes against the pathogen colonization, although some putative protective genes were involved in the endospore formation of *Bacillus* species. Thus, we cannot discard that the presence of *Bacillus* species may be important for the control of the pathogen.

The cross-talk between human immune system and gut microbiota could be an indirect (immune-mediated) colonization resistance mechanism against CDI. The innate and adaptive immune responses of the host are important to reduce the acute inflammation associated to CDI, as well as the recurrence of the disease (Hasegawa et al. 2011, 2012; Jarchum et al. 2012). Moreover, these immune-associated responses are at least in part dependent of beneficial bacteria that stimulate the

immune system, which in turn target the pathogen (Buffie & Pamer 2013). However, antibiotic treatments reduce microbial diversity and suppress the innate immune system, leading the host to an immunosupressed state, ideal for opportunistic pathogens as *C. difficile* (Brandl et al. 2008, Lawley & Walker 2013). Commensal members of Clostridia (as our protective group) exert a strong influence in development of the host immune system (Atarashi et al. 2011, Bibbò et al. 2014, Francino 2013). It has been described that Clostridia species promote the development of T-cell receptor intraepithelial lymphocytes (IEL) and immunoglobulin A (IgA-). The stimulation of the immune system by this commensal group seems to work through a gradient of SCFAs and secondary bile acids that are sensed by the epithelial cells, triggering the initiation of immunological signalling (Lopetuso et al. 2013, Umesaki et al. 1999). In relation with the immune system stimulation and the gut epithelial homeostasis, we described the products of some metabolic pathways related to amino acids metabolism (tryptophan or polyamines biosynthesis) as protective against CDI. *Clostridium* species are the most common amino acid fermenting bacteria of the human gut (Dai et al. 2011). The amino acids catabolism is an important process that produces as a result, metabolites as SCFAs, organic acids, phenolic and indolic compunds and amines, which have a strong impact on the gut epithelium physiology. Specifically, an increase in polyamine levels is associated with a major growth of intestinal mucosal cells and microorganisms, possibly contributing to restoration of the gut equilibrium during stress conditions, as well as they have important anti-inflammatory effects (Dai et al. 2011, Kibe et al. 2014). In a recent collaboration of our group we found that *C. difficile*-infected patients showed a deficiency in the production of polyamines respect to healty controls, supporting the possible protective role of these metabolites against CDI (Rojo et al. 2015 inpress).

In summary, antibiotic treatments deplete members of the gut microbiota that through one or various colonization resistance mechanisms (previously described) would be able to prevent CDI. These mechanisms are summarized in Figure 4.1.

The gut environment under antibiotic stress is characterized by a low abundance of SCFAs, high abundance of primary bile acids, high carbohydrate availability, a suppressed immune system and absence of competitors and inhibitors. All these factors contribute to *C. difficile* germination and growth. It is noteworthy that the biology of Clostridia members in the gut greatly contributes to create an adverse environment for *C. difficile* germination and colonization. Clostridia class, which are Gram-positive bacteria, constitutes a great part (10-40%) of the total bacteria of the gut (Hold et al. 2002, Manson et al. 2008). In addition, it has been recently proposed that Clostridia dominate the more active fraction of the intestinal microbiota and they are, as a consequence, more susceptible to antibiotic perturbations (Maurice et al. 2013). They populate a region in the intestinal mucosa close to the epithelium and therefore have a strong influence in physiologic, metabolic and immunological processes in the gut (Lopetuso et al. 2013, Nava et al. 2011).

In particular, Clostridia members metabolize diet carbohydrates and produce SCFAs that have several benefits for the host health, as a source of energy and stimulating the immune system. This microbial group also participates in bile acids metabolism, producing secondary metabolites with pathogenic inhibitory capability. Moreover, they would share the niche with *C. difficile* and compite with it. Thus, these findings support that species of Clostridiales group may be key in the gut equilibrium and specifically in colonization resistance against *C. difficile*. Interestingly, an *in vitro* study about the effect of some plant phenolic compounds and aromatic metabolites derived from their metabolization by intestinal bacteria, showed that those products inhibit the growth of pathogenic bacteria as *C. difficile*, while they enhance the growth of commensal species of *Clostridium* genus (Lee at al. 2006). Further studies are required to test the influence on the gut microbiota of these products *in vivo*. If this could be confirmed, they may be potential candidates for prebiotics or specific dietary supplements used for stimulating the growth of commensal gut bacteria, maintaining intestinal health and protecting against the pathogen infection.

It is very important to find out what is the exact colonization resistance mechanism(s) operating in the gut ecosystem and which microbial members are implicated. Thereby, it would be possible to design specific diets, prebiotics or probiotics for patients under antimicrobial therapies in order to prevent and fight CDI, especially recurrent cases.

