Interplay between gut microbiota and antibiotics

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Interplay between gut microbiota and antibiotics

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To my family

A mi familia

ABSTRACT

The human body is colonized by a vast number of microorganisms collectively defined as the microbiota. In the gut, the microbiota has important roles in health and disease, and can serve as a host of antibiotic resistance genes. Disturbances in the ecological balance, e.g. by antibiotics, can affect the diversity and dynamics of the microbiota. The extent of the disturbance induced by antibiotics is influenced by, among other factors, the class of antibiotic, the dose, and administration route. One of the most common consequences of excessive antibiotic use is the emergence of antibiotic resistant bacteria and the dissemination of the corresponding resistance genes to other microbial inhabitants of the gut community, in addition to affecting the colonization resistance and promoting the overgrowth of pathogens. These effects are particularly relevant for Intensive Care Unit (ICU) patients, which are frequently exposed to a high risk of hospital-acquired infections associated with antibiotic resistant bacteria.

Due to the important roles that members of the gut microbiota play in the host, including their role as potential hubs for the dissemination of antibiotic resistance, recent research has focused on determining the composition and function of gut microorganisms and the antibiotic resistance genes associated with them.

The objectives of the research described in this thesis were to study the diversity and dynamics of the gut microbiota and resistome in ICU patients receiving antibiotic prophylactic therapy, and to assess the colonization dynamics with antibiotic resistant bacteria focusing on the commensal microbiota as a reservoir of antibiotic resistance genes by using culture dependent and independent techniques. Furthermore, the genetic background involved in the subsistence phenotype was investigated to disentangle the links between resistance and subsistence.

Bacteria harbor antibiotic resistance genes that participate in a range of processes such as resisting the toxic effects of antibiotics, but could also aid in the utilization of antibiotics as sole carbon source, referred to as antibiotic subsistence phenotype. In chapter 2, the potential of gut bacteria from healthy human volunteers and zoo animals to subsist on antibiotics was investigated.

Various gut isolates of *Escherichia coli* and *Cellulosimicrobium* spp. displayed the subsistence phenotype, mainly with aminoglycosides. Although no antibiotic degradation could be detected, the number of colony forming units increased during growth in medium with only the antibiotic as a carbon source. By using different approaches to study the aminoglycoside subsistence phenotype, we observed that laboratory strains carrying the aminoglycoside 3'phosphotransferase II gene also displayed the subsistence phenotype on aminoglycosides and that glycosyl-hydrolases seem to be involved in the subsistence phenotype. As the zoo animals for which the subsistence phenotype was investigated also included a number of non-human primates, the applicability of Human Intestinal Tract Chip (HITChip) to study the gut microbiota composition of these animals was assessed, including a comparison with healthy human volunteers (Chapter 3). It was concluded that the HITChip can be successfully applied to the gut microbiota of closely related hominids, and the microbiota dynamics can therefore be quickly assessed by the HITChip.

In Chapter 4, a combination of 16S rRNA phylogenetic profiling using the HITChip and metagenomics sequencing was implemented on samples from a single ICU hospitalized patient that received antibiotic prophylactic therapy (Selective Digestive Decontamination - SDD). The different approaches showed a highly dynamic microbiota composition over time and the prevalence of aminoglycoside resistance genes harbored by a member of the commensal anaerobic microbiota, highlighting the role of the commensal microbiota as a reservoir of antibiotic resistance genes. As an extension of this study (Chapter 5), 11 ICU patients receiving SDD were followed using 10 healthy individuals as a control group to compare the diversity and dynamics of the gut microbiota and resistome by HITChip and nanolitre-scale quantitative PCRs, respectively. The microbial diversity of the healthy individuals was higher compared to ICU patients, and it was less dynamic compared to ICU patients under antibiotic treatment. Likewise, the levels of antibiotic resistance genes increased in ICU patients compared to healthy individuals, indicating that during ICU hospitalization and the SDD, gut microbiota diversity and dynamics are profoundly affected, including the selection of antibiotic resistance in anaerobic commensal bacteria.

This was further expanded in an extensive study focusing on colonization dynamics with antibiotic resistant bacteria as described in Chapter 6. This was performed in the same group of ICU-hospitalized patients receiving SDD therapy and showed that by using a range of culture media and selective conditions a variety of taxonomic groups could be isolated, including aerobic and anaerobic antibiotic resistant bacteria. The overall composition of the faecal microbiota detected by HITChip indicated mainly a decrease of *Enterobacteriaceae* and an increase of the enterococcal population. Since critically ill patients are susceptible to hospital-acquired infections and the control of the emergence of antibiotic resistance is crucial to improve therapeutic outcomes, an extended analysis of the *Enterococcus* colonization dynamics in this group of patients by cultivation and phenotypic and genotypic characterization of the isolates provided new information about carriage of antibiotic resistance and virulence factor encoding genes (Chapter 7). It also highlighted the opportunity for the exchange of resistance and virulence genes, which could increase the risk of acquiring nosocomial infections.

Next, chapter 8 described the implementation of high-throughput cultivation-based screening using the Microdish platform combined with high-throughput sequencing (MiSeq) using faecal samples from ICU patients receiving SDD. This allowed for the recovery of previously uncultivable bacteria, including a pure culture of a close relative of *Sellimonas intestinalis* BR72T that was isolated from media containing tobramycin, cefotaxime and polymyxin E. This strain could therefore represent a potential antibiotic resistance reservoir.

In conclusion, this thesis provides broad insight into the diversity and dynamics of the gut microbiota and resistome in ICU hospitalized patients receiving SDD therapy as well as the dynamics of colonization with antibiotic resistant bacteria. Especially our extensive study of the colonization dynamics of *Enterococcus* spp. during ICU stay reinforced the notion that SDD therapy does not cover this group of bacteria and highlights the importance of a critical control of the emergence of antibiotic resistance in enterococci and their spread and dissemination as known potential pathogens.

Furthermore, the extensive use of antibiotics could select for an increase in the rate of antibiotic resistance against aminoglycosides and beta-lactams, indicating that a control in the use of broad spectrum antibiotics needs to be considered. In addition, this thesis provides evidence regarding the possible genetic background involved in the subsistence phenotype, however, future studies on metabolic pathways could provide novel insight into the underlying mechanisms.

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CHAPTER 1

Introduction and thesis outline

INTRODUCTION

Infectious diseases represent the second most important cause of death worldwide (WHO, 2014). It has been estimated that 5-10% of patients develop an infection during hospital stay (Fauci, 2005). One of the most powerful tools for the treatment of infectious diseases is the use of antibiotics. However, infectious diseases caused by bacteria are increasingly difficult to control due to the evolution of antibiotic resistance. Furthermore, complex microbial communities residing in the gut play an important role in the selection, enrichment and spread of antibiotic resistance and represent an ideal reservoir for the transfer of antibiotic resistance genes to potential pathogens.

Antibiotic use and the emergence of antibiotic resistance

One of the major breakthroughs in the early 20th century has certainly been the discovery of antibiotics (Stokes and Gillings, 2011). Starting with penicillin found by Alexander Fleming in 1928 (Van Hoek *et al.*, 2011), the subsequent discoveries of new antibiotics changed the perspective in the therapy of infectious diseases (Wenzel, 2004). Indeed, after the introduction of antibiotics in the pharmaceutical industry in the 1950s, antibiotics have been used for the control of infections and the reduction of the associated morbidity and mortality (Davies and Davies, 2010). At the same time, the evolution of antibiotic resistance was considered improbable due to the assumption that the frequency of mutations leading to resistance in bacteria was minimal (Davies, 1994). Later on this turned out to be a wrong assumption as it was discovered that antibiotic resistance emerged before the first antibiotic, penicillin, was even characterized (Abraham and Chain, 1940). Antibiotics have been defined as natural, semi-synthetic or synthetic compounds that can either inhibit bacterial growth (bacteriostatic) or kill (bactericidal) bacteria.

Depending on their activity, they are used against a wide range of disease-causing bacteria, including Gram-positive and Gram-negative strains (broad-spectrum antibiotics) or against a specific group of bacteria (narrow-spectrum antibiotics) (Demain and Sanchez, 2009).

Nowadays, different classes of antibiotics are known and can be classified based on their mechanism of action (**Fig. 1**). In general, antibiotics interfere with important cellular processes and can, for instance, inhibit the bacterial cell wall synthesis (β-lactams and glycopeptides), inhibit the protein synthesis (aminoglycosides, macrolides, tetracycline and chloramphenicol), interfere with the synthesis of DNA and RNA (quinolones or rifampin) or modify the energy metabolism of the microbial cell, i.e. folate synthesis (sulfonamides and trimethoprim) (Neu, 1992).

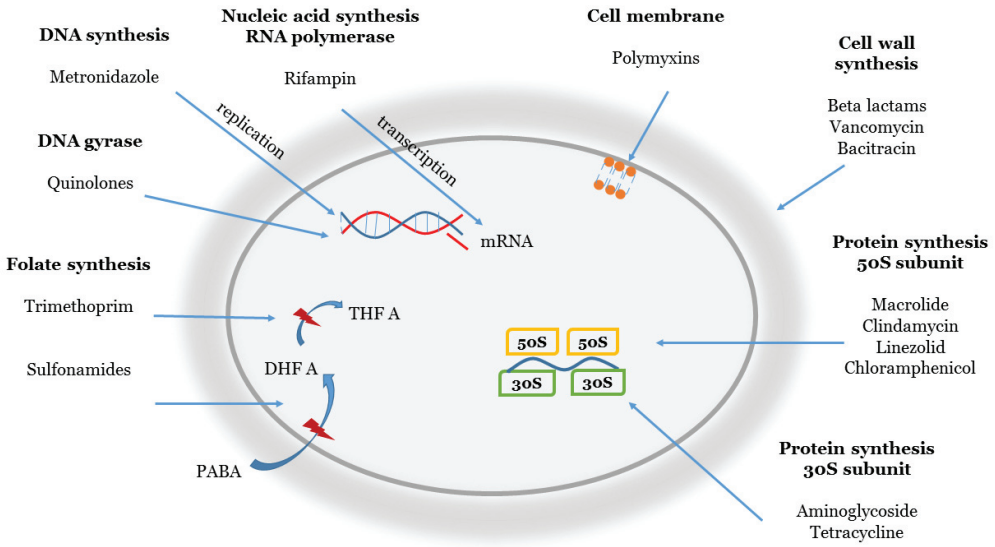

Figure 1. Mechanisms of action of antibiotics. The four main targets of antibiotics include the synthesis of cell wall and cell membrane, protein synthesis (30S and 50S ribosomal subunits), nucleic acid synthesis and folate synthesis. Adapted from Johnson (2011).

Over the years, the extended use of antibiotics, estimated to be 100-200 x10^6 kg/year worldwide (Wise, 2002; Anderson and Hughes, 2010), has led to an enormous increase of antibiotic resistance among pathogenic bacteria (Nikaido, 2009). In fact, large amounts of antibiotics are used not only for clinical purposes, but also in animal production as therapeutic agents as well as growth-promoters, resulting in a selective pressure for the emergence, enrichment and spread of antibiotic resistant pathogenic bacteria (Anderson and Hughes, 2012).

Analogous to the mechanism of actions, different mechanisms allow bacteria to become resistant to antibiotics. These mechanisms include a decrease in the permeability of the bacterial cell wall, enzymatic modification of antibiotics, degradation of antibiotics, modification of the target, overproduction of the target enzyme or the presence of efflux pumps in the bacterial cell (**Fig. 2**) (Alekshun and Levy, 2007).

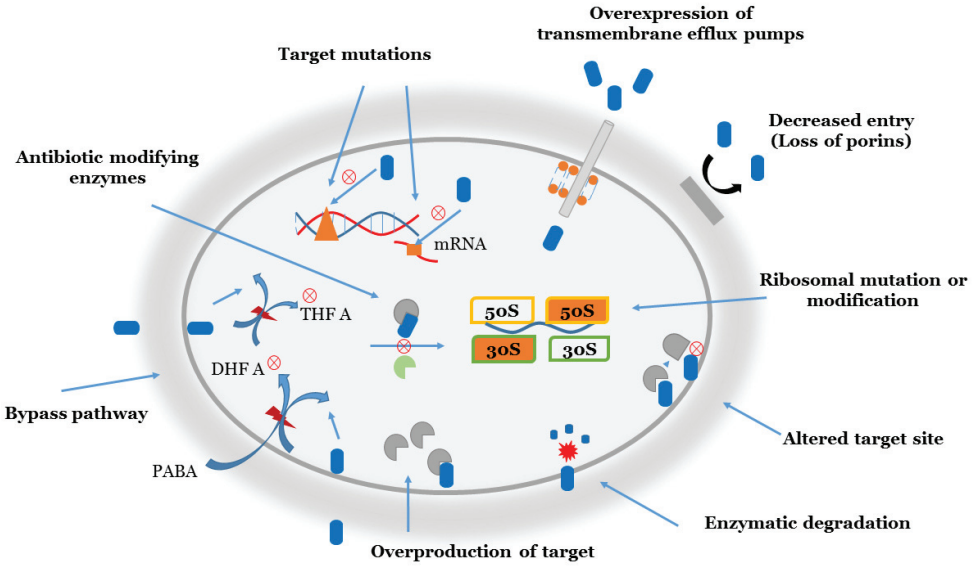

Figure 2. Mechanisms and target sites of defensive mechanisms used by bacteria to prevent detrimental effects caused by antibiotics. Adapted from Hawkey (1998).

Antibiotic resistance (AR) can be achieved by chromosomal DNA mutations (Martinez and Baquero, 2000) and/or by acquisition of new genetic material (mobile elements) from other bacteria through Lateral Gene Transfer (LGT), the latter of which is facilitated through three main pathways, including transformation, transduction and conjugation (Summers, 2006). In general, LGT requires two principle processes to occur: a) the physical movement of DNA from a donor to the recipient organism and b) the incorporation into the receiving cell and/or genome to allow stable inheritance (Stokes and Gillings, 2011). Such DNA acquisition can occur between different bacterial species and between hosts present in different environments (**Fig. 3**).

Many of the AR genes encountered in the environment are encoded on transferable mobile genetic elements that are highly homologous between pathogens and commensal bacteria, where commensal bacteria represent the majority of the microbial community present in the host and natural environments. It has been indicated that commensal bacteria could play an important role in the evolution and dissemination of genetic elements such as AR genes in the microbial communities inhabiting different ecosystems (Wang, 2009).

A range of factors can influence the acquisition of mobile elements containing AR genes such as selective pressures in the environment, non-specific and specific host factors and properties of the mobile genetic elements such as the production of anti-restriction proteins (van Hoek *et al.,* 2011). Convincing evidence for the transfer of AR genes between Gram-positive and Gram-negative commensal bacteria and between aerobic and anaerobic bacteria has been reported (Courvalin, 1994; Salyer *et al*., 2004 and Ojo *et al*., 2006).

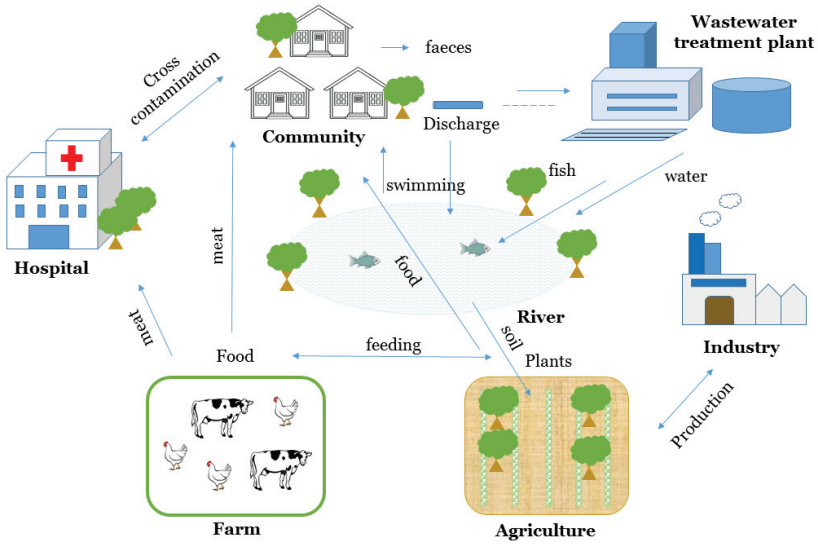

Figure 3. Schematic representation of the transmission of AR genes and resistant bacteria between community, hospital, wastewater plants, farms, agriculture and industry. Adapted from Davies and Davies (2010)

AR genes: the ecological context

Since the majority of antibiotics used for the treatment of infections have originated from natural environments, AR genes acquired by pathogens could similarly originate from the same sources (Martinez, 2008; Bhullar *et al.,* 2012). Natural habitats, such as soil, for example, represent a common reservoir of resistance genes (Dantas *et al.,* 2008). In hospital environments, the high concentrations of antibiotics used for clinical propose can select for resistant mutants which can serve as a reservoir of resistance genes. The selection of resistant mutants was thought to occur at concentrations between the minimal inhibitory concentration (MIC) of the susceptible wild type strain and the MIC of the resistant bacteria, and concentrations below the MIC of the susceptible strain should not inhibit the growth of the bacteria and hence not be selective.

However, a recent study has shown that the low antibiotic concentrations present in natural environments might actually contribute significantly to the emergence and maintenance of resistance (Gullberg *et al.,* 2011). It has been suggested that antibiotic-producing microorganisms could have provided the initial pool of genes from which the present antibiotic resistance genes derived (Benveniste and Davies, 1973). In fact, at the low concentrations encountered in natural environments antibiotics induce responses in their target microorganism, but like other compounds, become toxic at higher concentrations, the so-called hermetic effect (Martinez *et al*., 2009).

Recent work has shown that a large and diverse group of bacteria from soil, seawater and the gut microbiota from humans and farm animals was not only able to resist the toxic effects of antibiotics, but also they could utilize antibiotics as a sole carbon source, a phenotype commonly referred to as antibiotic subsistence (Dopazo *et al*., 1988; Dantas *et al.,* 2008; Barnhill *et al*., 2011; Xin *et al*., 2012). Controversially, Walsh *et al*. (2013) showed that soil bacteria could not utilize antibiotics as a carbon source since no degradation of antibiotics occurred. The fact that multidrug resistance elements participate in other processes such as detoxification of metabolic intermediates, signal trafficking and virulence, could perhaps explain why genes could not only play a role in resistance but also evolved into other functions.

Nonetheless, the genes involved in the antibiotic subsistence phenotype have not been identified and therefore, the relationship between resistance and subsistence remains unclear (Dantas & Sommer, 2012). Previous studies indicated that, for example, humans are continuously exposed to AR genes present in bacteria associated with retailed food (Wang *et al*., 2006).

Recently, Kluytmans and colleagues showed that extended-spectrum β-lactamase-producing *Escherichia coli* isolates from chicken meat and human faecal samples shared similar genetic mobile elements, virulence genes and genomic backbone (Kluytmans *et al.,* 2013).

Furthermore, an association has been established between the AR genes present in commensal bacteria from food animals, lagoon water, farm manures and exposure to growth-promoting antibiotics (Allen *et al*., 2010). In contrast, the relationship between the AR genes present in commensal bacteria from healthy humans and wild animals without recent antibiotic exposure is still unclear. Nonetheless, Kuiken and collaborators indicated that more than 70% of emerging infections originate from animals, especially wild animals (Kuiken *et al.,* 2005). Wild animals held in captivity in zoos could therefore serve as a reservoir for zoonotic pathogens and transfer their pathogens and resistance genes to humans through direct contact (handing and feeding activities) (Wang *et al.,* 2012); Bender and Shulman supported this claim, and reported that a human infectious disease outbreak in the period of 1990 to 2000 was associated with animal contact (Bender and Shulman., 2004).

Besides animal handling, the contamination of water and food with multidrug-resistant bacteria is one of the main sources of the spread of antibiotic resistance in humans and animals. Recent studies reported the presence of multidrug-resistant bacteria present in food and water systems, highlighting the potential risk for the human health after consumption, being the gut microbiota the most substantial reservoir of antibiotic resistance (Karumathil *et al*., 2016; Stange *et al.,* 2016).

Gut microbiota: Composition and functions

The human body coexists with a vast number of microbes, including bacteria, archaea, viruses and unicellular eukaryotes, commonly referred to as the microbiota (Neish, 2009). Among all external body surfaces, the gut harbours over 70% of the total microbes (Ley *et al*., 2006). The majority of the gut microbiota is dominated by anaerobes, followed by facultative anaerobes and aerobes, having as predominant phyla *Bacteroidetes* and *Firmicutes*, whereas *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria* and *Verrucomicrobia* represent only a minor proportion of the total microbial load (Eckburg *et al*., 2005).