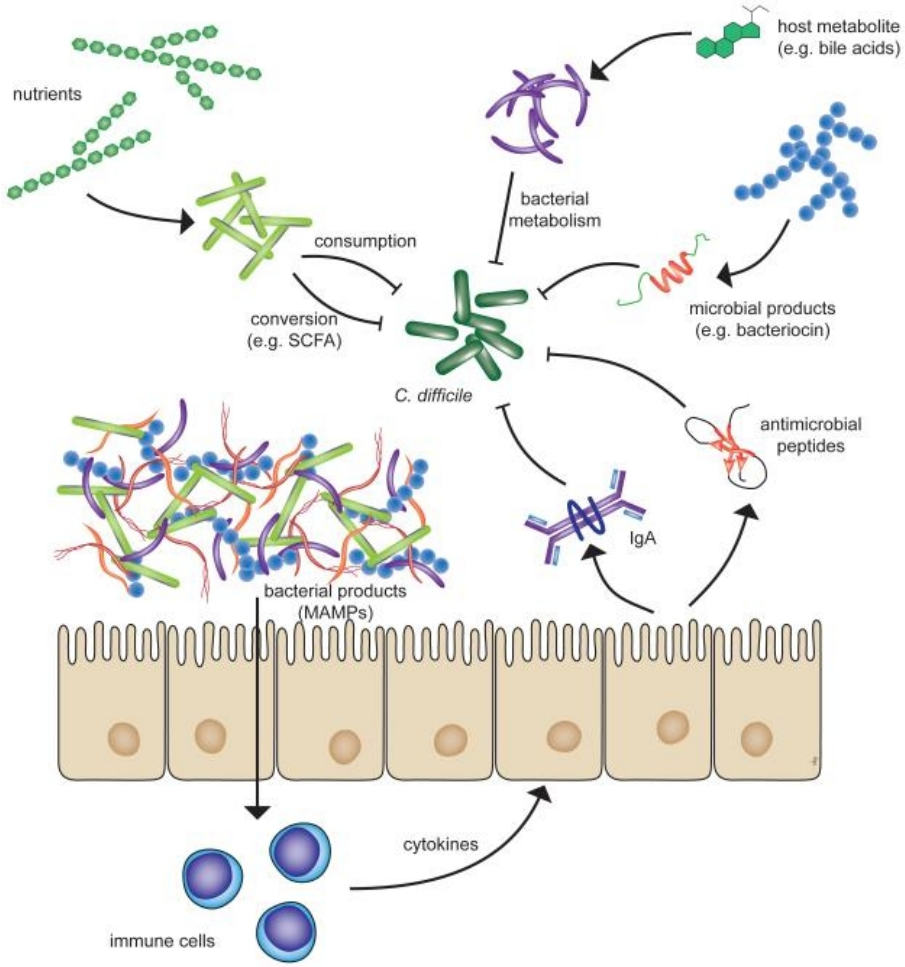


Figure 4.1. Possible mechanisms by which the gut microbiota can mediate colonization resistance against *C. difficile*. Direct inhibition can occur through competition for nutrients, the conversion of nutrients or host metabolites to compounds that are inhibitory to *C. difficile* or by the production of microbial products that inhibit *C. difficile*. Indirect control of *C. difficile* can occur via interactions between the microbiota and the host that results in the expression of host products that control *C. difficile* colonization and growth. Detection of microbial-associated molecular patterns (MAMPs) can trigger the host immune signaling cascades leading to the production of innate (e.g. antimicrobial peptides) or adaptive (e.g. IgA) immune effectors. SCFA, short-chain fatty acids (Adapted from Britton & Young, 2012).

5

CONCLUSIONS

5. CONCLUSIONS

1- Total and active human gut bacterial populations are strongly affected by broad-spectrum antibiotics, and the changes during therapies are associated to the initial microbial composition and the pharmacodynamic and pharmacological properties of the administered drug, together with the dynamics of the resistant microbial populations.

2- The use of these broad-spectrum antibiotics promotes drastic changes in the total microbiota with increase of Gram-negative bacteria as Bacteroidaceae and Enterobacteriaceae families. On the other hand, the active microbiota shows less variations than the total, since it contains the resistant bacteria, which take longer to be affected by the antibiotics.

3- Initial responses of the gut microbiota during antibiotic stress are directly related to avoid the antimicrobial damage, including high expression of antibiotic resistance genes. In contrast, later during treatment, the microbial responses are directed towards the renewal, repairing and maintenance of essential cell components.

4- During antibiotic therapies human gut bacteria present an attenuated metabolic status with lower protein production, being significantly altered the energy metabolism. Also, bacterial pathways related with the host physiology as the metabolism of bile acid, cholesterol, hormones or vitamins are strongly affected by antibiotics, possibly affecting human health. Moreover, specific bacterial groups can exert strong influence in the overall gut metabolic status and host interactions during antibiotic-associated disturbances, highlighting the role of minority community members.

5- The antimicrobial effect and the mode of action of antibiotics have a significant influence on the evolution of the gut microbiota during therapies, despite other influential factors as the initial composition, the diet, or the host immune status.

6- The survival strategies of the resistant bacteria during antibiotic therapies are reflected in the functional profile of the gut microbiome. A general mechanism that could confer advantage to bacteria for surviving under antibiotics is related to sugar metabolism, that counteracts the osmotic stress and favors the energy metabolism.

7- The genetic composition of the gut resistome after antibiotic therapies is partially determined by the resistance genes carried by the surviving bacteria and the type of administered antibiotic. Antibiotic use increases the abundance of resistance genes in the gut environment, including genes unrelated with the class of the administered antibiotics, specially the multidrug resistance genes.

8- *C. difficile* significantly influences the structure of the human gut microbiota, with over-abundance of specific intestinal genera (including opportunistic as *Enterococcus* and Clostridium clusters XI and XIVa) and under-representation of various commensal members (*Ruminococcus*, *Roseburia*, *Subdoligranulum*).

9- The alteration of sugar metabolism in the gut due to antibiotic therapy, could favor the infection by opportunistic pathogens, as *C. difficile*.

10- Members of Clostridiales order, principally from Lachnospiraceae and Ruminococcaceae families, play a key role in the gut ecosystem and seem to be involved in colonization resistance against *C. difficile*. The protectors members could avoid CDI by creating an unfavorable environment for the pathogen germination and growth, and also by stimulating the immune system.

11- Future studies focused on the colonization resistance mechanism(s) operating in the gut ecosystem and the responsible microorganisms, will allow to design diets, prebiotics and probiotics for patients under antimicrobial therapies in order to prevent infection by pathogens as *C. difficile*.

6

SHORT SPANISH VERSION

6. SHORT SPANISH VERSION

6.1. INTRODUCCIÓN

El cuerpo humano está poblado por complejas comunidades microbianas (definidas como microbiota) que han colonizado una gran variedad de regiones como la piel, las vías respiratorias, los órganos reproductores o el tracto gastrointestinal, entre otras (Charlson et al. 2011, Gao et al. 2007, Grice et al. 2009, Ma et al. 2012, Marchesi 2010, Ravel et al. 2011). La mayor cantidad de microorganismos y la diversidad más alta se encuentran en el tracto gastrointestinal donde el número total de microorganismos supera el número total de células humanas en un orden de magnitud (Savage 1977). Dentro del tracto, la diversidad va aumentando desde el estómago al recto, siendo el colon la región más densamente poblada (10^{13}-10^{14} células) (Leser & Molbak 2009, Marchesi 2011, Whitman et al. 1998).

La mayoría de relaciones que se dan entre las comunidades microbianas y el hospedador son de tipo mutualista por lo que ambos participantes se benefician (Dethlefsen et al. 2007). En el caso de la microbiota intestinal humana, los microorganismos participan en una gran variedad de funciones que benefician al hospedador como la digestión de alimentos de la dieta y obtención de energía, la síntesis de vitaminas y amino ácidos esenciales, el desarrollo y homeostasis del sistema inmune, la proliferación, diferenciación y mantenimiento del epitelio intestinal y la protección contra patógenos, entre otras (Backhed et al. 2004, Hattori & Taylor 2009, Hooper 2004, Leser & Molbak 2009, Montalto et al. 2009). Los microorganismos reciben por su parte condiciones estables de crecimiento en cuanto temperatura y pH así como una fuente constante de nutrientes (Savage 1977).

La mayor cantidad de microorganismos del intestino humano (en términos de biomasa), pertenecen al dominio bacteria, sin embargo se han encontrado principalmente 7 filos diferentes (Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia y Cyanobacteria), perteneciendo un 95% de las bacterias a Bacteroidetes y Firmicutes (Eckburg et al. 2005). En contraste al bajo número de filos se encuentra una gran diversidad a niveles filogenéticos más bajos, con más de 1000 especies descritas hasta el momento (Blaser & Falkow 2009, Claesson et al. 2009, Eckburg et al. 2005, Ley et al. 2008b, Rajilić-Stojanović et al. 2009). Además, la composición de la comunidad intestinal presenta una gran variabilidad interindividual, lo que ha impedido definir las especies que conforman el "core" del intestino humano. Sin embargo, dentro de un individuo, en ausencia de perturbaciones, hay una menor variabilidad en la composición microbiana lo que indica que la microbiota intestinal es única y relativamente estable durante largos períodos de tiempo (Ahmed et al. 2007, Durbán et al. 2012a, Eckburg et al. 2005, Green et al. 2006, Lozupone et al. 2012, Marchesi 2011, Robinson et al. 2010).

La composición de la microbiota se ve influenciada por una serie de factores determinísticos como el genotipo del hospedador, la dieta, la edad, las interacciones entre los miembros del ecosistema, así como por procesos estocásticos tales como el orden inicial de colonización o el ataque de fagos. Todos estos factores contribuyen a explicar la gran diversidad y variabilidad interindividual presente en la microbiota intestinal humana (Claesson et al. 2011, David et al. 2013, Dethlefsen et al. 2006, Filippo et al. 2010, Flint 2004, Koenig et al. 2011, McKnite et al. 2012, Palmer et al. 2007, Turnbaugh et al. 2008, Vallès et al. 2014).

Por otro lado, el conjunto de genes microbianos de este ecosistema se ha definido como microbioma intestinal y supera el número total de genes del genoma humano en dos órdenes de magnitud (Yang et al. 2009). A diferencia de la ausencia de un "core" de especies bacterianas en el intestino humano, la presencia de un gran número de genes compartidos entre individuos ha permitido definir un "core" funcional. Precisamente, debido al gran número de genes compartidos entre distintas especies intestinales existe una alta redundancia funcional en el microbioma intestinal (Lozupone et al. 2012, Qin et al. 2010, The Human Microbiome Project Consortium 2012, Turnbaugh et al. 2010). El estudio del microbioma ha permitido conocer que las funciones más abundantes en los genomas de las bacterias del intestino son importantes para el mantenimiento del equilibro microbiota-hospedador y que estas están relacionadas principalmente con el metabolismo de los carbohidratos, la producción

de energía, o la síntesis de amino ácidos esenciales y vitaminas (Gill et al. 2006, Kurokawa et al. 2007, Qin et al. 2010). Una de las funciones intrínseca de la microbiota consiste en proteger contra infecciones, conocida como capacidad de resistencia a la colonización por patógenos (Servin 2004). Esta capacidad opera a través de diferentes niveles de defensa, que se clasifican en dos tipos: directas e indirectas. Los mecanismos de protección directa funcionan a través de interacciones entre la microbiota y el patógeno, tales como la competencia por nutrientes o nichos, la exclusión metabólica donde el metabolismo de las bacterias intestinales crea condiciones desfavorables para el patógeno, o mediante la liberación de agentes antimicrobianos, como las bacteriocinas (Buffie & Pamer 2013, Lawley & Walker 2013, Lawley et al. 2012, Stecher & Hardt 2011). Por otro lado, los mecanismos de protección indirecta funcionan a través de la estimulación y mantenimiento del sistema inmune del hospedador, así como controlando la respuesta inflamatoria (Buffie & Pamer 2013, Kamada & Núñez 2014, Kawai & Akira 2009, Maslowski et al. 2009, Rakoff-Nahoum et al. 2004).