The number of microbial cells and their composition varies greatly along the gut, starting from 10^1 to 10^3 bacteria per gram in the stomach due to the short retention time of gastric content and acid pH, increasing to 10^4 to 10^8 per gram in the small intestine and ending in the large intestine. Here the rate of peristaltic movements decreases, facilitating the development of a complex and dense microbial community with 10^{11} to 10^{12} bacterial cells per gram of content (Sekirov *et al.,* 2010).

Starting from the moment of birth, the human gut microbiota becomes more diverse rapidly until reaching a relatively stable state during childhood. At old age the diversity decreases again (Claesson *et al.,* 2011; Scholtens *et al.,* 2012). Although it has been established that the human gut microbiota composition is unique per individual, a classification into a limited number of major constellations has been proposed, the so-called enterotypes. Each enterotype is defined by correlation networks and named according to microorganisms at central nodes within these networks, namely *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam *et al.,* 2011). Interestingly, Wu and colleagues showed that long-term dietary changes could contribute to shifts between different enterotypes (Wu *et al.,* 2011). A recent study based on phylogenetic analysis of the gut microbiota of a thousand western adults, indicated the presence of different groups of bimodally distributed bacteria that are in most cases either abundant or almost absent, and which could represent “tipping elements” of the gut microbiota that are indicators and/or drivers of the transition between alternative stable states of gut microbiota composition (Lahti *et al.,* 2014).

It has been well documented that the human gut microbiota plays an important role in a broad range of metabolic, nutritional, physiological and immunological processes within the host, and as such contributes to gut and systemic homeostasis (O’Hara and Shanahan, 2006). One important metabolic activity of the gut microbiota is the breakdown of dietary components that are not digested by the host’s own secreted enzymes, converting them through fermentation to short-chain fatty acids (SCFA) such as acetate, propionate and butyrate.

Particular interest has been attributed to butyrate as the main energy source for colonocytes (Hamer *et al.,* 2008). Changes in gut microbial composition have been found to correlate with inflammatory and metabolic disorders (O'Toole and Claesson*,* 2010) such as inflammatory bowel diseases (Frank *et al*., 2007), irritable bowel syndrome (Jeffery *et al.,* 2012), obesity (Ley *et al.,* 2006), cancer (Lupton, 2004) and diabetes (Larsen *et al.,* 2010).

Different internal and external factors can affect the composition and disrupt the ecological balance of the gut microbiota, including, for example, age, genetics and host immune response (internal factors), and geographic location, diet and administration of modulators of the gut microbiota such as prebiotics, probiotics and antibiotics (external factors).

The gut microbiota of other mammals resembles that of humans; however, more or less pronounced differences are observed between animals that differ, e.g. in terms of genetic background, anatomy and morphology of the gut, and dietary habits (Ley *et al.,* 2008). In fact, similar to what has been described for humans, also the gut microbiota in other mammals is affected by a range of different external or internal factors (Yildirim *et al.,* 2010, Moeller and Ochman, 2013). Recently, Moeller and colleagues, described the cospeciation of microbiota with hominids, further emphasizing the functional role of the microbiota for the specific needs of the host (Moeller *et al.,* 2016).

Interplay between gut microbiota and antibiotics

The gut microbiota of healthy adults remains generally stable over time (Martinez *et al.,* 2013). During antibiotic treatment, however, a disturbance in microbiota composition is established, the number of commensal bacteria is reduced and the colonization resistance barrier is broken, which can lead to an overgrowth of and colonization with potentially pathogenic bacteria (Schjørring and Krogfelt, 2011) (**Fig. 4**).

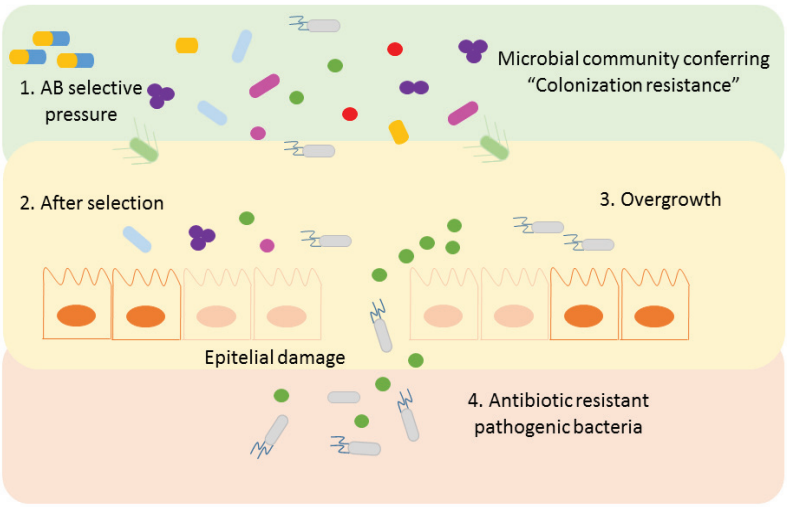

Figure 4. Schematic representation of the disrupted balance of the gut microbial community induced by antibiotics. The antibiotic selective pressure induces a disbalance in the commensal microbiota that normally provides colonization resistance (1). The resulting reduction in the commensal microbiota (2) is followed by overgrowth of and colonization with antibiotic resistant pathogenic bacteria (3, 4). Adapted from Kamada *et al.*, 2013.

One of the most important factors that influence the extent to which a given antibiotic will change and decimate the microbiota is the degree to which it is absorbed in the gut and thus its effective local concentration that directly acts on the microbiota, as well as the duration of the exposure. Due to the fact that different antibiotics induce specific effects on the gut microbiota, as reported previously (Young and Schmidt, 2004; Robinson and Young, 2010), a selective pressure of the antibiotic is maintained in this microbial environment, which contributes to the increase of antibiotic resistant bacteria.

Furthermore, previous studies showed that co-selection of AR determinants by other antimicrobial compounds such as antiseptics and heavy metals can further contribute to the occurrence of antibiotic resistance without antibiotic selective pressure (Baker-Austin *et al.,* 2006). The complexity and dynamics of the gut microbiota further increases the feasibility for the exchange of AR genes between commensals and pathogens (Kazimierczak and Scott, 2007). The hypothesis "Could the microflora of the human colon, normally considered innocuous or beneficial, be playing a more sinister role in human health as a reservoir for antibiotic resistance genes?" established by Salyers and collaborators is nowadays well accepted (Salyers *et al.,* 2004). A growing number of publications indicated that gut commensal bacteria, including aerobes and anaerobes, act as a donor of AR genes to bacteria that are transitory in the gut microbiota. The principal adverse effect is the increase of nosocomial pathogens resistant to antibiotics, which reduces the efficacy of antibiotic treatment, and thereby increases morbidity and mortality and the cost of hospitalization.

Antibiotic therapy: Control of gut colonization and overgrowth of nosocomial pathogens

Hospital-acquired infections represent a major cause of mortality and increase of health care cost around the world. In intensive care units (ICUs), critically ill patients are at continuous risk of acquired infections due to their vulnerable conditions (Vincent, 2003). One of the main concerns in this category of patients is that they are susceptible to colonization with antibiotic resistant bacteria due to the exposure to invasive procedures and antibiotic administration, which could increase the incidence of infection, reduce the efficacy of antibiotics and increase AR selection (van Duijn *et al.,* 2011). During invasive procedures, the skin and mucosa are disrupted allowing the translocation of bacteria into the bloodstream, causing bacteraemia or candidaemia, or into the oro-pharyngeal and nasal cavities causing ventilator associated pneumonia (VAP) (Thom *et al.*, 2010 and Carlet, 2012).

Not only colonization with antibiotic resistant bacteria, but also overgrowth of bacteria, defined as the presence of potential pathogens at high concentration (> 10^5 colony forming units/ml) could facilitate the bacterial translocation (Pierro *et al.,* 1998).

One of the most common factors associated with the risk of infections in ICU patients is the duration of ICU stay. An international study that focused on the prevalence and outcomes of infection in 1265 participating ICUs (14,414 patients in total) from 75 countries, showed that 51% of the patients were considered infected and 71% of them received antibiotics. The main origin of infections was respiratory and more than 50% of the isolates were Gram-negative bacteria followed by Gram-positive bacteria and a minor percentage of fungi. Likewise, the authors reported that a higher rate of infection was associated with prolonged stays in ICU (Vincent *et al.,* 2009).

It has been shown that broad spectrum antibiotic therapy affects the target bacteria as well as the entire microbial community (Jernberg *et al.,* 2010), increasing the pool of antibiotic resistant bacteria present in the gut. AR rates in European ICUs were recently studied, indicating that Gram-negative bacteria (e.g. *Escherichia coli* and *Klebsiella pneumoniae*) play the main role in the emergence and spread of infections, facilitating the exchange of resistance genes, while methicillin-resistant *Staphylococcus aureus* (MRSA) remained stable (van Duijn *et al.,* 2011).

Different measures have been established for the control of infections in ICUs such as standard care, strict hand hygiene to decrease the cross-transmission and the implementation of prophylactic antibiotic therapy (D'Amico *et al*., 1998; Liberati *et al.,* 2009). Two prophylactic antibiotic therapies, Selective Oropharyngeal Decontamination (SOD) and Selective Digestive Decontamination (SDD), have been used to prevent the colonization by Gram-negative bacteria, *Staphylococcus aureus* and yeast without disrupting the anaerobic microbiota, through the application of non-absorbable antimicrobial agents into the oropharynx and gastrointestinal tract.

Different combinations of antimicrobial agents have been used. The most frequent combination used in the SDD protocol includes the narrow spectrum antibiotic polymyxin E, the broad spectrum aminoglycoside tobramycin and the antifungal drug amphotericin B in the oropharynx (paste) and the gastrointestinal tract (suspension) applied four times daily, and a short course (first 3-4 days of ICU admission) of a broad spectrum systemic antibiotic, usually a third generation cephalosporin (cefotaxime or ceftriaxone). The SOD protocol includes only the application of the same topical antibiotic through the oropharynx, and is considered as an alternative therapy to prevent VAP (Melsen *et al.*, 2012).

SDD was introduced in 1984 as a method to reduce the rate of nosocomial infections in trauma patients (Stoutenbeek *et al.*, 1984). During the following years, several studies were conducted (http://www.clinicaltrials.gov, Bonten *et al.,* 2000), and the main conclusions were that SDD reduces the occurrence of VAP and that low levels of antibiotic resistance remain. The lack of evidence of patient outcome, however, and the unknown role in the development of AR led to a European consensus conference (European consensus conference, 1992), which recommended to not apply SDD in ICU patients until enough proof of the beneficial effect of the therapy has been established.

In 2001, van Nieuwenhoven and collaborators showed that during studies, special attention needs to be given to the design and methodology used, since an inadequate approach could introduce bias and overestimate the effects of the SDD treatment (van Niewenhoven *et al.,* 2001).

In the Netherlands, several additional studies were performed and showed that indeed the application of prophylactic antibiotic therapy decreased the incidence of VAP, with a low level of antibioticresistance remaining, and that the rate of mortality decreased compared with standard care (de Jonge *et al.*, 2003 and de Smet *et al.*, 2009). Later on, Melsen and colleagues showed that SDD therapy reduces the mortality in surgical and non-surgical patients, while SOD therapy showed a similar effect only in non-surgical patients (Melsen *et al.,* 2012).

While it is well established that SDD reduces the incidence of VAP, fewer studies were performed in order to study the effect of SOD in a short course application on the development of VAP. To this end, Schnabel and colleagues, reported a significant reduction of VAP during SOD/SDD therapy compared with the control group (Schnabel *et al.,* 2015). Based on these results and considering that only 30% of ICUs in the Netherlands implemented SDD-SOD therapy (Barends *et al.,* 2008), an evaluation of the trends of antibiotic resistant Gram-negative bacteria was needed, especially because the effect of both therapies on AR was still unclear. A study performed in 38 ICUs (17 used continuously SDD/SOD, 8 introduced SDD/SOD and 13 did not use SDD/SOD) during 2008-2012 indicated that a significant reduction in antibiotic resistant Gram-negative bacteria was associated with continuous or recent use of SDD/SOD as compared with no use (Houben *et al.,* 2013). Similarly, an evaluation on the trends of antibiotic resistant Gram-positive bacteria was performed in 42 Dutch ICUs from 2008-2013, indicating that a continuous use of SDD/SOD therapy was not associated with an increase of isolates of Gram-positive cocci. Although the introduction of SDD/SOD was associated with an increase in rate of isolation, it was not associated with antibiotic resistance (van der Brij *et al.,* 2016). A more recent survey performed in ICUs registered in the European Registry for Intensive Care (ERIC) showed that only 17% of them used SDD as a prophylactic therapy, and mainly ICUs in the Netherlands (13/23) and Germany (6/15) (Miranda *et al.,* 2015).

Furthermore, a number of studies was performed in order to determine the effect of SDD and SOD therapy on antibiotic resistance, all of them focussing on the target group for the therapy without considering the commensal microbiota.

Oostdijk and collaborators showed that both therapies contributed equally to low AR prevalence in Gram-negative bacteria in rectal and respiratory samples, however, an increase of ceftazidime resistant Gram-negative bacteria was observed after SDD therapy discontinuation (Oostdijk *et al.,* 2010).

In another study performed in 13 ICUs in the Netherlands, the rate of acquisition of respiratory tract colonization with Gram-negative antibiotic resistant bacteria was higher during SOD therapy compared to SDD (de Smet *et al.,* 2011). A recent meta-analysis of randomized control trials indicated that SOD therapy has similar effects as SDD in reducing mortality, in spite of the fact that SOD has been associated with a higher incidence of ICU-acquired bacteremia and antibiotic-resistant Gram-negative bacteria, while SDD increased the risk of antibiotic resistance (cephalosporins). Based on this outcome, the authors recommend the use of SOD as prophylactic antibiotic regimen in patients in the ICU (Zhao *et al.,* 2015).

These results raised questions with respect to the contribution of SOD and SDD on colonization with antibiotic resistant Gram-positive bacteria. In a trial performed in a non-endemic area, de Smet and co-authors (2009) reported low levels of MRSA and Vancomycin Resistant *Enterococcus* (VRE) during SOD therapy compared with the control group (no antibiotics). It is important to consider that the antibiotics included in SOD and SDD therapies do not target most Gram-positive bacteria. Therefore, increased rates of colonization and infection by the two main players of nosocomial infections, namely MRSA and VRE, can be expected. In Europe, Austria and Belgium studies have reported an increase of MRSA in SDD treated patients (Verwaest *et al.*, 1997; Lingnau *et al.,* 1998).

On the other hand, *Enterococcus* species, mainly *Enterococcus faecium* and *E. faecalis,* represent the third most common cause of bacteraemia, frequently associated with a high rate of antibiotic resistance. Usage of SDD therapy in combination with topical and enteral vancomycin has been effective to eradicate VRE where VRE is not endemic, however, Dahms and collaborators reported an increase of VRE colonization in ICU patients when SDD therapy was applied in combination with vancomycin or ceftazidime and vancomycin (Dahms *et al.,* 2000). Most recently, Benus and collaborators showed that during SDD therapy, an increase of enterococci was observed when compared to SOD or standard care (Benus *et al.,* 2010).

Interestingly, the presence and spread of high risk clonal complexes, especially the ones with the capacity to adapt to hospital environments, carrying antibiotic resistance and virulence genes, represent a growing problem around the world. In 2015, a spread of *E. faecalis* clonal complex (CC2) present in ICU patients receiving SDD therapy was reported in Spain (Muruzabal-Lecumberri *et al.*, 2015).

SDD and SOD therapies do not only have a short-term effect on the microbiota composition but also long term effects. It cannot be excluded that during SDD therapy, the concentration of antibiotics in faeces reach a high level due to the direct administration of antibiotics through a gastric tube providing a protective effect against overgrowth, but when the therapy is terminated, a recolonization occurs.

A recent emergence of polymyxin E (Colistin) resistance in *Enterobacteriaceae* has been reported after the introduction of SDD therapy (Halaby *et al.*, 2013 and Lubbert *et al.,* 2013). Similarly, Sanchez-Ramirez and collaborators reported that after three years of SDD application, a reduction in infections with antibiotic resistant bacteria, decrease in nosocomial infections and antibiotic consumption was observed compared with the control group; however, colonization by tobramycin and colistin resistant bacteria was observed during the study period (Sanchez-Ramirez *et al.,* 2015). In contrast, in the Netherlands, Wittekamp and collaborators showed that long-term use of SOD and SDD therapy was not associated with an increase of colistin and tobramycin-resistant Gram-negative bacteria (Wittekamp *et al.,* 2015).

So far, questions remain with respect to the direct health effects of SDD and SOD therapies during and after the ecological perturbations induced in terms of reduction of hospital-acquired infections and potential development of antibiotic resistance being the main goal from the public health perspective, but also in terms of microbial composition and functions.

Tools for studying the gut microbiota and resistome

The compositional and genetic complexity of the gut microbial ecosystem have increased the interest to understand its role and functions by using state of the art microbiological tools. For many years, the techniques used to study microbial diversity have been divided in culture dependent and independent methods. Both types of approaches contributed to a better understanding of the microbial composition and ecological perturbations induced for example by antibiotic administration.

By using culture dependent methods, microbiologists have been able to study only a small fraction of the complex community present in the gut, and it has been previously estimated that only 10% of the gut microbiota can be cultivated under standard conditions (Eckburg *et al.,* 2005). As a consequence, the diversity of the microbiota has been grossly underestimated based on cultivation-derived data. Generally, microbiologists use selective and non-selective media to culture specific functional groups of microorganisms or rather as many different microorganisms as possible, respectively. It has been noted, however, that many of the bacteria thriving in the gut environment may require special nutrients or other metabolic products that can be provided by other members of the gut microbiota, and thus can be classified as obligate syntrophs (Macfarlane and Gibson, 1994; Macfarlane *et al.,* 1994).

In addition, sampling methods, transportation, storage and cultivation technique used can lead to differences with respect to results reported by different studies (Macfarlane and Macfarlane, 2004, Tedjo *et al.,* 2015).

In the last years, a growing interest in innovative culture methods has been established, for example by using diffusion chambers to stimulate the growth of previously uncultured bacteria or by using rumen fluid or extract of fresh faecal material to better simulate the environmental conditions present in the gut (Kaeberlein *et al.,* 2002).

Browne *et al.* (2016) recently showed more than 10% of the gut bacteria are culturable by using a single growth medium to isolate spore-forming bacteria.

One of the advances in culturing techniques include the implementation of the micro-petri dish. Porous aluminium oxide (PAO) or PAO Chips, were introduced in 2005 (Ingham *et al.,* 2005) as a microbial culture support while agar functioned as a matrix supplying nutrients to the bacterial cells. It has been used in microbiology for different purposes, including cell counting and identification, growth and micro-colony imaging of microorganisms, and as a high throughput screening tool (Ingham *et al.,* 2007; Ingham *et al.,* 2012). Several studies have used cultivation techniques in order to detect the growth of common pathogens e.g. during SDD or SOD therapy. In contrast, strictly anaerobic bacteria, which represent the majority of the gut microbiota and comprise an important reservoir of antibiotic resistance in the gut (Shoemaker *et al.,* 2001; Sommer *et al*., 2009), have not been extensively explored by cultivation methods because their cultivation is time consuming and laborious and requires special equipment (Macfarlane, 1994).

Since culture dependent methods underestimate the microbial diversity present in the gut, molecular biological techniques (culture independent methods) have been introduced, allowing microbiologists to characterize more comprehensively the complex ecosystem present in the gut. By using the bacterial 16S ribosomal RNA (rRNA) gene as a genetic marker, an analysis of the phylogenetic groups present in the gut community can be established. In the 1990s, Polymerase chain reaction (PCR) was introduced to detect bacteria in complex communities by using specific primers. As one of the first examples, Matsuki *et al.* (1999) showed that a qualitative detection of bifidobacterial species present in faecal samples from healthy adults and breast-fed infants could be accomplished by 16S rRNA-gene-targeted species-specific PCR.

It has been noted that also cultivation-independent approaches are not without limitations, including, e.g., differences in the efficiency of extraction of DNA and RNA from different bacteria, which is related to difference in the susceptibility to

chemical enzymatic and/or mechanical lysis for some bacterial groups (Zoetendal *et al.,* 2001). Advances in molecular analysis include the quantitative analysis of microbial communities by Real-Time PCR by using genus- or species-specific primers to quantify specific groups of bacteria. Early examples include the analysis of microorganisms associated with the mucosa in the gastrointestinal tract (Huijsdens *et al.,* 2002), and the comparison of patients treated or not treated with antibiotics (Bartosch *et al*., 2004).