Algunos aspectos de la vida moderna, como el uso de medicamentos (fundamentalmente antibióticos), o las dietas ricas en grasa, están interfiriendo negativamente con la relación beneficiosa que existe entre la microbiota y el hospedador, lo que ha desencadenado un aumento en la incidencia de enfermedades asociadas a una microbiota alterada o disbiótica, como la obesidad, la alergia o el asma, entre otras (Blaser 2011, Francino 2013). En el caso de los antibióticos, aunque algunos tienen como diana patógenos específicos, la mayoría son de amplio espectro y son comúnmente usados para tratar la mayoría de infecciones (Nathan 2004). De esta manera, no sólo los patógenos sino también miembros comunes de la microbiota se ven afectados por la terapia antimicrobiana, así como los que están funcionalmente conectados a ellos, provocando una fuerte perturbación en la comunidad microbiana intestinal y en consecuencia en las funciones que realizan. Uno de los principales problemas del uso de antibióticos es que promueve la expansión de bacterias resistentes en el intestino, convirtiéndolo en un reservorio de genes de resistencias que podrían ser transferidas a patógenos (Jernberg et al. 2010, Löfmark et al. 2006, Modi et al. 2013, Sommer et al. 2009). Por otra parte, se ha comprobado que el uso de antibióticos afecta a funciones de la microbiota, como la estimulación del sistema inmune, el metabolismo energético o la resistencia a la colonización por patógenos (Brandl et al. 2008, Buffie et al. 2012, Dessein et al. 2009, Ng et al. 2013, Romick-Rosendale et al. 2009, Ubeda et al. 2013). Esta última función juega un papel esencial, ya que cuando la microbiota es suprimida por los antibióticos, patógenos oportunistas como *Salmonella* spp., *Klebsiella oxytoca* o *Clostridium difficile* pueden causar graves infecciones intestinales (Walk & Young 2008). *C. difficile* es el causante más común de la diarrea nosocomial y constituye actualmente un serio problema en salud pública. Su principal factor de riesgo es la terapia con antibióticos, especialmente la de amplio espectro (Denève et al. 2009, Kuijper et al. 2007). Además, la incidencia y virulencia de la enfermedad han aumentado drásticamente en los últimos años con la aparición de una cepa hipervirulenta (McFarland et al. 2002, Wilcox 1998). Por otra parte, la infección por *C. difficile* presenta una elevada tasa de recurrencia, y las actuales terapias basadas principalmente en la administración de antibióticos tienen un bajo éxito (Kelly & LaMont 2008, McFarland et al. 2002, Taur & Pamer 2014, Wilcox 1998). Esto indica una necesidad de nuevos enfoques que se dirijan hacia una búsqueda de terapias alternativas eficaces para la eliminación del patógeno.

En los últimos años el desarrollo de técnicas moleculares, como la secuenciación del gen que codifica para el 16S rRNA, las aproximaciones meta-"omicas" (metagenómica, metatranscriptómica, metaproteómica, etc) y la secuenciación de alto rendimiento, ha incrementado el conocimiento sobre la estructura, funciones y ecología de la microbiota intestinal, así como su papel en la salud y en la enfermedad (Andersson et al. 2008, Arumugam et al. 2011, Gill et al. 2006, Gosalbes et al. 2011, 2012; Kurokawa et al. 2007, Marchesi 2010, Qin et al. 2010, Verberkmoes et al. 2009). En el marco de esta nueva tecnología, se ha comenzado a estudiar recientemente con gran interés la interacción de los antibióticos con la microbiota intestinal, y su repercusión en la salud humana.

6.2. OBJETIVOS

El objetivo general de esta tesis consistió en caracterizar el efecto de la terapia con antibióticos en la microbiota intestinal

humana, así como los efectos de la infección del patógeno oportunista *Clostridium difficile*.

El primer objetivo específico consistió en analizar en profundidad, mediante diferentes aproximaciones ómicas, la evolución de la microbiota fecal de un individuo durante un tratamiento con antibióticos beta-lactámicos. La idea era evaluar los cambios estructurales (diversidad y composición de la microbiota total y activa) y funcionales (genes, RNAm, proteínas y metabolitos) del ecosistema microbiano como respuesta al estrés inducido por los antibióticos. Los principales resultados de este estudio se encuentran publicados en el artículo científico: "Gut microbiota disturbance during antibiotic therapy: a multi-omic approach" (capitulo 3.1).

El segundo objetivo específico de este proyecto consistió en estudiar el efecto de diferentes clases de antibióticos en la microbiota intestinal humana. Específicamente, queríamos comprobar si las diferencias en cuanto espectro, modo de acción y efecto antimicrobiano que presentaban los antibióticos utilizados, se reflejaban en los cambios asociados de la microbiota total, activa y en el contenido génico, de manera significativa. Además queríamos estudiar la evolución de los genes de resistencia (resistoma) y su relación con el tipo de antibióticos. Los principales resultados de este estudio se publicaron en el artículo "Differential effects of antibiotic therapy on the structure and function of human gut microbiota" (capítulo 3.2).

El tercer objetivo consistió en estudiar efecto de la infección por *C. difficile* en una microbiota alterada por los antibióticos. En primer lugar, se querían evaluar los cambios de la microbiota intestinal (estructurales y funcionales) debido a la infección por el patógeno. Por otra parte, se querían identificar microorganismos y funciones metabólicas asociadas a la infección, así como otras bacterias y funciones que podrían estar implicadas en la protección contra la colonización por este patógeno. Este trabajo se encuentra publicado en el artículo "Structural and functional changes in the gut microbiota associated to *Clostridium difficile* infection" (capítulo 3.3).

6.3. METODOLOGÍA

El total de pacientes incluidos en los estudios de esta tesis provienen del Departamento de Medicina Interna del hospital universitario Shleswig-Holstein en Kiel, Alemania, enmarcados en un proyecto europeo sobre la microbiota intestinal humana, el uso de antibióticos y la infección por *C. difficile* (ERA-NET project on *C. difficile*). Todos los pacientes recibieron terapia con antibióticos de amplio espectro durante su estancia en el hospital, pero no en los seis meses previos a su entrada. Todos los pacientes fueron sometidos a la detección de *C. difficile* mediante reacción en cadena de la polimerasa (en inglés, PCR) de los genes que codifican las toxinas *tcdA* y *tcdB*, así como el gen que codifica la triosa fosfato isomerasa (*tpi*) del patógeno. Se tomaron muestras de heces de cada uno de los pacientes antes, durante y después de la terapia con antibióticos y se mantuvieron congelados en tubos estériles a -80º hasta su procesamiento. Dentro del capítulo 3.3 en la tabla 1 se encuentra resumida la información referente a los pacientes de todos los estudios que comprende esta tesis.

En los diferentes estudios utilizamos dos tipos de aproximaciones, las basadas en el gen que codifica para el RNA ribosomal 16S y las "meta-omicas", seguidas por la pirosecuenciación del material obtenido con un secuenciador Roche GS FLX y química de Titanium (excepto para la metabolómica y metaproteómica).

Para caracterizar los cambios asociados a las terapias con antibióticos en la composición y diversidad de la microbiota total y la fracción activa, nos basamos en el gen del RNA ribosomal 16S (16S rDNA) y en el transcrito de dicho gen (16S rRNA), respectivamente. Para estudiar la microbiota total se extrajo el DNA total de las heces y se realizaron "PCRs" del gen que codifica para el 16S rRNA. En el caso de la fracción activa de la microbiota se realizó una extracción del RNA total de las heces, se procedió a su retrotranscripción, obtención de cDNA y posterior secuenciación. Las secuencias resultantes de ambos grupos se analizaron bioinformáticamente y se clasificaron taxonómicamente con la base de datos Ribosomal Database Project (RDP) (Cole et al. 2009). Para ambos grupos se realizaron varios análisis ecológicos, así como estadísticos (especificados en los materiales y métodos de los capítulos 3.1, 3.2 y 3.3). La mayoría de análisis se realizaron utilizando los programas QIIME (Caporaso et al. 2010) y R (R Development Core Team, 2011).

La caracterización del contenido génico de las muestras (metagenomas) se realizó mediante la extracción del DNA total y secuenciación directa de éste, lo que nos permitió describir el total de genes, y como cambian las funciones metabólicas para las que codifican durante las terapias antimicrobianas.

Por otro lado, el análisis de la expresión total de los genes en la comunidad microbiana de las muestras (metatranscriptómica), se basó en la extracción del RNA total, seguida de la eliminación del RNA ribosomal y purificación del RNA mensajero, a partir del que se obtuvo el correspondiente cDNA que fue secuenciado.

Para la obtención de los metaproteomas, se extrajeron de las muestras de heces las proteínas totales y se sometieron a una cromatografía de líquidos de ultra alta eficacia acoplada a un intrumento Orbitrap MS^2 (UPLC-LTQ Orbitrap-MS/MS) para la identificación de las proteínas. Finalmente, los meta-metabolomas se obtuvieron a través de la extracción de los metabolitos totales y se identificaron posteriormente mediante la aplicación de una cromatografía líquida de alta eficacia acoplada a un espectómetro de masas ESI-QTOF. Estas últimas dos aproximaciones ómicas se llevaron a cabo en diferentes laboratorios con los que colaboramos.

Las secuencias obtenidas para los metagenomas, metatranscriptomas y metaproteomas se procesaron bioinformáticamente y se anotaron funcionalmente contra la base de datos TIGRFAM, mientras que la información de los metabolitos fue también procesada y se clasificaron usando la base de datos MELTIN (Haft et al. 2003, Smith et al. 2005). Los metatranscriptomas y metaproteomas fueron mapeados sobre los metagenomas con los que comparten su anotación funcional. Se realizaron una serie de análisis bioinformáticos, ecológicos y estadísticos con los datos funcionales obtenidos a través de estas técnicas, utilizando, en la mayoría de los casos paquetes del programa R (R Development Core Team, 2011). Los detalles específicos de análisis de los metagenomas se encuentran en los capítulos 3.1, 3.2 y 3.3, mientras que los de los metatranscriptomas, metaproteomas y meta-metabolomas se encuentran en el capítulo 3.1.
Las secuencias procedentes de los diferentes estudios fueron depositadas en la base de datos pública "European Bioinformatics Institute database".

6.4. RESULTADOS Y CONCLUSIONES

En el capítulo 3.1 realizamos un seguimiento de la microbiota intestinal de un único paciente bajo terapia de antibióticos beta-lactámicos a través de la integración de diferentes herramientas moleculares (basadas en el 16S rRNA, metagenómica, metatranscriptómica, metaproteómica y meta-metabolómica), constituyendo así, el primer estudio multi-ómico donde se evaluó la respuesta de la microbiota al daño ocasionado por los antibióticos a varios niveles biológicos (Pérez-Cobas et al. 2013a).

A consecuencia de esta investigación llegamos a la conclusión de que la aplicación conjunta de esta serie de aproximaciones moleculares constituye una poderosa herramienta para una caracterización en detalle de la composición, diversidad, funcionalidad y ecología de la comunidad microbiana intestinal, así como su respuesta a perturbaciones como son las inducidas por el estrés antibiótico.

En primer lugar, describimos como los cambios en la composición de la microbiota intestinal debido al tratamiento se corresponden con el metabolismo de la droga y con la dinámica de crecimiento de las poblaciones bacterianas resistentes.

Por otro lado, encontramos que la respuesta inicial de la microbiota intestinal al estrés inducido por los beta-lactámicos está enfocada a evitar el efecto dañino directo de los antimicrobianos, con una alta expresión de genes codificantes de resistencias, como por ejemplo bombas exportadoras de drogas. Por el contrario, tras varios días de tratamiento la respuesta de la microbiota está más dirigida a la renovación, reparación y mantenimiento de componentes celulares esenciales.

Además, observamos que durante los tratamientos con antibióticos las bacterias intestinales presentan un metabolismo atenuado con una producción de proteínas más baja que en condiciones de normalidad, afectando a procesos biológicos tales como el metabolismo energético. No sólo se ven afectadas rutas metabólicas importantes para los

microorganismos sino también para la fisiología del hospedador, tales como el metabolismo de los ácidos biliares, el colesterol y las hormonas, al igual que la síntesis de vitaminas, lo que puede tener consecuencias negativas en la salud humana.

Una observación relevante de este trabajo fue el hecho de que algunos miembros específicos de la microbiota intestinal, especialmente grupos minoritarios pueden jugar un papel importante en el estado metabólico global del intestino y su equilibrio, cuando miembros mayoritarios de la microbiota han sido afectados por los antibióticos.