Moreover, 16S rRNA gene clone libraries have been used for phylogenetic analysis of the intestinal microbiota (Suau *et al*., 1999), however, this technique is time consuming and does not allow to comprehensively characterize complex microbial communities such as those residing in the gut at realistic costs. Therefore, other techniques based on molecular fingerprinting such as Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragments Length Polymorphism (T-RFLP) have been used in the past for rapid comparative analysis of microbial communities, for example to monitor the microbiota present in different regions in the gut (Zoetendal *et al.,* 2002) and to analyze the disruption of the microbiota during antibiotic treatment (Donskey *et al.,* 2003). More recently, the advent of a growing list of next generation sequencing technologies, including but not limited to pyrosequencing and Illumina sequencing, dramatically increased the possibilities to analyse large numbers of samples in the same sequencing run using sample-specific bar-coded primers. Early examples include the comparison of gut microbiota present in obese and lean twin pairs (Turnbaugh *et al.,* 2009) and the evaluation of the effect of a short course ciprofloxacin treatment in three healthy adults (Dethlefsen *et al.,* 2008). In addition to next generation technology sequencing based approaches, also DNA microarrays represent powerful tools designed for high-throughput screening of the gut microbiota. By using the Agilent platform, Palmer and collaborators designed for the first time a DNA microarray containing probes targeting 359 microbial species and 316 novel Operational Taxonomic Units (OTUs) (Palmer *et al.,* 2006; Palmer *et al.,* 2007).

More recently, Rajilic-Stojanovic and colleagues designed the Human Intestinal Tract Chip (HITChip) that contains 4800 oligonucleotides probes based on two hypervariable regions of the SSU rRNA gene of microorganisms detected in the human gastrointestinal microbiota (Rajilic-Stojanovic *et al.,* 2009). The HITChip has been extensively used to determine the diversity and dynamics of the gut microbiota in a broad range of different subject groups. A comparison between phylogenetic microarray (HITChip) and pyrosequencing-derived data was established for four faecal samples of elderly individuals, showing good correlation of both methods especially at higher taxonomic ranks (Claesson *et al.*, 2009).

Fluorescent In Situ Hybridization (FISH) is a useful technique when specific bacterial phylogenetic groups are targeted and allows to monitor the spatial organization of bacteria in the community. Nevertheless, some limitations have been encountered such as design of probes and the ability of the probes to reach the target side. Similar to FISH, for qPCR, target-specific primers are needed, and generally, both techniques are applied in combination with other more generic approaches to support the results (Kerckhoffs *et al.*, 2009). Recently, a high-throughput qPCR chip has been designed to study gut microbial diversity in combination with next generation sequencing (Hermann-Bank *et al.,* 2013). The majority of the molecular methods described above require the use of more or less specific primers or probes targeting a microbial group of interest.

In contrast, by using metagenomics, the repertoire of bacteria that can be studied is extended. Furthermore, metagenomics allows not only to identify the bacterial species but also their functional role in the microbial community. The introduction of metagenomics methods has turned on a new page for characterizing uncultivable organisms present in different environments (Martinez, 2008; Aminov, 2009). Functional metagenomics screening has also been used to study the function of several of the encoded genes, especially the flow of resistance genes and unknown genes that cannot be detected by PCR (Riesenfeld *et al.*, 2004; Sommer *et al.,* 2009). Targeted (PCR-based), functional and sequence-based metagenomics methods have been applied to study the resistome (Penders *et al.,* 2013).

The implementation of culture dependent and independent techniques including metagenomics and high-throughput sequencing have been increasing our knowledge in the study of the gut microbiome and resistome. Recently, Dubourg *et al.* (2014) implemented the integrated application of culture dependent and independent techniques to determine the impact of antibiotics on the gut microbiota in patients treated with broad-spectrum antibiotics. Similarly, Rettedal and collaborators (2014) showed that the combination of novel cultivation methods with high-throughput sequencing can allow scientists to identify and phenotypically characterize previously uncultivated species.

Research aim and thesis outline

In line with the above, the aim of the research described in this thesis was to increase our knowledge regarding the gut microbiota and associated resistome by using culture dependent and independent techniques, focusing on the diversity and dynamics of the gut microbiota induced by antibiotic treatment.

Chapter 1 provides an overview of the introduction of antibiotics as a powerful tool to fight nosocomial infections and the subsequent development of resistance, considering the emergence of antibiotic resistant genes from an ecological point of view. Furthermore, information is given on our current knowledge regarding the role of the gut microbiota as a reservoir of antibiotic resistance genes and the ecological implications of antibiotic administration in critical ill patients, including the different tools developed for the study of the gut microbiota and resistome.

It has been previously shown that antibiotics can not only act as a toxic compound, but also can be used as a single source of carbon by bacteria, which is referred to as the "Subsistence phenotype". **Chapter 2** describes different strategies that were implemented to study the subsistence phenotype in microorganisms present in faecal samples from humans as well as zoo animals.

The animals included in this initial study of subsistence also included a number of non-human primates. Therefore, in order to allow for deep and comprehensive analysis of the composition of gut microbiota in these animals, experiments were performed as reported in **Chapter 3** that investigated to what extent the Human Intestinal Tract Chip (HITChip) could also be applied for the characterization of gut microbiota composition in non-human primates.

In the gut microbiota, commensal bacteria play an important role in homeostasis with respect to a broad range of metabolic, nutritional, physiological and immunological processes, but can also act as a reservoir of antibiotic resistance genes.

The majority of the commensal bacteria is represented by anaerobes, however, few studies have been performed in this group of microorganisms due to the laborious and difficulties to cultivate them. In **Chapter 4,** culture independent techniques such as HITChip phylogenetic microarray, metagenomics-shotgun sequencing and functional metagenomics were applied to study the gut microbiota and resistome in a single ICU patient receiving prophylactic antibiotic therapy.

The analysis was further expanded in **Chapter 5** by studing the dynamics of the microbiota and resistome in eleven ICU patients receving prophylactic antibiotic therapy using HITChip phylogenetic microarray and nanolitre-scale quantitative PCRs, targeting a broad range of antibiotic and disinfectant resistance genes.

Using cultivation techniques, complementary information regarding the ecological consequences of antibiotic administration in critically ill patients can be established. In **Chapter 6** a range of cultivable aerobic and anaerobic bacteria was isolated and further characterized from eleven ICU patients receiving prophylactic antibiotic therapy, by using several complementary culture media, and the cultivable fraction was compared with the overall composition of the microbiota present in the samples as measured by using the HITChip.

Chapter 7 provides a more detailed acount of the dynamics of *Enterococcus* species colonization in ICU patients receiving prophylactic antibiotic therapies, including the identification of clonal complexes. Furthermore, carriage of antibiotic resistance and virulence factor encoding genes was determined, highlighting the opportunity for the exchange of resistance and virulence genes, which could increase the risk of aquiring nosocomial infections.

Chapter 8 describes the implementation of high-throughput cultivation-based screening using the PAO-based Microdish platform combined with high-throughput sequencing (MiSeq), which allowed the recovery previously uncultivable bacteria present in the gut of critical ill patients receiving antibiotic treatment.

Chapter 9 provides a general discussion of the results obtained from the studies described in this thesis, with emphasis on the different approaches implemented to study the microbiome and resistome.

Furthermore, this chapter provides an outlook and unanswered questions that should be included in the design of future studies.

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CHAPTER 2

Study of the aminoglycoside subsistence phenotype of bacteria residing in the gut of humans and zoo animals

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Abstract

Recent studies indicate that next to antibiotic resistance, bacteria are able to subsist on antibiotics as a carbon source. Here we evaluated the potential of gut bacteria from healthy human volunteers and zoo animals to subsist on antibiotics. Nine gut isolates of *Escherichia coli* and *Cellulosimicrobium* spp. displayed increases in colony forming units (CFU) during incubations in minimal medium with only antibiotics added, i.e. the antibiotic subsistence phenotype. Furthermore, laboratory strains of *E. coli* and *Pseudomonas putida* equipped with the aminoglycoside 3'phosphotransferase II gene also displayed the subsistence phenotype on aminoglycosides. In order to address which endogenous genes could be involved in these subsistence phenotypes, the broad-range glycosyl-hydrolase inhibiting iminosugar deoxynojirimycin (DNJ) was used. Addition of DNJ to minimal medium containing glucose showed initial growth retardation of resistant *E. coli*, which was rapidly recovered to normal growth. In contrast, addition of DNJ to minimal medium containing kanamycin arrested resistant *E. coli* growth, suggesting that glycosyl-hydrolases were involved in the subsistence phenotype. However, antibiotic degradation experiments showed no reduction in kanamycin, even though the number of CFU increased. Although antibiotic subsistence phenotypes are readily observed in bacterial species, and are even found in susceptible laboratory strains carrying standard resistance genes, we conclude there is a discrepancy between the observed antibiotic subsistence phenotype and actual antibiotic degradation. Based on these results we can hypothesise that aminoglycoside modifying enzymes might first inactivate the antibiotic (i.e. by acetylation of amino groups, modification of hydroxyl groups by adenylation and phosphorylation respectively), before the subsequent action of catabolic enzymes. Even though we do not dispute that antibiotics could be used as a single carbon source, our observations show that antibiotic subsistence should be carefully examined with precise degradation studies, and that its mechanistic basis remains inconclusive.

Keywords: Antibiotic resistance, antibiotic subsistence, antibiotic subsistence phenotype, aminoglycosides, single carbon source

Introduction

Antibiotic resistance is a global health problem, and resistance is prevalent in bacteria isolated from both human and animal sources (van den Bogaard & Stobberingh, 2000; Sommer *et al.*, 2009). Also, other natural habitats, for example soil, represent a common reservoir of resistance genes (Dantas *et al.*, 2008). Recent metatranscriptome analyses have revealed that antibiotic resistance genes are expressed in a broad range of natural habitats, even in the absence of obvious antibiotic selection pressure (Versluis *et al.*, 2015). Furthermore, metagenomic studies of ancient environments have revealed that antibiotic resistance is a natural phenomenon that predates the anthropogenic selective pressure of clinical antibiotic use (D'Costa *et al.,* 2011).

It has long been speculated that, for example in clinically relevant strains, genes conferring resistance to aminoglycoside antibiotics were derived from organisms producing aminoglycosides, suggesting that members of the *Actinomycetes* could have provided the initial pool of aminoglycoside resistance genes (Benveniste & Davies, 1973; Wright, 2007). Aminoglycosides are useful in the treatment of Gram-negative aerobic bacilli, staphylococci and other Gram-positive bacterial infections (Yao and Moellering, 2007). The initial site of aminoglycoside action is the outer bacterial membrane, where the cationic antibiotic molecules create fissures in the outer cell membrane. These fissures result in leakage of intracellular contents, and enhanced antibiotic uptake. Once inside the bacterial cell, aminoglycosides inhibit protein synthesis by binding to the 30S ribosomal subunit (Gonzalez *et al.*, 1998). Resistance to aminiglycosides is often due to enzymatic inactivation by acetyltransferases, nucleotidyltransferases and phosphotransferases. Other resistance mechanisms include loss of permeability, structural alteration of the ribosomal target and the presence of efflux pumps (Azucena and Mobashery, 2001). Streptomycin, a representative of aminoglycoside antibiotics produced naturally by bacteria, has been shown to participate in microbial survival pathways.

These pathways can be defined as the capacity of bacterial metabolism to modulate antibiotic resistance (Martinez and Rojo, 2011). This could indicate that aminoglycosides, apart from inhibiting bacterial growth, could stimulate the acquisition of aminoglycoside resistance genes. This can play an important role in the survival of microorganisms, as indicated for the acetyltransferase involved in aminoglycoside resistance in *Providencia stuartii* (Goldberg *et al.*, 1999; Barlow and Hall, 2002).

Recently a large and diverse group of bacteria from soil, seawater, and the gut of humans and farm animals were found to not merely resist the toxic effects of antibiotics, but also to use antibiotics including aminoglycosides as a single carbon source. This phenotype is commonly referred to as "antibiotic subsistence" (Dopazo *et al.*, 1988; Dantas *et al.*, 2008; Barnhill *et al.*, 2011; Xin *et al.,* 2012). In addition, the concept of bacteria subsisting on antibiotics has been referred to as "antibiotic-resistant extremophiles" (Gabani *et al.*, 2012) or "antibiotrophs" (Woappi *et al.*, 2014). These alternative terms depict the microorganisms as being able to subsist under harsh environmental conditions, e.g. elevated antibiotic concentrations or the use of antibiotics as the sole carbon source. In disagreement with the accumulating body of literature supporting the possibility of bacterial subsistence on antibiotics, Walsh *et al.* (2013) tested whether soil bacteria could subsist on antibiotics. As no degradation of antibiotics occurred, Walsh *et al.* (2013) concluded that soil bacteria could not utilise antibiotics (including streptomycin, trimethoprim, penicillin and carbapenicillin) as a carbon source.

To date, no genes have been identified that could enable bacteria to use antibiotics as a single carbon source, and therefore the relationship between antibiotic resistance and antibiotic subsistence remains unclear (Dantas & Sommer, 2012). To this end, and since the gut microbiota of humans and animals has been described as a reservoir of antibiotic resistance, we studied the potential of gut bacteria to display the antibiotic subsistence phenotype using a range of antibiotics.

Almost all of the bacteria able to subsist on antibiotics grew on an aminoglycoside, and therefore we focused on aminoglycosides to address-mechanistic aspects of the subsistence phenotype that could be readily approached using laboratory model organisms.

Materials and methods

Samples and antibiotics used

We evaluated the antibiotic subsistence phenotype of bacteria subsisting on a range of antibiotics: ampicillin, chloramphenicol, erythromycin, kanamycin, streptomycin and tetracycline (1mg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands). Faecal samples from two healthy human volunteers and six species of exotic zoo animals (Burgers 'Zoo - Arnhem, the Netherlands) with no previous antibiotic administration (6 months) were used as inocula (**Table 1**). Faecal samples from zoo animals were taken by the zookeepers following internal standard regulations. The samples were collected immediately after defecation into a sterile container, and then stored at 4°C (for 0.5-4 h) before being transferred to -80°C.

Isolating bacteria with the subsistence phenotype

Faecal samples (˜ 200 mg) were suspended in 5 ml of M9 minimal salts medium (Sigma-Aldrich) and centrifuged twice (5 min at 18,400 *g*) to prevent carry-over of dissolved carbon from the faecal material. Washed bacterial cells were then suspended in 5 ml of fresh M9 medium, and 50 µl inoculated into 5 ml M9 medium supplemented with 1 mg/ml of a single antibiotic (98-99% purity) and incubated at 37°C for 24 h. Then, the cultures were serially transferred twice to a fresh media with antibiotic, followed by plating on Luria Broth agar (LB agar), to quantify the bacterial growth based on enumeration of colony forming units (CFU) on the LB plates were counted after 8, 24, 48 h of incubation at 37°C. The subsistence phenotype criteria were identified based on a two-fold increase of CFUs over multiple transfers. A single colony was selected and tested to confirm the subsistence phenotype. Glucose (1 mg/ml) was used as a positive control, while M9 medium lacking any carbon source served as negative control for growth. All experiments were performed in duplicate.

Identification of bacterial isolates with the subsistence phenotype

Bacteria subsisting on antibiotics were selected for DNA amplification using the 27F and 1492R primers. PCR was carried out with FastStart Taq DNA polymerase (Roche) in a reaction mixture containing 10X Fast Taq buffer + $MgCl_2$, dNTPs (10mM each, Roche), 10pmol of both primers in a final volume of 49µl; finally add the template of DNA (1 µl). For the amplification reaction, after 5 min at 95°C, 35 identical cycles (30 s of denaturation at 95°C, 40 s of annealing at 52°C, 90 s of elongation at 72°C) were followed by a final elongation step of 7 min at 72°C. The amplified fragments were selected for partial sequence analysis of the 16S rRNA gene (~800bp) using the 1392R primer, and sequences were deposited in GenBank with accession numbers KT989026, KT989027, KT989028, KT989029, KT989030, KT989031, KT989032, KT989033, KT989034, KT989035 (**Table 1**). Furthermore, all isolates were tested for their antibiotic resistance phenotype by dilution agar test as recommended by Clinical & Laboratory Standards Institute (2014).

Experimental controls to differentiate between aminoglycoside resistance and the subsistence phenotype

In order to differentiate between antibiotic resistance and antibiotic subsistence, we used transformants containing a gene encoding aminoglycoside 3'phosphotransferase II (APH (3') II) (Berg et al., 1975), one of the most common aminoglycoside-modifying enzymes in prokaryotes, as a control. In detail, chemically competent cells of two different strains of *E. coli* (DH5α and TOP10) were transformed by heat-shock with cloning vectors pRSF-1b (Novagen, Billerica, MA, USA) and pCR-2.1TOPO (Invitrogen, Carlsbad, CA, USA) respectively, both containing an APH (3') II gene. Also, we used *Pseudomonas putida* TEC1 transformed with the cloning vector pUTmini-Tn5-Km1 (de Lorenzo *et al.,* 1990; Leprince *et al.,* 2012). Transformed and non-transformed strains were tested for their ability to resist and subsist on the aminoglycoside antibiotics kanamycin and neomycin using the protocol described above.

Effect of deoxynojirimycin (DNJ) on the aminoglycoside subsistence phenotype

To evaluate the involvement of glycosyl hydrolases (GH) in the subsistence phenotype on aminoglycoside we selected deoxynojirimycin (DNJ) (Laboratory of Organic Chemistry, Leiden University, The Netherlands), which is one of the simplest natural carbohydrate mimics that can competitively inhibit specific glycosidic enzymes (Hughes & Rudge, 1994). We tested the capacity of *E. coli* (DH5α) transformed with pRSF-1b plasmid-encoded APH (3') II gene, to grow on kanamycin or glucose (1mg/ml) as a single carbon source in the presence of DNJ (range of 0.00001-10 mM of DNJ) and monitored growth for 24 hours. All the experiments were performed in triplicate and used 96-well plates. Growth was measured by OD=600nm for 24 h continuously during incubation at 37°C with agitation at 75 rpm.

Kanamycin degradation by *Escherichia coli*

To investigate kanamycin degradation by *E. coli* we performed an LC-MS/MS analysis. The experimental control was carried out using *E. coli* (DH5α) with and without cloning vector pRSF-1b in the presence of kanamycin (99.25% Kanamycin A Sulfate, EvoPure™, GENTAUR Netherlands) (1mg/ml). An aliquot was taken at 0, 4, 8, 24 h and analysed in duplicate using LC-MS/MS. In detail, the samples were diluted hundred times in 0,065% heptafluorbutyric acid, with an expected concentration of 10 mg/L. Octamethylkanamycine was added as an internal standard to the diluted samples at a concentration of 10 mg/L. Fifty microliter of the diluted sample was injected using a 2690 separations module high-performance liquid chromatography (HPLC) system (Waters Corporation, USA) coupled to a Quattro Micro tandem mass detector (Waters-Micromass, Manchester, UK). For the analysis samples were separated using a Symmetry C18 (150 × 3 mm, 5 μm) chromatographic column from Waters (Milford, PA, USA) working at 30°C and at a flow rate of 0.4 ml/min.

The mobile phase was water containing 0.065% heptafluorbutyric acid (A) mixed on a gradient mode with methanol containing 0.065% heptafluorbutyric acid (B), as follows: initiated at 100% A, from 100% to 55% A in 5 min, from 55% to 40% A in 11.5 min, kept isocratic at 60% B for 5 min, from 60% B to 0% B in 1 min for equilibration of the column (initial conditions). The mass spectrometer was operated in electrospray positive mode, and data acquisition was in multiple reactions monitoring mode (MRM). Source settings were as follows: capillary voltage 2.7 kV, cone voltage 25 V, source temperature 120°C, desolvation temperature 400°C, cone nitrogen gas flow 60 L/h, desolvation gas flow 600 L/h. Argon was used as the collision gas at 3.2×10^{-3} mbar. Calibration was done by means of a calibration curve (0, 2, 5, 10 and 20 mg/L) in 0.065% heptafluorbutyric acid. Quantification of kanamycin in the samples was done on the calibrators by means of isotope dilution using octamethylkanamycin. The bacterial culture was also plated on LB agar for growth assessment (CFU/ml) as described above.

Results

Gut bacteria of human and zoo animals displayed subsistence phenotype

Nine isolates from human and animal faecal samples displayed subsistence phenotypes when cultivated with a single antibiotic as the sole carbon source: six on kanamycin, two on streptomycin and one isolate displayed the subsistence phenotype on both erythromycin and kanamycin (**Table 1**).

The subsistence phenotype was measured by plating and counting CFU increases, with a two-fold increase of CFUs used to identify the phenotype. The isolates were classified by partial sequence analysis of 16S rRNA genes, and seven isolates were identified as *E. coli* and three as *Cellulosimicrobium* sp. The *Cellulosimicrobium* sp. are members of the family *Promicromonosporaceae* within the *Actinobacteria*, and were most closely related to *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei* (**Table 1**), which are all related to human pathogens (Funke *et al*., 1995; Kaper *et al*., 2004; Petkar *et al*., 2011). All nine isolates were resistant to two or more of the following antibiotics: ampicillin, chloramphenicol, tetracycline, erythromycin, streptomycin and kanamycin (**Table 1**).

Table 1. Human and zoo animal faecal isolates with subsistence phenotype on antibiotics

Isolate (%16S rRNA gene identity)	**Source (Latin name)**	**Resistant to***	**Subsisting on***	**Accession number**
Escherichia coli (100)	Human 1 *(Homo sapiens)*	AMP, TET, E, KAN, STR	STR	KT989026
Escherichia coli (100)	Human 2 *(Homo sapiens)*	AMP, TET, KAN, STR, CL	KAN	KT989027
Cellulosimicrobium sp. (99)	Chimpanzee *(Pan troglodytes)*	AMP, TET KAN, STR	KAN	KT989030
Cellulosimicrobium sp. (100)	Chimpanzee *(Pan troglodytes)*	AMP, TET KAN, STR	STR	KT989029
Escherichia coli (100)	Baringo giraffe *(Giraffe camelopardalis rothschildi)*	TET, KAN, STR	KAN	KT989033
Escherichia coli (100)	Asian elephant (*Elephas maximus*)	AMP, TET, E, KAN, STR	KAN	KT989034
Escherichia coli (100)	Malayan sun bear *(Ursus malayanus)*	KAN, STR	KAN	KT989035
Escherichia coli (100)	Sumatran tiger *(Panthera tigris sumatrae)*	AMP, TET, E, KAN, STR	KAN, E	KT989032
Cellulosimicrobium sp. (99)	Warthog *(Phacochoerus africanus)*	AMP, KAN, STR	KAN	KT989028

Agent abbreviation*: AMP, Ampicillin; TET, Tetracycline; E, Erythromycin; KAN, Kanamycin; STR, Streptomycin; and CL, Chloramphenicol.

Experimental controls to differentiate aminoglycoside resistance and subsistence phenotype

Since nine isolates displayed the subsistence phenotype on aminoglycosides, mainly kanamycin, we included an experimental control in an attempt to differentiate between antibiotic resistance and antibiotic subsistence. This was performed by equipping laboratory strains with a plasmid-encoded APH (3') II gene. All transformants of *E. coli* and *P. putida*, but none of the non-transformed strains, displayed the subsistence phenotype on kanamycin and neomycin (**Table 2**). Growth of the strains on glucose was similar to that in the presence of aminoglycosides, whereas no growth was observed in M9 medium to which no carbon source was added (**Table 2**).

Table 2. Growth experiments (48 h, performed in duplicate) of non-resistant and resistant *E. coli* and *P. putida* strains on media containing no carbon source, glucose or aminoglycosides (kanamycin, neomycin) in M9 minimal salts medium.

	M9	M9 + Glucose 1 mg/ml	M9 + Kanamycin 1 mg/ml	M9 + Neomycin 1 mg/ml
Escherichia coli				
DH5α	-	+	-	-
DH5α + pRSF-1b	-	+	+	+
DH5α + pCR-2.1 TOPO	-	+	+	+
TOP10	-	+	-	-
TOP10 + pRSF-1b	-	+	+	+
TOP10 + pCR-2.1 TOPO	-	+	+	+
Pseudomonas putida				
TEC1	-	+	-	-
TEC1 + pUTmini-Tn5-Km1	-	+	+	+

Grey highlighted boxes indicate strains showing subsisting phenotype on aminoglycosides. The + indicates growth, - indicates no growth.

Effect of deoxynojirimycin (DNJ) on the aminoglycoside subsistence phenotype

In order to evaluate the involvement of GH in the subsistence phenotype on aminoglycoside, we tested the capacity of *E. coli* (DH5α) transformed with pRSF-1b plasmid- encoded APH (3') II gene to grow on kanamycin or glucose as a single carbon source in the presence of DNJ (range of 0.00001-10 mM). Cultivability was measured by plating and counting CFUs during 24 h. We found that in the presence of DNJ and glucose, the bacteria showed initial growth retardation which was then rapidly overcome (**Fig. 1A**). In contrast, adding DNJ to a minimal medium containing only kanamycin as a carbon source arrested growth completely. This suggested that glycosyl-hydrolases are required for the subsistence phenotype on kanamycin (**Fig. 1B**).

Kanamycin degradation by *Escherichia coli*

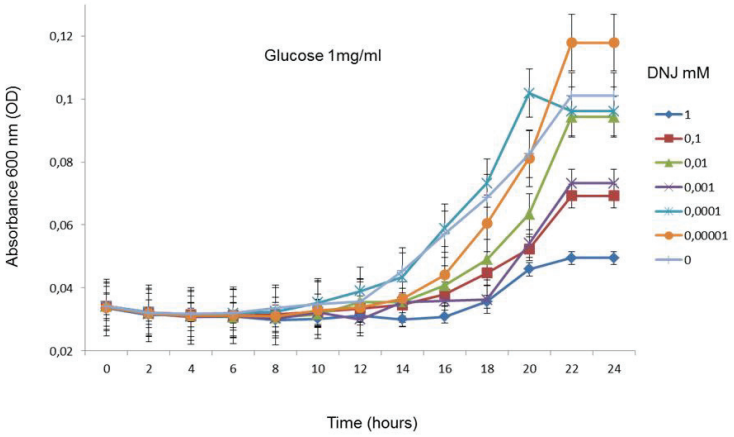
Finally, we studied kanamycin degradation by *E. coli* (DH5α) in the presence or absence of the plasmid encoded APH (3') II gene using 1 mg/ml of high purity kanamycin (Evopure, 99.25%) in M9 medium. Bacterial growth was calculated using the plate counting method, and kanamycin was measured by LC-MS/MS. It was observed that the number of CFUs increased during the first 8 h, although no degradation of the antibiotic was observed (**Table 3**).

Table 3. Concentration of kanamycin and colony forming units (CFUs) obtained in M9 minimal media with kanamycin (EvoPure™-1mg/ml) with and without resistant E. coli during LC-MS/MS experiments over time.

Samples	Kanamycin concentration (mg/L)				Colony forming units (CFU/ml)			
Time (Hours)	0	4	8	24	0	4	8	24
MM + KAN	978	1021	1066	1399	-	-	-	-
MM + *Ec* + KAN	923	977	1005	1306	6.6E+07	5.4E+07	5.4E+07	4.0E+07
MM + *Ec-p* + KAN	907	984	1008	1266	4.2E+07	6.8E+07	2.2E+08	1.1E+09

MM, minimal media; KAN, kanamycin; Ec, *Escherichia coli*; Ec-p, *Escherichia coli*-plasmid encoding aminoglycoside 3' phosphotransferase II gene.

A



B

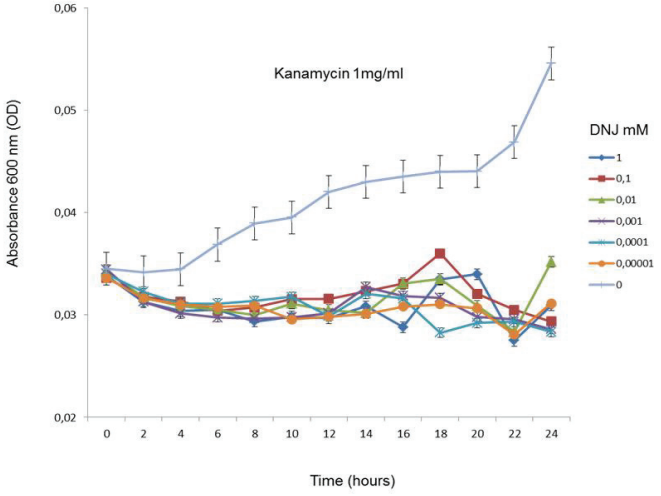


Figure 1. Growth dynamics (in triplicates) of transformed E. coli in M9 medium containing glucose (1 mg/ml) (**A**) and kanamycin (**B**) in the presence of different concentrations of DNJ (in mM).

Discussion

We observed that two groups of bacteria, *E. coli* and *Cellulosimicrobium* spp., present in the gut microbiota of healthy human volunteers and zoo animals, displayed the subsistence phenotype on aminoglycosides and erythromycin as a single carbon source. The subsistence phenotype was defined as an increase of CFUs over multiple transfers compared to the inoculum incubated in the same media without a carbon source. In order to avoid the presence of residual carbon sources, we included a pre-washing step to prevent carry-over of dissolved carbon from the faecal material and used new sterile glass material and freshly prepared media.In addition, we included serial two-fold dilutions of glucose and kanamycin (1 – 0.0625 mg/ml) and observed the subsistence phenotype at all antibiotic concentrations including those more similar to amounts found in natural habitats (Trieu-Cuot and Courvalin, 1986) (data not shown).

Subsistence phenotypes were found previously in *P. fluorescens* isolates obtained from lake sediments, which were described to utilize benzylpenicillin as a carbon, nitrogen and energy source (Johnsen, 1977). Soil bacteria from the orders *Pseudomonadales* and *Burkholderiales* have also been isolated based on their capacity to grow on a range of antibiotics as a single carbon source (Dantas *et al.,* 2008). In another environment including clinical and nonclinical samples, Barnhill *et al.* (2011) observed that multi-resistant *Salmonella* spp. were also able to subsist on antibiotics, highlighting the potential prevalence of the antibiotic subsistence phenotype in a clinical context. Xin *et al.* (2012) showed that two members of the Enterobacteria group (e.g. *Klebsiella pneumoniae* and *Escherichia fergusonii*) isolated from faecal material of healthy volunteers were able to subsist and bio-degraded chloramphenicol as a sole carbon source. However, all the strains in the study were chloramphenicol susceptible, which indicates that the subsistence and resistance mechanisms were independent in this particular case.

In our study, since the majority of the bacteria seemed to subsist on aminoglycosides, we studied laboratory strains of *E. coli* and *P. putida* with a plasmid-encoded APH (3') II gene in order to differentiate aminoglycoside resistance and the subsistence phenotype. Our results showed that a common resistance gene facilitates the subsistence phenotype on aminoglycosides, and these results indicated that resistance and subsistence mechanism might be linked. Similar subsistence phenotypes were obtained with *Pseudomonas putida* TEC1 using the cloning vector pUTmini-Tn5-Km1 (de Lorenzo *et al.,* 1990; Leprince *et al*., 2012), which similarly contains an APH (3') II gene.

Previous studies have shown that kanamycin is stable under culture conditions for at least a week (Ryan *et al.,* 1970). Stability has been attributed to its structure where a six-aminocyclitol ring is attached to aminosugar side chains through glycosidic bonds. We hypothesized that an intrinsic metabolic capacity to break down and utilize phosphorylated aminoglycosides is present in various bacteria.
In the genomes of *E. coli* and *P. putida* a multitude of genes predicted to encode glycosyl hydrolases (GH) exist (40 – 50 in *E. coli* and 26 in *P. putida*), with typically between 20-22 GH gene families annotated in *E. coli*. The encoded enzymes could potentially be involved in breaking the glycosidic bonds in the aminoglycosides, releasing an accessible carbon source. Due to the large number of GH encoding genes though single and combinatorial gene knockouts would not be numerically feasible. It is also likely that this approach may not deliver the necessary result due to potential functional redundancy of these enzymes. In our study we showed that a specific glycosyl-hydrolase inhibiting iminosugar (DNJ) abolishes the subsistence phenotype on aminoglycosides. This suggests that glycosyl-hydrolase activity could be necessary for the hydrolysis of the glycosidic bond and subsequent release of the aminosugars from the aminoglycoside, and hence indicates an involvement of GH in the antibiotic subsistence phenotype.

Since we found several indications of aminoglycoside subsistence phenotypes in line with previous observations, we applied the LC-MS/MS method to study kanamycin degradation. However, no degradation of kanamycin was observed in our study. Our findings thus align with the previous observations by Walsh *et al.* (2013) suggesting that due to the lack of antibiotic degradation, the subsistence phenotype cannot be linked to the use of the antibiotic as a sole carbon source.

So far, no genes have been identified in the catabolic pathways of kanamycin (http://www.ebi.ac.uk/chebi/chebiOntology.do?chebiId=CHEBI:6104). However, Stancu and Grifoll (2011), showed that several groups of Gram-positive and Gram-negative bacteria (including members of the *Enterobacteriaceae* family), displayed particular metabolic capabilities such as hydrocarbon degradation since these were able to grow on Poeni crude oil as a single carbon source. In addition, they show that Gram-negative bacteria possessed between two and four catabolic genes involved in degradation of saturated, monoaromatic and polyaromatic hydrocarbons. Interestingly, these groups of bacteria were resistant to hydrophilic antibiotics such as ampicillin and kanamycin, and cellular and molecular modifications were induced by the antibiotic.

Since subsistence phenotypes on a range of antibiotics are readily observed, it is possible that antibiotic resistance genes frequently allow not only resistance, but also simultaneously facilitate antibiotic subsistence. Dantas and Sommer (2012) investigated the connection between subsistomes and resistomes, and indicated that thus far not a single gene involved in antibiotic subsistence has been identified. Although active aminoglycoside efflux pumps have been observed in *E. coli* (Mingeot-Leclercq *et al.*, 1999), it is hypothesised that this mechanism is not actively involved in the *E. coli* clones subsisting on the antibiotics. This is because such activity would hinder accumulation of the drugs in the cytoplasm, where they are required for catabolism to occur.

Another potential subsistence mechanism that we considered was ribosomal protein mutations in spontaneous kanamycin resistant *E. coli* strains. It has been indicated that resistance to kanamycin and neomycin by ribosomal protein mutation is uncommon since this antibiotic binds to multiple sites on 30S and 50S ribosomal subunits, and high level resistance cannot be achieved by a single mutation (Kucers *et al.*, 1997). However, aminoglycoside modifying enzymes encoded by plasmids including the acetyltransferases, adenyltransferases and phosphotransferases encoded by plasmids (Neu, 1992) may inactivate antibiotics (i.e. by acetylation of amino groups, adenylation and phosphorylation of hydroxyl groups), before the subsequent action of the catabolic enzymes.

Based on our results we conclude that gut bacteria isolated were not able to degrade kanamycin and utilise it as a carbon source. Nevertheless, we observed that the presence of an aminoglycoside resistance gene supports the aminoglycoside subsistence phenotype, and GH seem to be required. This could indicate a possible link between the resistance and the subsistence phenotype. In addition, as we only tested one type of aminoglycoside modifying enzyme, we cannot assume that all the aminoglycoside modifying enzymes act in the same way. The different mechanisms of enzymatic modification could have different consequences. Further studies of kanamycin degradation linked to the evaluation of the subsistence phenotype and other aminoglycoside modifying enzymes may therefore provide further insight to the underlying subsistence mechanism.

Bacteria need to adapt to the growth medium in order to be able to metabolize the nutrients, and during the lag phase they are not completely inactive. They grow in size and develop primary metabolites (such as proteins, enzymes and RNA) as well as coenzymes and division factors required for making new cells. These factors together with the mechanisms involved in antibiotic resistance could also be hypothesised to facilitate the antibiotic subsistence phenotype.

It also may well be that bacteria simply need to be resistant to the antimicrobial in order to be able to exploit trace levels of non-toxic breakdown products. Future analyses including experimental evolution of antibiotic subsistence will help to further unravel the possible mechanisms involved in this phenotype. Nevertheless, since we were able to identify a bacterial strain that displayed the subsisting phenotype with both aminoglycoside (kanamycin) and macrolide (erythromycin) antibiotics, expansion of future studies to include resistance genes and metabolic pathways of macrolides as well as aminoglycosides could be of special interest.

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CHAPTER 3

Application of the Human Intestinal Tract Chip to the non-human primate gut microbiota

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Abstract

The human intestinal microbiota is responsible for various health-related functions, and its diversity can be readily mapped with the 16S ribosomal RNA targeting Human Intestinal Tract (HIT) Chip. Here we characterize distal gut samples from chimpanzees, gorillas and marmosets, and compare them with human gut samples. Our results indicated applicability of the HITChip platform can be extended to chimpanzee and gorilla faecal samples for analysis of microbiota composition and enterotypes, but not to the evolutionary more distant marmosets.

Keywords: Microbiota, phylogenetic profile, enterotypes, non-human primates.

Introduction

The human body is colonized by vast numbers of different microbes, most of which are found in the gastro-intestinal (GI) tract. These microbes have been referred to as the intestinal microbiota and are proposed to constitute a virtual organ with a range of beneficial functions (Backhed*, et al.*, 2005, Gill*, et al.*, 2006, Murphy*, et al.*, 2010). For example, intestinal microbiota can play a role in health by interacting with the host at the GI mucosa, modulating the host immune response (Ashida*, et al.*, 2012). The extensive study of the human microbiota composition has further resulted in the possible distinction of a limited number of well-balanced host-microbial symbiotic states, the so-called enterotypes (Arumugam*, et al.*, 2011, Koren*, et al.*, 2013).

Different techniques have been developed to analyse the composition and dynamics of the intestinal microbiota. Most recent technical advances to study microbiota composition include the implementation of next generation technology (NGT) sequencing as well as ribosomal RNA targeted microarrays for the high throughput and comprehensive profiling of intestinal microbiota. Our laboratory has implemented the Human Intestinal Tract (HIT) Chip, (Rajilic-Stojanovic*, et al.*, 2009). The HITChip is a well-validated phylogenetic array produced by Agilent Technologies (Palo Alto, CA) for human GI tract samples, with over 4,800 tiling oligonucleotides targeting the V1 or the V6 region of the 16S rRNA gene from 1,132 microbial phylotypes present in the human GI tract (Rajilic-Stojanovic*, et al.*, 2011, van den Bogert*, et al.*, 2011). The HITChip provides highly reproducible (median Pearson's correlation of 0.99), broad and deep analysis of the intestinal microbiota, comparable to new generation technology sequencing (Claesson*, et al.*, 2009). It can be used to assign enterotypes and also to distinguish low and high gene count subjects (Arumugam*, et al.*, 2011, Le Chatelier*, et al.*, 2013). In addition, we have implemented other microarrays including the Mouse Intestinal Tract (MIT) Chip and Pig Intestinal Tract (PIT) Chip to analyse the composition of the microbiota in frequently used animal models (Geurts*, et al.*, 2011, Haenen*, et al.*, 2013).

The interest to determine the composition of the intestinal microbiota of different animal species lead us to the question about the applicability of HIT Chip to determine the composition of evolutionarily related non-human primates. In fact, intestinal microbiota composition is linked to evolutionary relatedness of the intestine. As an example, the colonic microbiota from different animals is more similar than the small intestinal and large intestinal microbiota of the same animal (Muegge*, et al.*, 2011). Findings also suggest that for chimpanzees and humans intestinal bacteria patterns evolved before their split into evolutionarily separate ways (Degnan*, et al.*, 2012). Furthermore, for wild great apes the composition of intestinal microbial communities resembles the phylogenies of their host and contains species-specific signatures (Ochman*, et al.*, 2010).

The intestinal microbiota profiles in non-human primates give insight into co-evolution of microbiota with phylogenetic closely related hosts and how gut types, environments and food habits are associated with divergence. The possibility to use the HITChip for non-human samples, as is presented herein, provides a cost-efficient and fast alternative to screen GI tract microbiota composition of chimpanzees and gorillas as compared with NGT sequencing based techniques, especially when performed at comparable depth of around 200.000 sequencing reads per sample (Clasesson, *et al.*, 2009, van den Bogert, *et al.*, 2011, Hermes, *et al.*, 2014). This could lead to advances in microbiota-related research questions in primatology, in relation to evolution, health, disease, diets and environmental factors. Most importantly, The HITChip provides robust data that allow for relative quantification at different taxonomic levels with extremely high reproducibility, and allows for analysis of single samples with short processing times, as opposed to large pools of barcoded PCR products as normally employed for NGT-based approaches (Hermes, *et al.*, 2014).