En el capítulo 3.2 realizamos seguimientos de la microbiota intestinal de cuatro individuos que estaban siendo tratados con antibióticos de diferentes clases. Los antibióticos presentaban los dos tipos de efecto antimicrobiano: bactericidas y bacterioestáticos. Además, también presentaban diferentes modos de acción: inhibidores de la replicación celular, inhibidores de la síntesis de proteínas e inhibidores de la síntesis de la pared celular. En este trabajo, a través del uso de aproximaciones basadas en el 16S rRNA y metagenómica evaluamos el efecto de estas propiedades en el modelado de la microbiota total, la fracción activa y en el contenido genético total (potencial funcional) de la microbiota. Además, analizamos posibles respuestas funcionales de la microbiota al estrés con antibióticos, incluyendo la evolución de los genes de resistencia a lo largo de los tratamientos (Pérez-Cobas et al. 2013b).

En este trabajo observamos que el carácter único de la microbiota intestinal humana se refleja en los cambios asociados a antibióticos, encontrándose grandes diferencias entre individuos en cuanto a la diversidad, composición y potencial genético de la comunidad microbiana intestinal durante terapias similares.

Por otro lado, las propiedades de los antibióticos como el efecto antimicrobiano (bactericida o bacteriostático) y el modo de acción también resultaron ejercer una influencia significativa en la composición de la fracción total y activa de la microbiota. Así, a pesar de que una gran variabilidad es introducida por factores como la composición inicial de la microbiota, la dieta o el estado de salud del hospedador, tratamientos con antibióticos similares moldean la microbiota de una manera similar, dirigiendo el ecosistema intestinal hacia una estructura final donde comparten ciertas características.

En cuanto a las respuestas funcionales al estrés antibiótico destacan el transporte y metabolismo de azúcares, que podrían conferir ventaja a las bacterias para sobrevivir en condiciones adversas, como la que generan los antibióticos. Este efecto beneficioso puede deberse al papel de los transportadores de azúcares en contrarrestar el estrés osmótico, así como en favorecer el metabolismo energético de estas bacterias, lo que lleva a un mayor crecimiento poblacional. Además, los cambios en el perfil funcional del microbioma intestinal en respuesta a los antibióticos, se corresponden con las estrategias de supervivencia de las bacterias resistentes al tratamiento. Por ejemplo, un incremento en miembros de la familia Enterobacteriaceae durante una de las terapias con antibióticos, se correspondió con un aumento en la abundancia relativa de genes que participan en la síntesis de la membrana externa de bacterias Gram-negativas, como las pertenecientes a esta familia. La membrana externa es importante para la obtención de nutrientes y confiere resistencia contra los antibióticos, dándole ventaja a este grupo de bacterias durante condiciones de estrés.

En cuanto al análisis del resistoma observamos que la toma de antibióticos provoca un aumento en la abundancia relativa de los genes de resistencia. El incremento ocurre no sólo en aquellos genes que confieren resistencia contra el antibiótico administrado sino también en genes de resistencias a otros antibióticos como parte de un efecto colateral. El ejemplo más claro que encontramos de este efecto, fue el aumento en abundancia de genes que confieren resistencia a múltiples antibióticos. Con respecto a la composición genética del resistoma intestinal después de terapias con antibióticos, vimos que es un proceso complejo definido principalmente por los genes de resistencia de las bacterias que sobreviven, así como de las propiedades del antibiótico administrado.

En el capítulo 3.3 de una manera similar a los dos estudios previos realizamos un estudio de seguimiento de tres individuos tratados con antibióticos de amplio espectro, pero en este caso los pacientes presentaron una infección por el patógeno intestinal *C. difficile*. También usamos la pirosecuenciación del gen 16S rRNA y la metagenómica para caracterizar los cambios estructurales y funcionales debidos al tratamiento con antibióticos pero fundamentalmente a la infección por *C. difficile*. Por otra parte, realizamos un análisis comparativo de este grupo de pacientes infectados con los pacientes de los dos estudios previos, que a pesar de haber recibido tratamiento con antibióticos no desarrollaron infección (Capítulos 3.1 y 3.2). Estas

comparaciones estadísticas nos permitieron identificar bacterias y funciones posiblemente relacionadas con la infección por *C. difficile* y otras implicadas en la protección contra la infección por el patógeno. Además, hicimos uso de redes bayesianas de correlación para identificar otras bacterias y genes también posiblemente implicados en la resistencia a la colonización, basándonos en una asociación (correlación positiva) con las bacterias y genes previamente identificados como candidatos a ser protectores (Pérez-Cobas et al. 2014).

En esta investigación encontramos una influencia significativa de la infección por *C. difficile* en la estructura de la microbiota intestinal con una alta representación de géneros bacterianos específicos (generalmente microorganismos oportunistas como algunos del género *Enterococcus* y los grupos Clostridium XI y XIVa) y una baja abundancia de miembros comensales como *Ruminococcus*, *Roseburia*, *Subdoligranulum*, *Blautia* o *Coprococcus*.

El análisis de los metagenomas indicó que los antibióticos introducen un desequilibrio en el metabolismo de los azúcares de la dieta por parte de la microbiota intestinal y que esta alteración podría favorecer la infección por *C. difficile*.

Tras la identificación de bacterias protectoras en el análisis comparativo y la aplicación de redes bayesianas se encontró que miembros del orden filogenético Clostridiales, principalmente de las familias Lachnospiraceae y Ruminococcaceae, que juegan un papel esencial en el ecosistema intestinal, podrían estar implicadas en la resistencia a la colonización por *C. difficile*.

La identificación de rutas metabólicas candidatas a participar en la resistencia a la colonización por *C. difficile* sugiere que los miembros protectores de la microbiota podrían crear (como parte de su metabolismo y funciones naturales) condiciones desfavorables para la germinación y crecimiento del patógeno y además podrían participar en la estimulación del sistema inmune del hospedador, evitando así la infección.

Finalmente, es de destacar que las redes bayesianas de correlación que han sido poco utilizadas en este campo constituyen unas herramientas prometedoras en la exploración de asociaciones ecológicas en la comunidad microbiana del intestino. En nuestro estudio fueron utilizadas para ampliar el grupo de bacterias y genes candidatos a proteger contra la infección por *C. difficile*.