Materials and methods

A total of 12 human faecal samples were obtained from healthy volunteers. The non-human primates samples were collected from three chimpanzees (*Pan troglodytes*), two Western gorillas (*Gorilla gorilla*), which are kept at Burgers' Zoo (Arnhem, The Netherlands) and five marmosets (New world monkeys - *Callithrix jacchus*), kept at animal facilities of Erasmus University (Rotterdam, The Netherlands).

DNA isolation was done using a modified repeated beating method (Salonen*, et al.*, 2010). Amplification for 16S rRNA gene, *in vitro* transcription and labelling, and hybridizations were carried out as described previously (Rajilic-Stojanovic*, et al.*, 2009). Data analysis was performed using a microbiome R-script package (https://github.com/microbiome) in combination with a custom designed database as previously described (Jalanka-Tuovinen*, et al.*, 2011, Lahti*, et al.*, 2011). The reproducibility of obtained hybridization signals was determined by calculating the Pearson's linear correlation of the logarithm of spatially normalized signals of two independent hybridizations. Multivariate statistics using a Principal Component Analysis (PCA) to analyse the positive and negative correlations between the bacterial populations and the different species of non-human primates compared with faecal samples of healthy humans were performed using CANOCO 5.0 (Ter Braak, *et al*; 2012). Enterotypes were determined based on HITChip profiles of non-human primate samples and the HITChip data for all MetaHIT samples (n=124) classified by Arumugam *et al* (2011). The MetaHIT samples were used as a training set, for which the optimal number of clusters k was three as based on the Calinski-Harabasz (CH) index to determine the optimal number of clusters in each data set, and the silhouette score was calculated for each data set of clusters generated by partition around medoids (PAM) clustering (Arumugam*, et al.*, 2011). The QIIME pipeline (http://quime.org/) was used to compare our data obtained by HITChip analysis with high-throughput amplicon sequencing data from another study (454 sequencing) (J.Ritali, University of Helsinki, personal communication).

Results and Discussion

HITChip profiling of chimpanzee and gorilla intestinal microbiota composition

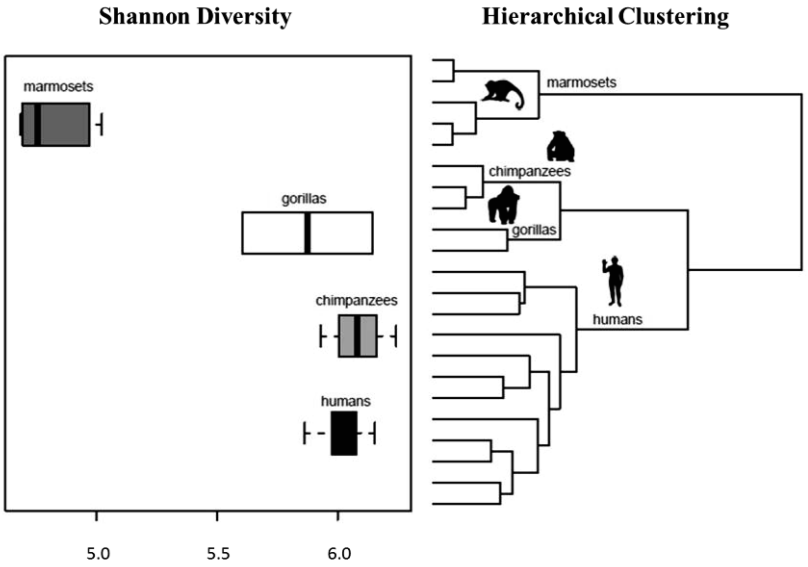
The applicability of the HITChip for chimpanzees, gorillas and marmosets was evaluated, and profiles were compared with those obtained from faecal samples of healthy human individuals. Hybridization to phylotype-specific probes and high reproducibility was obtained for chimpanzee and gorilla samples, as calculated by the Pearson's linear correlation of the logarithm of spatially normalized signals of two independent hybridizations (values of 0.98-0.99). In contrast, when comparing overall signal intensity as a percentage of that observed for human controls, and taking into account error propagation based on average and standard deviation, the marmoset samples had a lower overall signal intensity (41.4 +/- 14) as compared to samples from chimpanzees (85.3 +/- 21) and gorillas (59.0 +/- 22), indicating that only a small fraction of RNA had been hybridized. Based on these results we can speculate that the intestinal microbiota of chimpanzees and gorillas, but not that of marmoset, are sufficiently related to that of humans to warrant meaningful application of the HITChip.

Clustering of faecal microbiota composition byhost phylogeny

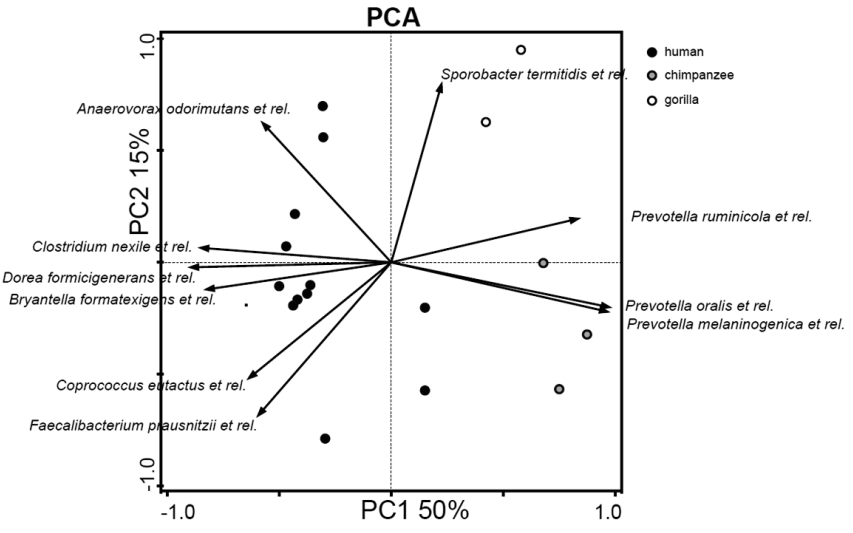
The faecal microbiota profiles from the different host species clustered separately using Hierarchical Cluster Analysis (**Fig. 1A**). The results also indicated that per host species, individuals have high similarity scores as calculated by Pearson's correlation, which reflects the influence of host on the microbiota composition (humans 0.80 +/- 0.03, chimpanzees 0.92 +/- 0.01, gorillas 0.85 and, marmosets 0.89 +/-0.06). In addition, correlations between chimpanzee and humans samples (0.80 +/- 0.04) and between gorilla and chimpanzee (0.88+/- 0.04) were higher as compared to what was observed for the respective correlations between humans and gorilla (0.69 +/-0.04).

The microbial diversity scores calculated by the Shannon Index indicated that the diversity in chimpanzee samples is in the range of that of healthy human faecal profiles. The gorilla samples had a high variability in the diversity index; and very low diversity scores were observed for the more distantly related marmosets (**Fig. 1A**), which is in line with the low hybridization signals.

To analyse the positive and negative correlations between members of the bacterial communities and the different host species; we performed multivariate statistics using PCA. The results indicated that humans, chimpanzees and gorillas have distinct faecal microbial community signatures clustering in different quadrants of the plot. More specifically, profiles of humans and apes were largely separated along PC1, which explains 50% of the variation of the compositional data, while the distinction between chimpanzees and gorillas could in part be explained by PC2 that accounts for 15% of the total variation (**Fig. 1B**).



A



B

Figure 1. Microbiota comparison of humans, chimpanzees, gorillas and marmosets

(A) Diversity index (Shannon), average within host similarity and Pearson clustering of samples that were analysed using the HITChip. (B) PCA analyses of human, chimpanzee and gorilla samples. Percentages indicate the variation in microbial profiles explained by principle components PC1 and PC2. Arrows and bacterial names indicate association of bacterial groups with the principle components.

HITChip profiles of microbial communities in humans, chimpanzees and gorillas

In order to examine the differences in faecal bacterial community composition between chimpanzees, gorillas and healthy humans in more detail, we compared the relative abundance of microbial taxa in the different samples at phylum and at genus level. The phylum-level composition in intestinal microbial communities indicated that for all hosts the two most prevalent phyla were *Firmicutes* and *Bacteroidetes,* which contributed up to 90% of bacterial abundance in all samples. The percentage of *Firmicutes* and *Bacteroidetes* varied in each species; 84.2% in humans, 33.3% in chimpanzees and 66.0% in gorillas for *Firmicutes* and 8.2% (humans), 62.2% (chimpanzees) and 21.3% (gorillas) for *Bacteroidetes*. For chimpanzees similar results were obtained previously, when the distal intestinal microbiota was studied of individual chimpanzees from two communities in Gombe National Park in Tanzania (Degnan*, et al.*, 2012). Furthermore, gorilla samples had a high prevalence of *Proteobacteria* (7.0%) than in chimpanzee (1.2%) and human samples (1.0%), whereas *Actinobacteria* were more prevalent in humans samples (2.7%) as compared to chimpanzees (0.3%) and gorillas (1.0%) derived samples.

Significant differences of bacterial populations were observed between the different hosts when examining the data at higher taxonomic resolution, i.e. at genus level. A remarkable variation was found with respect to the relative abundance of *Faecalibacterium prausnitzii et rel*., which were especially high (16.0 ± 8.7%) in humans versus chimpanzee (5.0 ± 1.5%) and gorilla (1.3 ± 0.8) samples. In addition, *Bacteroides* were more abundant in humans than in chimpanzees and gorillas derived samples. A possible explanation for this could be the high protein fat Western diet habits of humans that can enrich for proteolytic *Bacteroides* spp. (David*, et al.*, 2014; Thomas*, et al.*, 2011). Another notable difference–that was observed concerned high relative abundance of *Prevotella melaninogenica et rel*. in the tested chimpanzees samples (52.5±9.8%) as compared to human (2.8±5.7%) and gorilla (14.7±1.9%) samples.

This could also be the effect of diet as increased abundance of *Prevotella* has been associated with a vegetarian diet and long-term consumption of high fibre diet (David*, et al.* 2014). With respect to gorilla samples, we observed high relative abundance of *Sporobacter termitidis et rel.* (8.8 ± 4.5%) as compared to chimpanzee (4.4 ±3.3%) and human (4.0 ± 2.8%) samples.

Finally, we screened for possible enterotypes in all samples, as a previous report indicated that they are not exclusive to humans but also occur in chimpanzees (Moeller*, et al.*, 2012). Among human samples, enterotypes 1 and 3 were found. Interestingly, the chimpanzee samples all fell within the *Prevotella* dominated enterotype 2, whereas the gorilla samples all fell within the *Ruminococcus* dominated enterotype 3. The enterotypes observed for the chimpanzees in this study are similar to those described previously by Ochman *et al.*, 2010, even that they are not similar to those observed within our human set of samples, this support that indeed particular enterotypes can changes over the time.

Comparable microbiota signatures using HITChip analyses 454 sequencing

In order to compare our HIT Chip data analysis with microbiota profiles obtained by 454 pyrosequencing from the same animal species, albeit from different individuals, we analysed our chimpanzee and gorilla microbiota profiles together with samples obtained by Muegge and co-workers (2011) using the Qiime pipeline. This comparison indicated that the bacterial composition of the chimpanzees and gorillas from our study and that of Muegge *et al.* (2011) have high similarity at family level. Because of these two techniques have a different level of resolution and in some cases, some Operational Taxonomic Units (OTUs) cannot be assigned to specific genera, we used the higher level of taxonomic resolution to compare our results.

More specifically, Pearson correlations between family-level relative abundances in chimpanzees and gorillas reported by Muegge *et al.* (2011) and those observed by us amounted to 0.98 and 0.97, respectively, showing strong correlation of microbiota composition.

This high similarity reinforces the notion that the HITChip is a viable alternative for the currently used high throughput sequencing techniques to screen microbiota composition and allows forsimultaneous comparison of the relative abundance of specific groups of intestinal bacteria at different levels of taxonomic resolution.

Conclusions

Diverse factors including geography, diet, disease state, sex and host physiology can affect the composition of the intestinal microbiota. Degnan *et al.* (2012) found that the geographical distribution, sex and age are associated with the long term composition and diversity of the intestinal microbiota in chimpanzees from Gombe National Park (Tanzania), but that their microbiota remains distinct from those of other great apes including other subspecies of chimpanzees (Degnan *et al.*, 2010). This is in line with previous studies indicating that the host genetic background is a selective pressure that favours inter-individual and inter-specific divergence of intestinal microbiota composition (Ley*, et al.*, 2008, Ochman*, et al.*, 2010).

Based on the data reported here, we conclude that apart from human GI tract samples, the HITChip can be used for microbiota profiling of chimpanzee and gorilla faecal samples. Even though there are microbiota differences between the animal species, the 16S rRNA targeted probes used on the HITChip array hybridize the majority of 16S rRNA genes of chimpanzee and gorilla microbiota. Profiling the distinct faecal microbial communities of the different animals using the HITChip provides a simple and robust alternative for high throughput sequencing. We believe this contributes to advances in microbiota related research questions in primatology, in relation to evolution, health and disease, diets and environmental factors.

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CHAPTER 4

Effects of selective digestive decontamination (SDD) on the gut resistome

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Abstract

Objectives. Selective digestive decontamination (SDD) is an infection prevention measure for critically ill patients in intensive care units (ICUs) that aims to eradicate opportunistic pathogens from the oropharynx and intestines, while sparing the anaerobic flora, by the application of non-absorbable antibiotics. Selection for antibiotic resistant bacteria is still a major concern for SDD. We therefore studied the impact of SDD on the reservoir of antibiotic resistance genes (i.e. the resistome) by culture-independent approaches.

Methods. We have evaluated the impact of SDD on the gut microbiota and resistome in a single ICU-patient during and after ICU-stay by several metagenomic approaches. We also determined by quantitative PCR the relative abundance of two common aminoglycoside resistance genes in longitudinally collected samples of 12 additional ICU patients who received SDD.

Results. The patient microbiota was highly dynamic during hospital stay. The abundance of antibiotic resistance genes more than doubled during SDD use, mainly due to a 6.7-fold increase of aminoglycoside resistance genes, in particular *aph(2")-Ib* and an *aadE*-like gene. We show that *aph(2")-Ib* is harboured by anaerobic gut commensals and is associated with mobile genetic elements. In longitudinal samples of 12 ICU-patients, the dynamics of these two genes ranged from a ~10^{4} fold increase to a ~10^{-10} fold decrease in relative abundance during SDD.

Conclusions. ICU hospitalization and the simultaneous application of SDD has large, but highly individualized, effect on the gut resistome of ICU patients. Selection for transferable antibiotic resistance genes in anaerobic commensal bacteria could impact the risk of transfer of antibiotic resistance genes to opportunistic pathogens.

Keywords: Intensive care medicine, antibiotic resistance, metagenomics

Introduction

Infections are a major threat to hospitalised patients, especially to those treated in Intensive Care Units (ICUs), where infections are associated with increased morbidity, mortality and health care costs (Cosgrove, 2006; Vincet *et al.*, 2009). Selective decontamination of the digestive tract (SDD) is an infection prevention measure that reduces ICU-acquired respiratory tract infections and bacteraemia and improves survival of ICU patients (De Jonge *et al.*, 2003; de Smet *et al.*, 2009), through eradication of potential pathogenic microbes in the oropharynx and the digestive tract, while leaving the anaerobic microbiota undisturbed (van der Waaij *et al.*, 1990). SDD involves the administration of non-absorbable antibiotics (colistin and tobramycin) and an antifungal (amphotericin B) in the oropharynx and intestinal tract during the ICU stay, in combination with intravenous administration of a third-generation cephalosporin (usually cefotaxime) during the first 4 days in ICU. Despite the reported benefits of SDD, this intervention is currently not widely used, primarily because of concerns that it may select for antibiotic resistant bacteria in the patient's microbiota (van der Meer *et al.,* 2013).

However, a recent meta-analysis of 64 clinical studies failed to demonstrate that SDD increased the number of infections caused by antibiotic resistant pathogens (Daneman *et al.*, 2013). An important limitation of all studies included in this meta-analysis is that they relied on conventional culture techniques, which are unable to capture anaerobic commensals, such as Clostridia and *Bacteroidetes*. Anaerobic bacteria constitute the majority of the gut microbiota and can carry a large reservoir of antibiotic resistance genes, i.e. the resistome (Sommer *et al.*, 2009; Qin *et al.*, 2010). Antibiotics may select for antibiotic resistance genes carried by gut commensal bacteria and thereby facilitate horizontal gene transfer to opportunistic pathogens (Shoemaker *et al.,* 2001). Consequently, to fully evaluate the safety of SDD in ICU-settings, its effect on the patient gut resistome needs to be assessed.

Here, we describe the dynamics of the gut microbiota and the resistome in detail in a single patient admitted to the ICU after a traffic accident and who received SDD for 17 days. Samples were taken at days 4, 14 and 16 in ICU, at day 28 (during post-ICU hospitalisation) and at day 313 (270 days after hospital discharge). We subsequently studied the dynamics of two aminoglycoside resistance genes in the gut microbiota of 12 ICU-patients who received SDD. Our data indicate that SDD can have large, but highly individualized effects on the patient resistome.

Materials and methods

Patient information

The patient who was the main subject of this study had no previous history of hospitalisation and disease. Upon ICU admission, the patient presented with an acute neurological trauma due to a basal skull fracture after a traffic accident. Additional screening for trauma showed no abnormalities. Microbiological surveillance of the patient was performed according to conventional culturing techniques on an almost daily basis. Rectal cultures were grown on blood agar, MacConkey agar and malt agar. Sputum cultures were grown on blood agar containing optochin, MacConkey agar, malt agar, and *Haemophilus* chocolate agar. Blood samples were monitored in a BD BACTEC FX machine according to standard laboratory practice. Culture-based diagnostics failed to detect any pathogenic, antibiotic resistant bacteria at any time point in any sample (**Table S1**). The patient received SDD, with 1000 mg of cefotaxime intravenously four times daily for 4 days and an oropharyngeal paste containing polymyxin E, tobramycin and amphotericin B (each at a concentration of 2%) and administration of a 10 ml suspension containing 100 mg polymyxin E, 80 mg tobramycin and 500 mg amphotericin B via a nasogastric tube, four to eight times daily throughout ICU stay. Additional information on the antibiotic therapy that the patient received throughout the study period is provided in **Table S2**.

Strains and growth conditions

Escherichia coli EP1300-T1^{R} (Epicentre, Madison, WI, USA) was used for fosmid library construction (further described below) and *E. coli* TOP10 (Invitrogen, Life Technologies Europe BV, Netherlands) for other genetic manipulations. *E. coli* was grown in Luria Broth (LB; Oxoid) at 37°C. Antibiotics were used at the following concentrations: chloramphenicol 12.5 mg/L, ampicillin 100 mg/L, tobramycin 25

mg/L, tetracycline 10 mg/L, erythromycin 500 mg/L, colistin 50 and 10 mg/L, cefotaxime 25 mg/L and cefazolin 32 mg/L.

Faeces collection and isolation of high molecular weight DNA

Faeces from the patient described above were collected upon defecation and stored at 4°C between 30 min and 4h, after which the faeces were transferred to -80°C. For DNA isolation, an aliquot of approximately 15 g of faecal matter was defrosted and homogenised in PBS (138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl) by vigorous vortexing and layered on a Nycodenz AG gradient (Axis-Shield PoC, Oslo, Norway). The cellular fraction of the faecal matter was then separated via centrifugation at 16,000 *g* for 6 min. After removal of the upper layer, the bacterial cellular fraction was recovered and washed three times in PBS, as described previously (Jones *et al*., 2007). High molecular weight DNA was extracted from the cell pellet as described previously (Ogilvie *et al.,* 2013), with minor modifications. Briefly, the recovered cells were lysed with lysozyme (10 mg/mL; Sigma Aldrich, St Louis, MO, USA) and mutanolysin (100 U/mL) (Sigma Aldrich), followed by proteinase K (50 mg/mL; Sigma Aldrich) digestion and addition of 2.5% n-lauryl sarcosine (Sarkosyl; Sigma Aldrich). Proteins were precipitated with 10 M ammonium acetate and DNA extracted with chloroform by using phase-lock tubes (5 Prime, Gaithersburg, MD, USA) and ethanol precipitation. The quantity and purity of DNA was measured using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA).

Phylogenetic profiling of the gut microbiota

The faecal DNA isolated above was used to phylogenetically profile the gut microbiota using HITChip analysis, as described previously (Rajilic-Stojanovic *et al.,* 2009).