En conclusión, los antibióticos ejercen una gran influencia negativa en el ecosistema intestinal, viéndose afectada la composición, diversidad y actividad de la microbiota, y como consecuencia las funciones beneficiosas que las bacterias intestinales aportan al hospedador. Este es el caso de la capacidad de resistencia a la colonización por patógenos como *C. difficile*, una función que se ve debilitada durante las terapias antimicrobianas. Los antibióticos causan grandes variaciones en la abundancia relativa de microorganismos clave en el ecosistema intestinal, viéndose afectado el estado metabólico global del intestino y en consecuencia la fisiología del hospedador. Además, son frecuentes drásticas reducciones en la diversidad microbiana durante los tratamientos, incluyendo una pérdida definitiva de algunas especies. La ausencia de bacterias protectoras favorece la germinación y el crecimiento de patógenos oportunistas como *C. difficile*. Así, en nuestro estudio proponemos un grupo de microorganismos y funciones que podrían estar implicadas en evitar la infección de este patógeno. Futuros estudios permitirán clarificar si los taxones identificados están realmente implicados en la resistencia a la colonización y a través de qué mecanismo biológico. De esta manera será posible diseñar dietas, prebióticos y probióticos para pacientes bajo terapia antibiótica con el fin de prevenir y tratar la infección por *C. difficile*, especialmente los casos recurrentes.

7

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7. BIBLIOGRAPHY

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8

APPENDICES

8.1

PUBLICATIONS RELATED TO THIS THESIS

8.1 PUBLICATIONS RELATED TO THIS THESIS

METATRANSCRIPTOMIC APPROACH TO ANALYZE THE FUNCTIONAL HUMAN GUT MICROBIOTA.

Gosalbes MJ, Durbán A, Pignatelli M, Abellan JJ, Jiménez-Hernández N, **Pérez-Cobas AE**, Latorre A, Moya A.

PLoS One. 6(3):e17447. 2011.

ABSTRACT

The human gut is the natural habitat for a large and dynamic bacterial community that has a great relevance for health. Metagenomics is increasing our knowledge of gene content as well as of functional and genetic variability in this microbiome. However, little is known about the active bacteria and their function(s) in the gastrointestinal tract. We performed a metatranscriptomic study on ten healthy volunteers to elucidate the active members of the gut microbiome and their functionality under conditions of health. First, the microbial cDNAs obtained from each sample were sequenced using 454 technology. The analysis of 16S transcripts showed the phylogenetic structure of the active microbial community. Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Prevotellaceae, and Rickenellaceae were the predominant families detected in the active microbiota. The characterization of mRNAs revealed a uniform functional pattern in healthy individuals. The main functional roles of the gut microbiota were carbohydrate metabolism, energy production and synthesis of cellular components. In contrast, housekeeping activities such as amino acid and lipid metabolism were underrepresented in the metatranscriptome. Our results provide new insights into the functionality of the complex gut microbiota in healthy individuals. In this RNA-based survey, we also detected small RNAs, which are important regulatory elements in prokaryotic physiology and pathogenicity.

METAGENOMICS OF HUMAN MICROBIOME: BEYOND 16S rDNA.

Gosalbes MJ, Abellan JJ, Durbán A, **Pérez-Cobas AE**, Latorre A, Moya A.

Clinical Microbiology and Infection.18 Suppl 4:47-9. 2012.

ABSTRACT

The gut microbiota presents a symbiotic relationship with the human host playing a beneficial role in human health. Since its establishment, the bacterial community is subjected to the influence of many different factors that shape its composition within each individual. However, an important convergence is observed at functional level in the gut microbiota. A metatranscriptomic study of healthy individuals showed homogeneity in the composition of the active microbiota that increased further at functional level.

FUNCTIONAL CONSEQUENCES OF MICROBIAL SHIFTS IN THE HUMAN GASTROINTESTINAL TRACT LINKED TO ANTIBIOTIC TREATMENT AND OBESITY.

Hernández E, Bargiela R, Diez MS, Friedrichs A, **Pérez-Cobas AE**, Gosalbes MJ, Knecht H, Martínez-Martínez M, Seifert J, von Bergen M, Artacho A, Ruiz A, Campoy C, Latorre A, Ott SJ, Moya A, Suárez A, Martins dos Santos VA, Ferrer M.

Gut Microbes. 4(4):306-15. 2013.

ABSTRACT

The microbiomes in the gastrointestinal tract (GIT) of individuals receiving antibiotics and those in obese subjects undergo compositional shifts, the metabolic effects and linkages of which are not clearly understood. Herein, we set to gain insight into these effects, particularly with regard to carbohydrate metabolism, and to contribute to unravel the underlying mechanisms and consequences for health conditions. We measured the activity level of GIT carbohydrate-active enzymes toward 23 distinct sugars in adults patients (n = 2) receiving 14-d β-lactam therapy and in obese (n = 7) and lean (n = 5) adolescents. We observed that both 14 d antibiotic-treated and obese subjects showed higher and less balanced sugar anabolic capacities, with 40% carbohydrates being preferentially processed as compared with non-treated and lean patients. Metaproteome-wide metabolic reconstructions confirmed that the impaired utilization of sugars propagated throughout the pentose phosphate metabolism, which had adverse consequences for the metabolic status of the GIT microbiota. The results point to an age-independent positive association between GIT glycosidase activity and the body mass index, fasting blood glucose and insulin resistance ($r(2) \geq 0.95$). Moreover, antibiotics altered the active fraction of enzymes controlling the thickness, composition and consistency of the mucin glycans. Our data and analyses provide biochemical insights into the effects of antibiotic usage on the dynamics of the GIT microbiota and pin-point presumptive links to obesity. The knowledge and the hypotheses generated herein lay a foundation for subsequent, systematic research that will be paramount for the design of "smart" dietary and therapeutic interventions to modulate host-microbe metabolic co-regulation in intestinal homeostasis.

CLOSTRIDIUM DIFFICILE HETEROGENEOUSLY IMPACTS INTESTINAL COMMUNITY ARCHITECTURE BUT DRIVES STABLE METABOLOME RESPONSES.