Metagenomic shotgun sequencing and sequence analysis

DNA library construction and sequencing was performed by BGI (Shenzhen, China) using 91-nt paired end sequencing on an Illumina HiSeq 2000 system as described elsewhere (Qin *et al.*, 2012). Between 125 and 221 million high-quality reads were generated for the five samples. Using SOAPdenovo (http://soap.genomics.org.cn), sequence data was assembled into contigs larger than 500 nt for which between 78.6 and 89.0% of the reads could be used in the assemblies. Further details on the results of the metagenomic shotgun sequencing and *de novo* assembly are provided in **Table S3**. We used BLAST to detect the presence of antibiotic resistance genes in the different assemblies of each sample (Altschul *et al.*, 1997). We initially extracted a set of antibiotic resistance gene sequences from the Resistance Determinants DataBase (RED-DB) (http://www.fibim.unisi.it/REDDB). To reduce redundancy in this database, we first clustered the nucleotide sequences using CD-HIT with a threshold of 99% identity (Fu *et al.,* 2012). The clustered resistance gene database was used as a query in a local BLAST search on each assembled sample. All hits with a nucleotide identity of 90% or higher and covering > 50% of the query length were considered to be resistance genes that were encoded on the assembled contigs.

Relative quantification of the resistance genes per sample was performed as follow. First, the average sequencing depth over the entire assembly was calculated, and then the coverage of the individual contigs, determined using soap.coverage (http://soap.genomics.org.cn), encoding resistance genes was divided by the average sequencing depth over the entire assembly. Data were then log-2 transformed and plotted onto a heatmap using Multi Experiment Viewer (http://www.tm4.org/mev/). Non-transformed abundance data for each resistance gene are provided in **Table S4**.

Construction of fosmid libraries

The construction of fosmid libraries was performed using the CopyControl Fosmid Library Production Kit (Epicentre) according to the manufacturer's instructions with slight modifications. Size selection of approximately 40 kb DNA fragments was performed using PFGE using the CHEF-DR II system (Bio-Rad) with the following settings: initial switch time 0.1 s, final switch time 10 s, 4 V/cm, and running time 17 h. The DNA was excised from the gel at the height of a 40 kb marker (Fosmid Control DNA, Epicentre), recovered using GELase (Epicentre) and end-repaired using the End-It kit (Epicentre). DNA was then purified using SureClean (Bioline, London, UK), and used for ligation. Packaged phage extracts were diluted in 0.5 mL phage dilution buffer and added to 5 mL of phage-resistant EP1300-T1 *E. coli* for 1 h at 37°C. Serial dilutions of the transduced *E. coli* were plated on LB-agar plates containing chloramphenicol. Libraries were harvested by scraping the plates and suspending the colonies in LB containing 20% glycerol and chloramphenicol, and frozen in liquid nitrogen and stored at -80°C.

Identification and characterization of antibiotic-resistant clones in fosmid libraries

The fosmid libraries were plated in 10-, 100- and 1000-fold dilutions on LB agar with chloramphenicol supplemented with tobramycin, ampicillin, tetracycline, erythromycin, cefotaxime, colistin and cefazolin and incubated at 37°C overnight. A vector-only control (*E. coli* EP1300-T1^R with the fosmid library vector pCC1FOS) was also included and only produced colonies on plates supplemented with chloramphenicol but not on plates containing chloramphenicol and the other antibiotics. Quantification of resistant clones was performed in duplicate by plating serial dilutions of the libraries on LB agar supplemented with chloramphenicol in addition to the antibiotic of interest. The total number of clones in the library was determined by plating on LB with chloramphenicol only.

To ensure that resistant clones were due to the fosmid insert and not because of spontaneously occurring mutations, five clones per library and per antibiotic were randomly selected from plates and were restreaked on LB plates containing the appropriate antibiotics. After overnight growth of the restreaked clones, clones were picked and subsequently cultured in LB broth containing the appropriate antibiotics for fosmid isolation. Fosmids were induced to high-copy by the CopyControl Fosmid Autoinduction solution from Epicentre prior to fosmid isolation to increase total DNA yield. Fosmids were purified using the Qiagen (Venlo, The Netherlands) Mini-prep kit. The elution buffer was heated to 70°C prior to elution of the fosmids from the column. The isolated fosmids were then used to transform chemically competent EP1300-T1^R *E. coli* by heat shock. The transformed clones were restreaked on LB agar with chloramphenicol in addition to the antibiotics used for the initial resistance screening. A clone of EP1300-T1^R *E. coli* freshly transformed with the fosmid vector pCC1FOS was used as a control throughout. All phenotypes for the selected clones were reconfirmed while *E. coli* with pCC1FOS remained unable to grow.

To assess fosmid insert diversity, fosmids of the selected clones were digested with MslI (New England Biolabs, Ipswich MA, USA). Differences in restriction patterns were used as indicators for the diversity among the isolated clones. The most prominent clones were subsequently selected for transposon mutagenesis to functionally identify the resistance determinants.

In order to identify the resistance genes on the fosmids that were responsible for causing the resistant phenotype in *E. coli*, transposon mutagenesis was performed using the EZ-Tn5 <KAN-2> and EZ-Tn5 <TET-1> *in vitro* transposon mutagenesis kits (Epicentre). Transposon mutagenesis was carried out according to the manufacturer's instructions with the exception that 5 mM $MgCl_2$ was added to LB agar when using the EZ-Tn5 <TET-1> kit. After *in vitro* transposon mutagenesis, between 100 and 300 transposon mutants were streaked on LB agar with chloramphenicol and LB agar with chloramphenicol and ampicillin, tobramycin, or tetracycline to screen for the loss of the resistant phenotype.

For each *in vitro* transposon mutagenesis, between one and five clones could be identified that lost their resistance phenotype due to transposon insertion. Sequencing primers TET-1 FP-1 Forward Primer, TET-1 RP-1 Reverse Primer, KAN-2 FP-1 Forward Primer and KAN-2 RP-1 Reverse Primer (provided by Epicentre) were used to sequence along the transposon insertion sites and thereby identify the resistance genes. Partial sequences from the inserts cloned into the different fosmid and transposon insertion sites were obtained by standard Sanger sequencing. To identify the resistance genes based on the transposon insertion site sequences, we used the RED-DB (http://www.fibim.unisi.it/REDDB/). For each resistant phenotype, several clones that had lost their resistant phenotype upon transposon insertions were analysed. After analysis of the transposon insertion site sequences, we identified the same resistance determinant per antibiotic resistant clone (tobramycin, ampicillin and erythromycin) and therefore only chose one fosmid clone per antibiotic to be fully sequenced subsequently. We identified two different tetracycline resistance determinants, and two representative fosmids were selected for sequencing.

Fosmids were pooled and sequenced via Illumina sequencing on a HiSeq 2000 system using the Illumina CASAVA pipeline version 1.8.2 generating paired end reads (read length 101bp) with an average insert size between 265bp and 384bp. Assembly was performed using the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). The DNA sequences of the regions where the faecal DNA was cloned into the fosmid backbone and of the resistance gene previously obtained by Sanger sequencing were also used to assemble the fosmid insert.

Finally, fosmid insert sequences (ISs) were closed by sequencing of PCR products that spanned the gaps between the contigs in the assembly of each IS. Taxonomic classification and identification of putative source organism of fosmid ISs was performed using WebCARMA (Gerlach *et al.*, 2011). Annotation of the fosmid ISs was generated using the prokaryotic annotation pipeline offered by Integrative Services for Genome Analysis (Hemmerich *et al.*, 2010).

Annotations were visualised using the Geneious software package (http://www.geneious.com/). The ACLAME server was used to identify and classify putative mobile genetic elements within the fosmid sequences (Leplae *et al.,* 2010). IS elements were identified by IS Finder (Siguier *et al.,* 2006).

Quantification of *aph(2")-Ib* and the *aadE*-like gene in ICU patient microbiota by quantitative PCR (qPCR)

To further determine the effect of the ICU hospitalization and SDD on the relative abundance of *aph(2")-Ib* and the *aadE*-like aminoglycoside resistance gene in the gut microbiota of patients, faecal samples were collected from 12 patients that were hospitalized in the ICU for 9 days or longer. Two or three faecal samples per patient were collected during their ICU stay. DNA was isolated from 200 mg stool samples using the repeated mechanical bead beading method combined with the QIAmp DNA stool Minikit (QIAgen) as described elsewhere (Salonen *et al*., 2010). The DNA samples were used in qPCRs to quantify the copy number of *aph(2")-Ib* and the *aadE*-like gene. All qPCRs were carried out in MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems), sealed with optical adhesive film (Applied Biosystem), and using a StepOnePlus™ Real-Time PCR cycler (Applied Biosystems) with StepOnePlus software version 2.2 (Applied Biosystems). Total reaction volume was 25 µl using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer's instructions with a primer concentration of 200 nM and 1ng DNA. Primers were designed for the targeted resistance genes *aph(2")-Ib* (forward primer: 5'-GAAAAGGATGCCCTTGCATA-3'; reverse primer: 5'-TCACCAGAGCATCTGAAAACA-3') and the *aadE*-like gene (forward primer: 5'-GCATGATTTCCTGGCTGATT-3'; reverse primer: 5'-CCACAATTCCTCTGGGACAT-3') using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/).

The universal primers for 16S rRNA genes were previously described by Gloor *et al.* (2010) and PCR conditions were previously described by van den Bogert *et al.* (2011).

Melting curves were included for each qPCR run. Relative abundance of the resistance genes was calculated using the $2^{-\Delta\Delta CT}$-method with 16S rRNA as the universal housekeeping gene (Livak *et al.,* 2001). The relative abundance of the resistance genes in the first faecal sample that was collected during ICU hospitalisation was normalised to 1, and subsequent samples were compared to this first sample. The qPCRs were performed with three technical replicates.

Statement of ethical approval

The protocol for this study was reviewed and approved by the institutional review board of the University Medical Center Utrecht (Utrecht, The Netherlands) under number 10/0225. Informed consent for faecal sampling during hospitalization was waived. Written consent was obtained for the collection of faecal samples after hospitalization.

Data availability

Metagenomic shotgun sequence reads are deposited in the Sequence Read Archive (European Nucleotide Archive) with the primary accession number PRJEB3977. Assemblies can be accessed through MG-RAST with accession numbers 4508944.3, 4508945.3, 4508946.3, 4508947.3 and 4508948.3. Fosmid sequences are deposited at Genbank under accession numbers KF176928 - KF176932.

Results

We first monitored the dynamics of the resistome and the gut microbiota composition in a previously healthy patient that was hospitalised in our hospital's ICU after a traffic accident. The patient had no history of hospitalization or antibiotic use. The patient received SDD from the first day in ICU for 17 days, after which the patient was transferred to the neurology department, where he remained hospitalised until hospital discharge, 43 days after admittance. Faecal samples were collected at four time points during hospital stay (day four, 14 and 16 in ICU and day 28 in the neurology ward) and at day 313 (270 days after hospital discharge) (**Fig. 1a**). Diagnostic cultures were performed throughout the patient's stay in hospital and did not yield growth of antibiotic resistant bacteria (**Table S1**). The antibiotics administered to the patient during hospital stay are shown in **Fig. 1a** (further details are provided in **Table S2**). No antibiotics were prescribed following hospital discharge. Culture-independent techniques were used to profile the diversity of the gut microbiota and its resistome at the five time points at which faeces were collected during and after hospitalization.

Phylogenetic profiling of patient gut microbiota

16S rRNA gene-based profiling of the gut microbiota revealed that, during hospitalization, the most prevalent groups were *Bacteroidetes* (from 29 to 67% of the total microbiota) and *Clostridium* clusters XIVa and IV (from 21 to 69% of the total microbiota), which are all common inhabitants of the intestinal microbiota of healthy humans (**Fig. 1b**; the full data set is provided as **Tables S5** and **S6**) (Rajilic-Stojanovic *et al.*, 2007 and 2009). The relative abundance of these three groups fluctuated considerably during hospitalization. Unusually, at day 28 (11 days after ICU discharge and discontinuation of SDD), Bacilli represented 10% of the microbiota, which was mainly caused by an increased abundance of enterococci (5.1% of the microbiota).

Enterococci are usually quantitatively minor species in the healthy gut microbiota but can become more prominent during hospitalization (Qin *et al.*, 2010; Ubeda *et al.*, 2010). At other points in time, Bacilli were less abundant (≤1%). The composition of the patient's microbiota had markedly changed at day 313 (270 days after hospital discharge). At this time point, the gut microbiota consisted almost exclusively of bacteria belonging to the phylum *Firmicutes*, and was dominated by *Clostridium* cluster XIVa (87.5% of the total microbiota). *Bacteroidetes* were present at only 0.67% (**Fig. 1b**). As this patient had not received antibiotics during 270 days after hospital discharge, this may well reflect the normal, undisturbed state of this particular individual's microbiota.

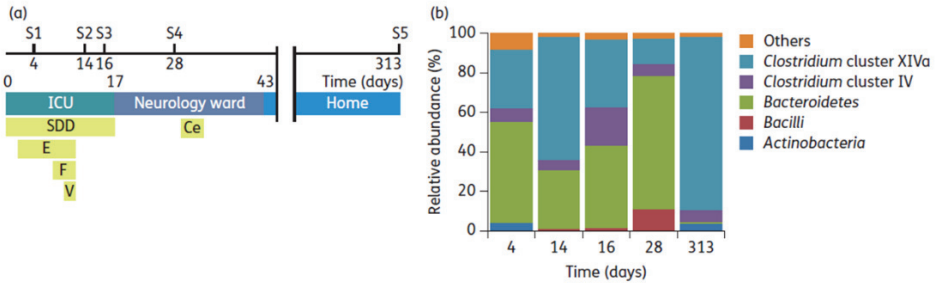

Figure. 1. Patient history and gut microbiota composition. (a) The timeline indicates the major events throughout the patient's hospital stay and the times at which faeces were collected. Yellow boxes indicate the antibiotics (E: erythromycin, F: flucloxacillin, V: vancomycin, Ce: cefazolin) that were administered to the patient. Further details are provided in the Methods section. Diagnostic culturing was performed for rectum, sputum, throat, urine and blood samples and no antibiotic resistant bacteria were found at any point in time (**Table S1**). (b) The patient's gut microbiota composition was monitored by a 16S rRNA-based microarray profiling approach (HITChip). The bars indicate the relative abundance of the most dominant bacterial phyla in the gut microbiota at the time points indicated on the x-axis. The colours code for the different phyla and classes as displayed in the figure key. Low-abundance phyla and classes are grouped together as "Others". Detailed information on the relative abundances of all phyla and classes detected by HITChip analysis are provided in the supplemental data **Table S5**.

Expansion of the resistome during ICU stay

The resistome of the patient substantially expanded during ICU stay and administration of SDD, this was most pronounced at days 14 and 16 (**Fig. 2a**). The reservoir of resistance genes had decreased at day 28, but genes conferring resistance to several classes of antibiotics were still detectable in the absence of antibiotic selective pressure at day 313 (270 days after hospital discharge). Specifically, there was a 6.7-fold increase in the relative abundance of aminoglycoside resistance genes at day 16, compared to the first sampling point at day four and the last sampling point at day 313 (**Fig. 2**).

Due to the inclusion of an aminoglycoside (tobramycin) and a β-lactam antibiotic (cefotaxime) in the SDD regimen, we focused on genes that were predicted to confer resistance to these antibiotics. In particular, the aminoglycoside resistance genes *aph(2")-Ib*, *aph(3')-IIIa* and an *aadE*-like gene increased in abundance during ICU stay (**Fig. 2b** and **Tables S4** and **S7**). In addition, the copy number of the β-lactam resistance gene *cblA* rose during ICU stay but increased further after ICU discharge (day 28; **Fig. 2b** and **Tables S4** and **S7**). Notably, the abundance of aminoglycoside resistance genes was lower at day 28 and had dropped even further at day 313 (**Fig. 2a**), although aminoglycoside resistance genes remained the most abundant class of resistance genes in the resistome at this point in time.

In addition to aminoglycoside resistance genes, genes conferring resistance to macrolides and tetracycline were the most abundantly present classes of resistance genes. The abundance of macrolide and tetracycline resistance genes remained relatively stable throughout hospital stay, but dropped sharply upon hospital discharge. The observed high levels of macrolide resistance genes throughout hospitalization may have been triggered by the usage of erythromycin, which the patient received to enhance gastric emptying during ICU stay. Tetracyclines were not administered to this patient and the high prevalence of these resistance genes is in line with the previously reported high abundance of tetracycline resistance genes in healthy individuals (Forslund *et al.,* 2013; Hu *et al.,* 2013).

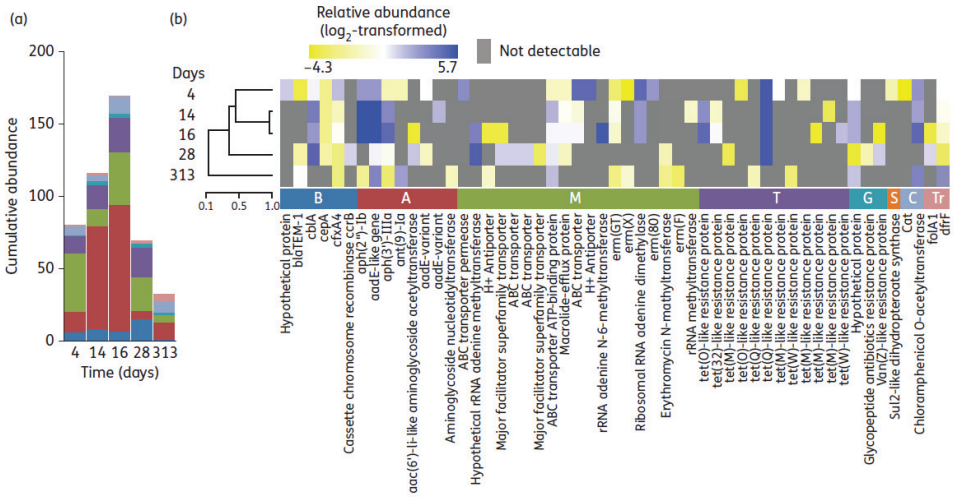

Figure. 2. Resistome dynamics determined by shotgun metagenomic sequencing.

(a) Cumulative abundance of antibiotic resistance gene families in metagenomic assemblies during ICU stay (day 4, 14 and 16), further hospitalisation (day 28) and 270 days after hospital discharge (day 313). The cumulative abundance of each resistance gene family represents the summed coverage data for resistance genes (normalised to average sequencing depth per assembly) per resistance gene family. Resistance gene families are indicated by the coloured bars which are coded as in panel (b). **(b)** Heat map of the relative abundance (log2-transformed and normalised to average sequencing depth per assembly) of antibiotic resistance genes that are present in the patient's gut microbiota during and after hospitalisation. Cluster analysis was performed using standard Pearson's correlation. Colour codes indicate resistance gene families (B: β-lactams; A: aminoglycosides; M: macrolides; T: tetracyclines; G: glycopeptides; S: sulphonamides; C: chloramphenicols; Tr: trimethoprim).

Resistance genes on mobile genetic elements in anaerobic gut commensals

Metagenomic shotgun sequencing and subsequent assembly generally resulted in contigs of limited size, precluding assessment of the genetic context of the identified resistance genes (data not shown). We therefore constructed fosmid libraries (with inserts of approximately 40 kbp) to functionally screen for antibiotic resistance genes and to further explore the genetic context of these genes.

Five fosmid libraries were constructed in E. coli from metagenomic DNA obtained from the faeces samples used for metagenomic sequencing. The total size of these libraries ranged from 0.8 to 2.6 Gbp (**Table S8**). Libraries were screened for clones that were resistant to ampicillin, cefotaxime, cefazolin, tobramycin, erythromycin, tetracycline and colistin (**Figure S1**). No clones that were resistant to colistin or cefotaxime were isolated, but we were able to isolate resistant clones for the other antibiotics.

The number of clones resistant to tobramycin, ampicillin, or erythromycin increased during ICU stay. At day 28 the number of tobramycin- and, to a lesser extent, erythromycin-resistant clones had decreased, whereas the number of ampicillin-resistant clones remained relatively stable, confirming the trends observed by metagenomic shotgun sequencing. The number of tetracycline-resistant clones was relatively stable throughout the monitored period. At day 313, tetracycline was the only antibiotic for which resistant clones could be isolated (**Figure S1**). From the resistant clones, five genes were identified that conferred resistance against tobramycin, ampicillin, erythromycin and tetracycline in *E. coli*. The identified genes were: *aph(2")-Ib* (conferring resistance to tobramycin), *cblA* (ampicillin), *ermBP* (erythromycin), and *tetW and tetO* (tetracycline). Sequencing of the vector/insert junction of ten clones in which resistance genes were identified showed that identical resistance determinants were present within different clones and genetic backgrounds.

Subsequently, the inserts of one selected fosmid clone per resistance gene were sequenced to characterize the genetic context of the resistance genes and to predict the bacterial sources of the cloned ISs. This revealed that the cloned resistance genes were harboured by anaerobes from the phyla *Firmicutes* (*Subdoligranulum*, Clostridia), *Bacteroidetes* (*Bacteroides uniformis*) and *Actinobacteria* (**Fig. 3**), which are all common members of the human gut microbiota (Rajilic-Stojanovic *et al.*, 2007; Qin *et al.*, 2010). In all sequenced fosmid inserts, the antibiotic resistance genes were associated with IS elements or genes of putative phage or plasmid origin, including genes that are predicted to be involved in plasmid replication and mobilization (**Fig. 3**). This suggests that the antibiotic resistance genes are located on mobile genetic elements that are harboured by anaerobic gut commensals.

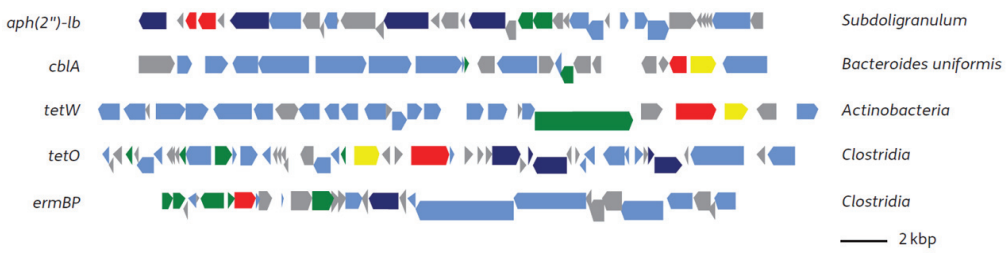

Figure. 3. Fosmid ISs for resistant clones identified by functional metagenomics. To identify and classify putative mobile genetic elements within the ISs of antibiotic-resistant fosmid clones, the ACLAME and ISFinder servers were used (Leplae et al., 2010; Siguier et al., 2006). Red arrows indicate antibiotic resistance genes. Light blue arrows indicate genes predicted to be of plasmid origin. Dark blue arrows indicate genes predicted to be of plasmid origin and putatively to be involved in plasmid mobilization and conjugation. Green arrows indicate genes to be of phage origin and yellow arrows indicate genes identified as IS-elements. The origins of the cloned resistance genes were predicted using CARMA3 (Forslund et al., 2013).

Heterogeneous effects of SDD on abundance on aminoglycoside resistance genes in ICU patients

Because metagenomic sequencing demonstrated an increasing abundance of aminoglycoside resistance genes in the patients' microbiota during ICU hospitalization, we decided to perform qPCRs to determine the levels of two aminoglycoside resistance genes in 12 additional, ICU-hospitalised patients who received SDD and from whom multiple faecal samples were collected during ICU stay.

The metagenomics DNA samples of the patients of whom the resistome was profiled by metagenomics shotgun sequencing and functional metagenomics was also included. Notable, none of the studied patients was treated therapeutically with an aminoglycoside (**Figure 1** and **Figure S2**). Consequently, the patient's only exposure to aminoglycoside antibiotics was due to the use of tobramycin in SDD. The two targeted aminoglycoside resistance genes that were targeted by qPCR were *aph(2")-Ib*, which was identified in our functional metagenomic screen, and the *aadE*-like gene, which was the most abundant aminoglycoside resistance gene found by metagenomic shotgun sequencing. The qPCR data indicated that the relative abundance of both resistance genes is highly divergent among the different patients. The copy number of the resistance genes changed between 1.5×10^{4} and 8.1×10^{-8}-fold (for *aph(2")-Ib*) and 1.0×10^{2} and 4.5×10^{-11}-fold (for the *aadE*-like gene) relative to the first sampling point during ICU stay (**Fig. 4**). Our findings indicate that the effect of SDD, and ICU hospitalisation in general, is highly individualized and that both a strong enrichment and a complete eradication of aminoglycoside resistance genes can be the result of SDD.

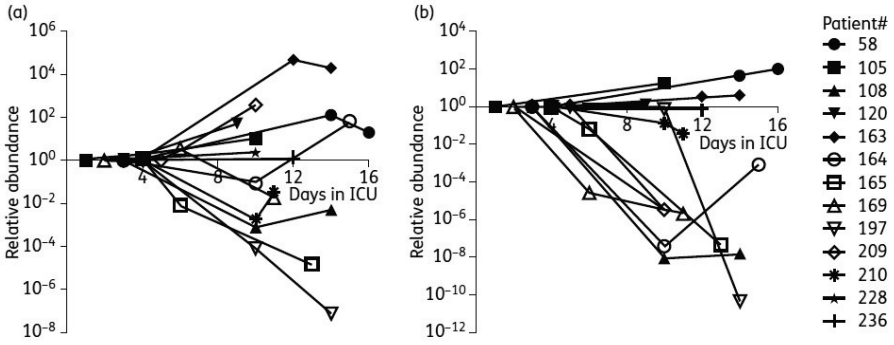

Figure. 4. Relative abundance of the aminoglycoside resistance genes *aph*(2")-*Ib* and *aad*E-like in ICU patients receiving SDD.

The relative abundance of *aph*(2")-*Ib* (A) and the *aad*E-like gene (B) was determined by qPCR from faecal samples of 13 patients during ICU hospitalisation. The relative abundance of both resistance genes was determined for all sampling time points relative to the 16S rRNA gene and the change throughout ICU stay was calculated relative to the first sampling point during ICU stay. The resistome of patient 58 was previously characterized by metagenomic shotgun sequencing and functional metagenomics in this study. Information on the antibiotic use of other patients is provided in **Fig. S2**

Discussion

The prophylactic use of antibiotics in SDD is one of the most successful interventions to reduce patient morbidity and mortality in ICU, but whether SDD will lead to the selection of antibiotic resistant bacteria is a topic of considerable controversy (de Smet *et al.*, 2009; Oostdijk *et al.,* 2010; Daneman *et al.,* 2013; van der Meer *et al.,* 2013). With this study, in which several metagenomics approaches were combined, we provide data indicating that the patient gut microbiota, and the resistance genes carried by the gut microbiota, can be profoundly affected by ICU hospitalization and SDD. Our functional metagenomics analyses indicate that the identified antibiotic resistance genes are all carried by anaerobic gut commensal and are associated with mobile genetic element.

Based on sequence analysis of a fosmid insert confering resistance to tobramycin, the aminoglycoside resistance determinant *aph(2")-Ib* was harboured by a strain from the genus *Subdoligranulum*. This genus belongs to *Clostridium* cluster IV and is commonly present in the microbiota of healthy individuals (Holmstrom *et al.*, 2004; Qin *et al.*, 2010). Interestingly, strict anaerobes such as *Bacteroidetes* and Clostridia are generally thought to be intrinsically resistant to aminoglycosides, because these bacteria lack an electron transport system that is needed for the energy-driven uptake of aminoglycosides into the cell (Bryan *et al.,* 1979). Nevertheless, aminoglycoside resistance genes can be readily identified in several *Clostridium* isolates (including strains that were isolated from human faeces) by either comparative genomic hybridisation (Janvilisri *et al.*, 2010) or by sequence analysis of publicly available *Clostridium* genomes (data not shown). These observations not only show that *Clostridium* and closely related genera may serve as a reservoir for aminoglycoside resistance genes, but also suggest that these resistance genes may have a, hitherto unrecognised, function in Clostridia. Alternatively, the resistance genes may form part of a larger genetic element that confers a fitness benefit to Clostridia and are retained for this reason.

In all sequenced fosmid inserts, we found evidence for the presence of IS elements and genes of putative phage or plasmid origin, including genes that are predicted to be involved in plasmid replication and mobilization. This observation suggests that these resistance genes may be part of larger genetic elements that can be mobilised and/or which have been acquired through horizontal gene transfer. Evidence for the extensive transfer of antibiotic resistance genes in the gut microbiota has been observed before in *Bacteroidetes* and *Firmicutes* (Shoemaker *et al.,* 2001; Jones *et al.*, 2010). Consequently, the enrichment of antibiotic resistance genes in the patient's gut microbiota during SDD and their association with mobile genetic elements is a cause of concern as this may facilitate transfer of resistance genes to aerobic nosocomial pathogens. In fact, our experimental design, using functional metagenomics, proved that these resistance genes can be expressed and are functional in *E. coli*, which is a common cause of hospital-acquired infections.

Based on our findings in a single patient, we subsequently determined the relative abundance of two aminoglycoside resistance genes (*aph(2")-Ib* and the *aadE*-like gene) in 12 other ICU patients who were hospitalized in the ICU for at least 9 days and who received SDD during this period. The relative abundance of both genes appeared very dynamic, indicating highly variable effects of SDD on the studies aminoglycoside resistance genes in individual patients. This may result from differences between the studied patients with respect to the bacterial hosts that carry the antibiotic resistance genes. For instance, the *aph(2")-Ib* gene, which was harboured by the Gram-positive bacterium *Subdoligranulum* in the patient in which we characterized the resistome by metagenomic approaches, can also be harboured by Gram-negatives such as *E. coli* (Chow *et al.,* 2001). In addition, the *aadE*-like gene is also found in the genome sequences of both Gram-positive and Gram-negative gut commensals such as *Faecalibacterium prausnitzii* and *Bacteroides uniformis* (data not shown). In patients that carry *aph(2")-Ib* in a Gram-negative host, such as *E. coli*, the relatively copy number of this gene may rapidly decrease during SDD due to the action of colistin, as this antibiotic specifically targets Gram-negative bacteria, while not inhibiting the growth of Gram-positive bacteria.

This study suggests that ICU hospitalization and SDD may have a large effect on the gut microbiota and the resistome. Previous, culture-based studies failed to demonstrate that SDD increased the prevalence of colonisation by antibiotic-resistant bacteria in the ICU (Daneman *et al.,* 2013). This observation indicates that the selection for resistance among anaerobic gut commensals during ICU stay may not directly impact on the resistance levels in aerobic bacteria, possibly because these are eradicated by other components of SDD. However, once patients are discharged from the ICU and SDD has been discontinued, the expanded resistome of the patients' gut microbiota may facilitate transfer of resistance genes to aerobic pathogens, once these recolonize the patient gut. This mechanism might explain the previously observed increase in antibiotic-resistance among *Enterobacteriaceae* after cessation of SDD (Oostdijk *et al.,* 2010).

Notably, microbiological cultures that were routinely performed in our diagnostic laboratory failed to yield the growth of any antibiotic-resistant bacterium throughout the period in which this patient was hospitalized. This discrepancy between traditional culture approaches and metagenomics analysis is likely to be due to antibiotic resistance genes being mostly carried by strictly anaerobic gut commensals, which are effectively impossible to culture in routine diagnostic settings. We note that the introduction of metagenomic shotgun sequencing as a tool in clinical diagnostics will allow the comprehensive identification and quantification of the resistome in individual patients. Although such approaches are currently still restricted by the costs of metagenomic shotgun sequencing and subsequent data analysis, our findings highlight the potential of these approaches as a future monitoring tool for the assessment of the impact of antibiotics on the gut resistome and to guide personalized antibiotic treatment. Most importantly, our findings indicate that the benefits of SDD on patient outcome and infections rates must be carefully balanced against the potential collateral selection and amplification of antibiotic resistance genes among anaerobic gut commensals.

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Supplementary data:

Table S1 to S8, Figure S1 and S2 are available at JAC online ((http://jac.oxfordjournals.org/).

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CHAPTER 5

Gut microbiota and resistome dynamics in intensive care patients receiving selective digestive tract decontamination

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ABSTRACT

Objectives. To determine the dynamics of the gut microbiota and resistome of ICU-patients during and after SDD.

Methods. Feces were collected during and after ICU stay (38 samples from eleven patients) and from ten healthy subjects (twice, with a one-year interval). Gut microbiota and resistome composition were determined using 16S rRNA phylogenetic profiling and nanolitre-scale quantitative PCRs, targeting 81 antimicrobial resistance genes (ARGs).

Results. Compared to the microbiota of healthy subjects, the microbiota of ICU patients was significantly less diverse. The microbiota of ICU patients was characterized by a reduction of butyrate-producing bacteria (up to 19-fold) and *Escherichia coli* (108-fold), while abundance of enterococci was 42-fold higher, all compared to healthy subjects. During ICU stay, the abundance of eleven ARGs, mostly associated with *E. coli*, were reduced, whereas the abundance of four ARGs, which were associated with Gram-positive cocci and included the staphylococcal *mecA* gene, significantly increased in the patients' microbiota.

Conclusions. SDD suppresses both butyrate-producing bacteria and *E. coli* and selects for Gram-positive cocci and their associated resistance genes.

Keywords: Anti-Bacterial Agents; Antibiotic Prophylaxis; Drug Resistance, Microbial; Intensive Care;
Microbiome

Introduction

The human gut microbiota comprises 10^{13} - 10^{14} bacterial cells that belong to hundreds of different species. The gut microbiota plays an important role in numerous metabolic, physiological, nutritional and immunological processes of the human host (Sekirov *et al.*, 2010). In healthy individuals, the gut microbiota mostly consists bacteria that have a commensal or mutualistic relationship with the human host. In critically ill patients, however, intestinal overgrowth by multi-drug resistant opportunistic pathogens, such as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter*), *Escherichia coli* or *Clostridium difficile*, is a common event and an important risk factor for the subsequent development of nosocomial infections (van der Waaij, 1989; Boucher *et al.*, 2009; Ubeda *et al.*, 2010; Buffie *et al.*, 2013; Britton *et al.*, 2014). To lower the risk of nosocomial infections with opportunistic pathogens in ICU patients, Selective Digestive tract Decontamination (SDD), have been implemented as prophylactic antibiotic therapy (van der Waaij *et al.*, 1990).

During SDD therapy, a paste containing colistin, tobramycin, and amphotericin B is applied to the oropharynx and a suspension of colistin, tobramycin, and amphotericin B via a nasogastric tube. These antibiotics are applied until ICU discharge. In addition, a third-generation cephalosporin (usually either cefotaxime or ceftriaxone) is administered intravenously during the first 4 days of ICU stay. Previous studies indicated that SDD lowers patient mortality during ICU stay in settings with a low prevalence of antibiotic resistance and lower the costs associated with ICU hospitalization (de Jonge *et al.*, 2003; de Smet *et al.*, 2009). However, selection for resistance to the antimicrobial agents used in SDD remains a major concern, in particular because the gut microbiota of patients is exposed to high quantities of antibiotics (Wunderink, 2010; Philips, 2014). However, based on the conventional culture results of clinical trials, there was no evidence for increased antibiotic resistance due to the implementation of SDD (Daneman *et al.*, 2013).

The patient gut is not only a potential source for opportunistic pathogens, but also forms a large reservoir for antibiotic resistance genes, termed the gut resistome (Sommer *et al.*, 2009; Wunderink, 2010; Daneman *et al.*, 2013; van Schaik, 2015). The use of antibiotics may favor the selection for antimicrobial resistance genes (ARGs) among members of the gut microbiota, thus increasing the likelihood of horizontal spread of ARGs between commensals and opportunistic pathogens co-residing in the gut (Salyers *et al.*, 2004). During ICU stay, the gut resistome of patients is primarily monitored by the cultivation of resistant bacteria from rectal swabs or faeces, as part of routine diagnostics. However, methods that rely on microbial culture capture only a fraction of the gut resistome, since anaerobic gut commensals, which are the main reservoir of ARGs in the gut microbiota, are difficult to culture (Sommer *et al.*, 2009; , Qin *et al.*, 2010; Buelow *et al.*, 2014). Thus, culture-independent methods need to be employed to assess the impact of topical antibiotic prophylaxis on the microbiota and resistome of ICU patients.

Here, we used the 16S ribosomal RNA (rRNA) gene-targeted Human Intestinal Tract Chip (HITChip) and nanolitre-scale quantitative PCR (qPCR) targeting a broad range of ARGs, to determine the dynamics of gut microbiota composition and resistome in patients receiving SDD during ICU hospitalization. We contrast these findings in ICU patients with the composition of the microbiota and resistome of healthy subjects.

Methods

The protocol for this study was reviewed and approved by the institutional review board of the University Medical Center Utrecht (Utrecht, The Netherlands). Patients included in the study did not receive antibiotics prior to ICU admission. All patients received selective digestive tract decontamination (SDD), during ICU stay until ICU discharge. SDD consists of 1000 mg of cefotaxime intravenously four times daily for four days, an oropharyngeal paste containing polymyxin E, tobramycin and amphotericin B (each in a 2% concentration) and administration of a 10 mL suspension containing 100 mg polymyxin E, 80 mg tobramycin and 500 mg amphotericin B via a nasogastric tube, four to eight times daily throughout ICU stay. All patients received additional antibiotics during ICU stay ranging from 2-11 antibiotic courses.

Faecal samples of patients were collected at different time points during hospitalization by nursing staff (**Fig. S1**). Faeces were collected upon defecation and stored at 4°C for 30 min to 4 h, after which the samples were transferred to -80°C.Routine surveillance for colonization with aerobic Gram-negative bacteria in ICU patients was performed through culturing of rectal swabs on sheep blood agar and MacConkey agar. All suspected Gram-negative colonies were analyzed by Maldi-TOF for species identification.

Faecal samples of healthy subjects were collected as part of the 'Cohort study of intestinal microbiome among Irritable Bowel Syndrome patients and healthy individuals' (CO-MIC) study at two time-points with a one-year interval between sampling. None of the individuals in this cohort received antibiotics. The protocol for this study was reviewed and approved by the Ethics Committee of Gelderse Vallei Hospital (Ede, The Netherlands). All included patients and subjects were ≥18 years of age.

DNA of faecal samples of patients and healthy subjects was isolated as described elsewhere (Salonen *et al.*, 2010).

Gut microbiota profiling by HITChip

Gut microbiota composition profiles were determined using the HITChip, as described previously (Rajilić-Stojanović *et al.*, 2009). The full-length 16S rRNA gene was amplified from fecal DNA, and PCR products were further processed and hybridized to the microarrays as described previously (Jalanka-Tuovinen *et al.*, 2011). Data analyses were performed using R (www.r-project.org), including the microbiome package (https://github.com/microbiome). Bacterial associations in the different patient groups and healthy subjects were assessed using Principal Component Analysis (PCA) as implemented in CANOCO 5.0 (Ter Braak *et al.,* 2012). Differences in microbiota composition in the study groups at the genus-like level were assessed by the Wilcoxon test for unpaired data (healthy vs ICU) and the Mann-Whitney test for paired data (different time points within healthy and ICU groups). All P-values were corrected for false discovery rate (FDR) by the Benjamini and Hochberg method, and corrected P-values below 0.05 were considered significant.

qPCR analysis

To sensitively quantify the levels of *E. coli* in samples, the number of *E. coli* 16S rRNA gene copies relative to total 16S rRNA gene copies were determined by quantitative PCR using previously described primers for *E. coli* (Furet *et al.*, 2009) using serial dilutions of genomic DNA of *E. coli* DH5α to generate a standard curve and total 16S rRNA (Gloor *et al.*, 2010). The qPCR analysis for the quantification of antibiotic resistance genes was performed using the nanoliter-scale 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA), according to the manufacturer's instructions, with the exception that an annealing temperature of 56°C was used. Faecal DNA was first subjected to 14 cycles of Specific Target Amplification using a 0.18 μM mixture of all primer sets, excluding the 16S rRNA primer sets, in combination with the Taqman PreAmp Master Mix (Applied Biosystems), followed by a 5-fold dilution prior to loading samples onto the Biomark array for qPCR.

Thermal cycling and real-time imaging was performed on the BioMark instrument, and Ct values were extracted using the BioMark Real-Time PCR analysis software. Primers were designed for the ARGs that are most commonly detected in the gut microbiota of healthy individuals (Forslund *et al.*, 2013; Hu *et al.*, 2013) and clinically relevant ARGs, including genes encoding extended spectrum β-lactamases (ESBLs), carbapenemases, and proteins involved in vancomycin resistance A total of 81 antimicrobial resistance genes and 14 resistance gene classes (**Table S1**) were used and also 10 genes encoding transposases, and a gene encoding an integrase as representatives of mobile genetic elements (Zhu *et al.,* 2013). Primer design was performed using Primer3 (Untergasser *et al.,* 2012) with its standard settings with a product size of 80 – 120 bp and a primer melting temperature of 60°C.