Rojo D, Gosalbes MJ, Ferrari R, **Pérez-Cobas AE**, Hernández E, Oltra R, Buesa J, Latorre A, Barbas C, Ferrer M, Moya A.

ISME Journal (*in press*). 2015.

ABSTRACT

Clostridium difficile-associated diarrhoea (CDAD) is caused by *C. difficile* toxins A and B and represents a serious emerging health problem. Yet, its progression and functional consequences are unclear. We hypothesised that *C. difficile* can drive major measurable metabolic changes in the gut microbiota and that a relationship with the production or absence of toxins may be established. We tested this hypothesis by performing metabolic profiling on the gut microbiota of patients with *C. difficile* that produced (n=6) or did not produce (n=4) toxins and on non-colonised control patients (n=6), all of whom were experiencing diarrhoea. We report a statistically significant separation (p-value<0.05) among the three groups, regardless of patient characteristics, duration of the disease, antibiotic therapy, and medical history. This classification is associated with differences in the production of distinct molecules with presumptive global importance in the gut environment, disease progression and inflammation. Moreover, while severe impaired metabolite production and biological deficits were associated with the carriage of *C. difficile* that did not produce toxins, only previously unrecognised selective features, namely, choline- and acetylputrescine-deficient gut environments, characterised the carriage of toxin-producing *C. difficile*. Additional results demonstrated that the changes induced by *C. difficile* become marked at the highest level of the functional hierarchy, namely the metabolic activity exemplified by the gut microbial metabolome regardless of heterogeneities that commonly appear below the functional level (gut bacterial composition). We discuss possible explanations for this effect and suggest that the changes imposed by CDAD are much more defined and predictable than previously thought.

8.2

OTHER PUBLICATIONS

8.2. OTHER PUBLICATIONS

METAGENÓMICA DEL MICROBIOMA INTESTINAL HUMANO.

Pérez-Cobas AE, Gosalbes MJ, Latorre A, Moya A.

Revista de la Sociedad Española de Microbiología. Número 54. Especial "Taxonomía, Filogenia y Diversidad". 2012.

RESUMEN

A lo largo de la evolución los mamíferos han establecido simbiosis con microorganismos que han colonizado diferentes regiones del cuerpo humano tales como la piel, las mucosas, el tracto gastrointestinal, etc. Al conjunto de ellos se le denomina «microbiota». La mayor densidad de microorganismos se concentra en el tracto gastrointestinal, constituyendo de hecho uno de los ecosistemas más complejos de toda la biosfera. La microbiota gastrointestinal participa en una serie de funciones esenciales para el hospedador, como la digestión de polisacáridos de la dieta, el metabolismo de las grasas, la síntesis de vitaminas esenciales, la renovación del epitelio intestinal, el desarrollo del sistema inmune y la protección contra patógenos. Debido a su importancia, alteraciones en la microbiota tienen una gran repercusión en la salud humana, estando relacionados con enfermedades de gran impacto, como la obesidad, la colitis o el cáncer, por citar algunas. La metagenómica estudia los genomas de los microorganismos de un determinado hábitat sin necesidad de aislarlos y cultivarlos. El uso de esta técnica combinada con las nuevas tecnologías de secuenciación masiva ha permitido un gran avance en el estudio de la ecología y la diversidad de las comunidades microbianas de prácticamente cualquier ambiente.

SUCCESSION OF THE GUT MICROBIOTA IN THE COCKROACH *BLATTELLA GERMANICA*.

Carrasco P, **Pérez-Cobas AE**, van de P, Baixeras J, Moya A, Latorre A.

International Microbiology. 17:99-109. 2014.

ABSTRACT

The cockroach gut harbors a wide variety of microorganisms that, among other functions, collaborate in digestion and act as a barrier against pathogen colonization. *Blattabacterium*, a primary endosymbiont, lives in the fat body inside bacteriocytes and plays an important role in nitrogen recycling. Little is known about the mode of acquisition of gut bacteria or their ecological succession throughout the insect life cycle. Here we report on the bacterial taxa isolated from different developmental instars of the cockroach *Blattella germanica*. The bacterial load in the gut increased two orders of magnitude from the first to the second nymphal stage, coinciding with the incorporation of the majority of bacterial taxa, but remained similar thereafter. Pyrosequencing of the hypervariable regions V1–V3 of the 16S rRNA genes showed that the microbial composition differed significantly between adults and nymphs. Specifically, a succession was observed in which *Fusobacterium* accumulated with aging, while *Bacteroides* decreased. *Blattabacterium* was the only symbiont found in the ootheca, which makes the vertical transmission of gut bacteria an unlikely mode of acquisition. Scanning electron microscopy disclosed a rich bacterial biofilm in third instar nymphs, while filamentous structures were found exclusively in adults.

DIET SHAPES THE GUT MICROBIOTA OF THE OMNIVOROUS COCKROACH *BLATELLA GERMANICA*.

Pérez-Cobas AE, Maiques E, Angelova A, Carrasco P, Moya A, Latorre A.

FEMS Microbiology Ecology (*in press*). 2015.

ABSTRACT

The gut microbiota of insects contributes positively to the physiology of its host mainly by participating in food digestion, protecting against pathogens, or provisioning vitamins or amino acids, but the dynamics of this complex ecosystem is not well understood so far. In this study, we have characterized the gut microbiota of the omnivorous cockroach *Blattella germanica* by pyrosequencing the hypervariable regions V1–V3 of the 16S rRNA gene of the whole bacterial community. Three diets differing in the protein content (0, 24 and 50%) were tested at two time points in lab-reared individuals. In addition, the gut microbiota of wild adult cockroaches was also analyzed. In contrast to the high microbial richness described on the studied samples, only few species are shared by wild and lab-reared cockroaches, constituting the bacterial core in the gut of *B. germanica*. Overall, we found that the gut microbiota of *B. germanica* is highly dynamic as the bacterial composition was reassembled in a diet-specific manner over a short time span, with no-protein diet promoting high diversity, although the highest diversity was found in the wild cockroaches analyzed. We discuss how the flexibility of the gut microbiota is probably due to its omnivorous life style and varied diets.

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