The universal primers for 16S rRNA genes were previously described (Gloor *et al.,* 2010). Forward and reverse primers were evaluated in silico for cross hybridization using BLAST (Altschul *et al.,* 1990) and were cross-referenced against ResFinder (Zankari *et al.,* 2012) to ensure the correct identity of the targeted genes. All primers that aligned with more than 10 nucleotides at their 3' end to another primer sequence were discarded and redesigned. Additionally, all primer sets were aligned to all resistance genes that were targeted in this PCR analysis to test for cross hybridisation with genes other than the intended target resistance gene. Primers that aligned with more than 10 nucleotides at their 3' end sequence with a non-target resistance gene were discarded and redesigned. Finally all primers were cross-referenced A reference sample consisting of pooled fecal DNA from different patients was loaded in a series of 4-fold dilutions and was used for the calculation of primer efficiency.

All primers whose efficiency was experimentally determined to be between 80% and 120% were used to determine the normalized abundance of the target genes. The detection limit on the Biomark system was set to a CT value of 20, as recommended by the manufacturer. In addition, to assess primer specificity we performed melt curve analysis using the Fluidigm melting curve analysis software (http://fluidigm-melting-curve-analysis.software.informer.com/).

All PCRs were performed in triplicate and sample-primer combinations were only included in the analysis when all triplicate reactions resulted in a CT-value below the detection limit. After completion of the nanolitre-scale qPCR assays, the presence of the transferable colistin resistance gene *mcr*-1 was evaluated. To detect and quantify *mcr*-1, we developed primers (qPCR-*mcr*1-F: 5'-TCGGACTCAAAAGGCGTGAT-3' and qPCR-*mcr*1-R: 5'-GACATCGCGGCATTCGTTAT-3') for use in a standard qPCR assay. The *mcr*-1 gene was synthesized based on the sequence described previously (Liu *et al.*, 2016) by Integrated DNA Technologies (Leuven, Belgium) and used as a positive control in our assays. The qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Leusden, The Netherlands) and a StepOnePlus instrument (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) with 5 ng DNA in the reaction and the following program: 95°C for 10 min, and subsequently 40 cycles of 95°C for 15 sec, 56°C for 1 min.

For each sample, the normalized abundance of resistance genes was calculated relative to the abundance of the 16S rRNA gene (CTARG – CT16S rRNA), resulting in a log2-transformed estimate of ARG abundance. Statistical analysis was performed using GraphPad Prism (La Jolla, CA). The Mann-Whitney test was used to test for differences in the normalized abundance of ARGs between the different groups of patients and healthy subjects.

Fisher's exact test was used to test for differences between groups in the number of samples in which each ARG could be detected. For resistome analysis only ARGs were considered and the remaining genes on mobile genetic elements (MGEs) were analyzed separately. Seven MGE-genes were detected by qPCR but no significant differences could be observed between patients and healthy subjects (data not shown). Therefore we decided to not include this set of genes in the subsequent analyses. Cumulative abundance was calculated based on the sum of delta-delta CT values (2^(-(CTARG – CT16S rRNA)) per resistance gene family. Visualization of the qPCR data in the form of a heat map was generated using Microsoft Excel. Correlations between resistance genes and bacterial taxa were calculated using Pearson's *r*.

Results

Patient data

The included patients (n = 11) were treated with SDD during their hospitalization in the ICU of the University Medical Center Utrecht (Utrecht, The Netherlands). The patients were acutely admitted to the ICU and had not been hospitalized, or treated with antibiotics, 6 months prior to ICU hospitalization. A total of 38 faecal samples were collected during ICU stay, and, if possible, after transfer to a medium care ward.

The faecal samples of patients were categorized in order to monitor in detail the dynamics and diversity of the gut microbiota and resistome into the following, mutually exclusive groups: “early ICU” samples (the first faecal sample during ICU stay of a patient, collected no later than five days after ICU admission; n = 10), “during ICU” samples (samples collected more than five days after ICU admission and before the final ICU sample; eleven samples from four ICU patients), “final ICU” samples (the patient’s last faecal sample collected during ICU stay, ranging from 9 to 40 days (median 13.5 days) after the start of ICU-hospitalization; n = 10) and seven “post ICU” samples from six patients, collected after ICU discharge during hospitalization in a medium-care ward (**Fig. S1** includes detailed information on sampling time points and antibiotic usage of the ICU patients in this study).

During ICU stay, routine surveillance by conventional microbiological culture was performed on all patients. *E. coli* could be cultured from five patients within one day of ICU admission and from one patient after nine days of ICU stay (total=6 out of 73 rectal swabs). Antibiotic resistance phenotypes of these isolates indicated that one patient had an extended-spectrum beta-lactamase (ESBL) phenotype and was resistant to tobramycin. The other *E. coli* strains were susceptible to cephalosporins and aminoglycosides. All strains were susceptible to colistin.

Microbiota dynamics in ICU patients and healthy subjects

Global changes in the gut microbiota of healthy subjects and ICU patients were visualized in a Principal Component Analysis (**Fig. 1A**). The microbiota profiles of healthy subjects clustered together, indicating that they had stable and broadly comparable microbiota profiles, which were clearly distinct from the microbiota profiles of patients during and after ICU stay. These profiles covered a larger area in the PCA plot, indicating that the differences in the microbiota composition of patients are larger than in healthy subjects. The diversity of the microbiota, as quantified by Shannon's diversity index (**Fig. 1B**), was highest in the healthy subjects at both time points (5.95 ± 0.15 at the first sampling time-point, 5.86 ± 0.24 at the second sampling time point), and was significantly lower in the "during ICU" (5.08 ± 0.36) and "final ICU" (4.93 ± 0.40) groups, but not in the "early ICU" group (5.66 ± 0.33).

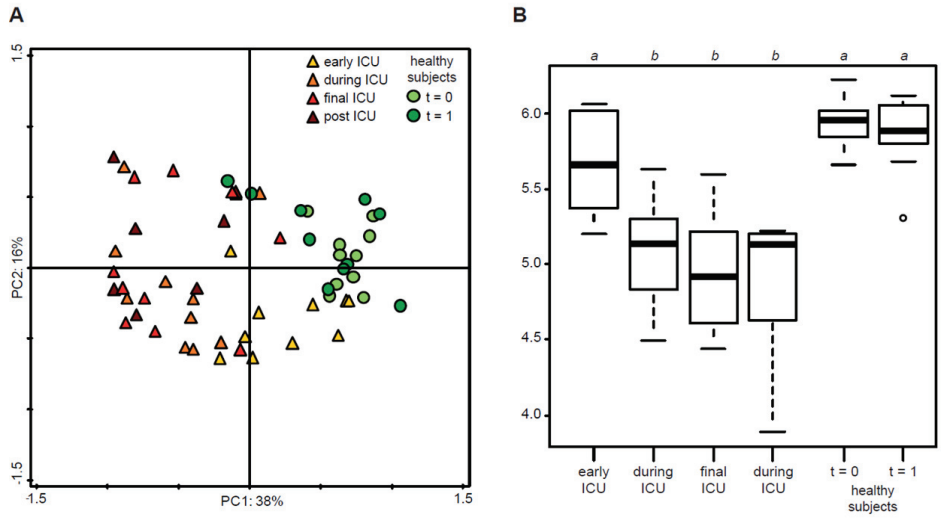

Figure 1: Dynamics of gut microbiota composition and diversity in ICU patients and healthy subjects. **Panel A**: Principal Component Analysis (PCA) of gut microbiota composition of ICU patients and healthy subjects, sampled at two time-points with a one-year interval (t=0 and t=1). **Panel B**: Diversity of the microbiota of ICU patients and healthy subjects. Diversity of the microbiota was estimated by the Shannon diversity metric. Diversity is shown in box plots with whiskers extending from the 25th percentile to 75th percentile and outliers; lines within each box indicate median diversity of a sample group. Differences in diversity between groups are significant (Student's t-test, $p < 0.01$) if the letters at the top of the graph are different.

Compared to healthy subjects, the microbiota of patients during ICU hospitalization was characterized by an increase in Bacilli, particularly of *Enterococcus* and *Granulicatella* groups (**Fig. 2**). The abundance of *Enterococcus* and *Granulicatella* was 42- and 34-fold higher, respectively, in the "final ICU" group than in the healthy subjects. Conversely, levels of several anaerobic commensal bacteria in the Firmicutes phylum, were reduced in the "during ICU" and "final ICU" groups, compared to healthy subjects. The most affected groups of bacteria were the butyrate producers *Faecalibacterium prausnitzii et rel*. (16.2-fold lower abundance in the "final ICU" group versus healthy subjects), *Eubacterium rectale et rel*. (10.7-fold lower), and *Roseburia intestinalis et rel.* (10.6-fold lower).

We performed quantitative PCRs to accurately determine the abundance of *E. coli*, one of the primary targets of SDD, in the gut microbiota of patients and healthy subjects (**Fig. 3**). The abundance of *E. coli* in the "final ICU" samples was significantly lower compared to the "early ICU" group and the healthy subjects (325-fold and 108-fold, respectively). The decrease in *E. coli* abundance during ICU stay in nine patients for which both "early ICU" and "final ICU" samples were collected, ranged from 9.4-fold (patient #180) to 7.6 x 104-fold (patient #108), with a 301-fold decrease as median value. The abundance of *E. coli* rebounded in four of six patients after cessation of SDD and transfer to a medium-care ward, reaching levels that were comparable to, and, in one patient, surpassing those found in healthy individuals.

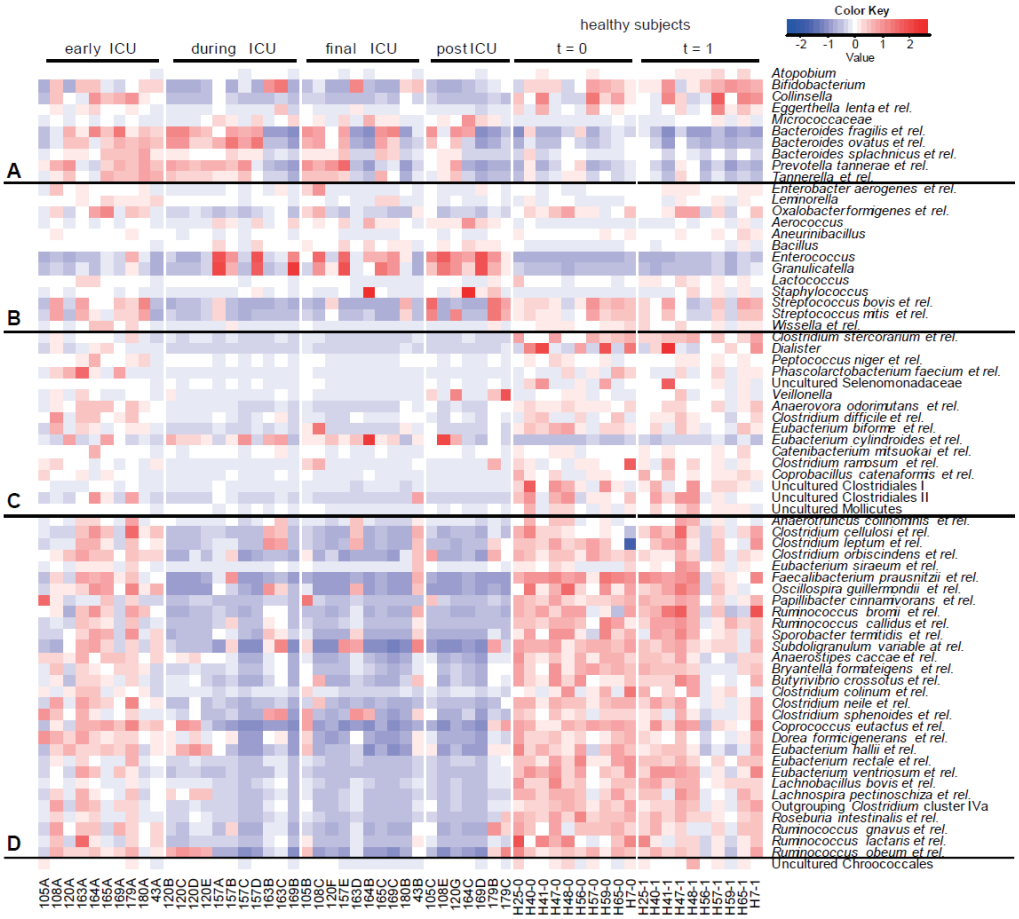

Figure 2. Differences in bacterial composition of the gut microbiota. The bacterial genus-like groups that are significantly different (Kruskal-Wallis $p < 0.03$; FDR < 0.05) among the sample groups are shown. Colors indicate the differences in log-10 abundance compared to the average abundance of a given taxon in the entire data set. Bacterial groups are indicated as follows: A: *Actinobacteria* and *Bacteroidetes*; B: Bacilli and Proteobacteria; C: Other Firmicutes; D: *Clostridium* clusters IV and XIVa.

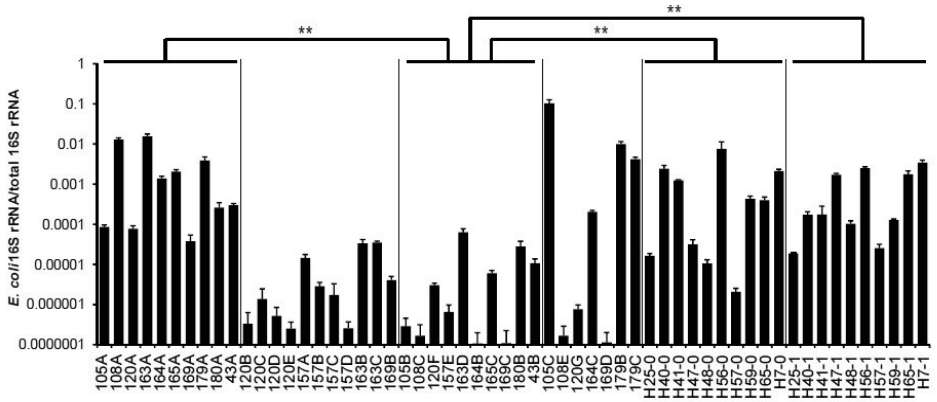

Figure 3. Quantification of *E. coli* 16S rRNA gene copies relative to total 16S rRNA gene copies. The quantification was performed by qPCR with three technical replicates. Error bars indicate standard deviation. The order of the samples is identical to panel (A). Statistical differences between the "final ICU" group vs the "early ICU" group and the healthy controls were analyzed by Student's t-test with correction for multiple testing (**: FDR < 0.01).

Resistome dynamics in ICU patients and healthy subjects

A total of 48 unique ARGs conferring resistance to 14 different classes of antimicrobials were detected in the DNA isolated from faecal samples of hospitalized patients and healthy subjects (**Fig. S2**). The number of detected resistance genes per sample ranged between 6 and 38. Thirteen resistance genes were detected in >80% of healthy subjects and critically ill patients and these include tetracycline resistance genes (*tetO, tetQ*, *tetM*, *tetW*), the bacteroidal β-lactam resistance gene *cblA*, and two aminoglycoside resistance genes (*aph(3′)-III* and an *aadE*-like gene).

Genes encoding for extended-spectrum beta-lactamases (ESBLs) were not detected in healthy subjects. In four samples of three ICU patients, ESBL genes could be detected (n = 1 for $bla_{CTX\text{-}M}$, n = 2 for bla_{CMY}, n = 2 for bla_{DHA}; a single patient sample (#179B) was positive for both bla_{CMY} and bla_{DHA}). Three of the four ESBL-positive samples were collected after ICU discharge and cessation of topical antibiotic prophylaxis.

The carbapenemase *bla*$_{KPC}$ was detected in a single patient (patient #180), in the first sample collected after 5 days in ICU. The *bla*$_{AMPC}$ β-lactamase was present in 41.3% of samples, including 9 of 11 patients and 8 of 10 healthy subjects, whereas the *bla*$_{TEM}$ β-lactamase was present in 27.6% of samples, corresponding with 6 of 11 patients and 4 of 10 healthy subjects, respectively. None of the samples were positive for the carbapenemases *bla*$_{NDM}$ and *bla*$_{OXA}$, or for the recently described (Liu *et al.*, 2016) transferable colistin resistance gene *mcr-1* (data not shown).

Among resistance genes that are associated with Gram-positive pathogens, the staphylococcal methicillin resistance gene *mecA* was detected in 13 samples from 8 of 11 patients, but not in samples of healthy subjects. The vancomycin resistance gene *vanB* was present in 5 samples from 3 of 11 patients and 6 samples from 4 of 10 healthy subjects.

Resistome dynamics during ICU stay

To assess resistome stability, we plotted the average abundance of detected ARGs in healthy subjects at the two time sampling points and the average abundance of ARGs of the nine patients for which both "early ICU" and "final ICU" samples were available. Based on linear regression fitting of the different ARGs the resistome appeared more stable in healthy subjects (r = 0.96) than in ICU patients (r = 0.56) (**Fig. 4**). When comparing the presence of individual ARGs in the "final ICU" group versus the "early ICU" group and samples from healthy subjects, four ARGs were found to be enriched (**Fig. 5**), while eleven ARGs were reduced (**Fig. 6**) in abundance at the end of ICU stay. Increased abundance was demonstrated for genes contributing to aminoglycoside resistance (*aac(6')-Ii*), resistance to erythromycin (*ermC*), methicillin resistance in staphylococci (*mecA*), and non-susceptibility to antiseptics (*qacA*). Decreased abundance of ARGs in the "final ICU" group was demonstrated for eleven genes, which were involved in β-lactam resistance (*bla*$_{AMPC}$), chloramphenicol resistance (*cat*), the efflux of toxic compounds (*acrA*, *acrF*, *macB*, *mdtF*, *mdtL*, *mdtO*, *tolC*), resistance to polymyxins (*arnA*) and tetracycline resistance (*tetW*).

Abundances of *bla*$_{AMPC}$, *acrA, acrF, macB, mdtF, tolC* and *arnA* were highly correlated with each other ($r \geq 0.9$) and with levels of *E. coli* ($r = 0.86$ for *arnA*, $r \geq 0.95$ for the other ARGs), as determined by qPCR.

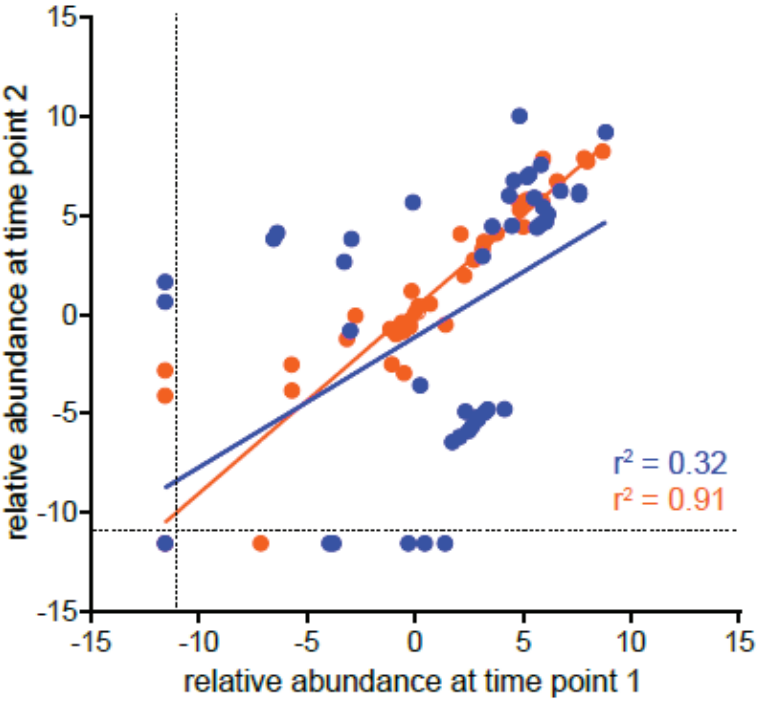

Figure 4: Dynamics of the resistome in ICU patients and healthy subjects. The averages of log2-transformed abundances for 48 ARGs (normalized to the 16S rRNA gene) that were detected in samples of nine patients for which both early ICU (time point 1)and final ICU (time point 2) samples were collected (blue circles) and for the healthy subjects (orange circles; n = 10, sampled twice with a one year interval) are plotted to depict resistome dynamics over time. Trend lines and correlation coefficients are shown. The dashed lines indicate the detection limit of the qPCR assay.

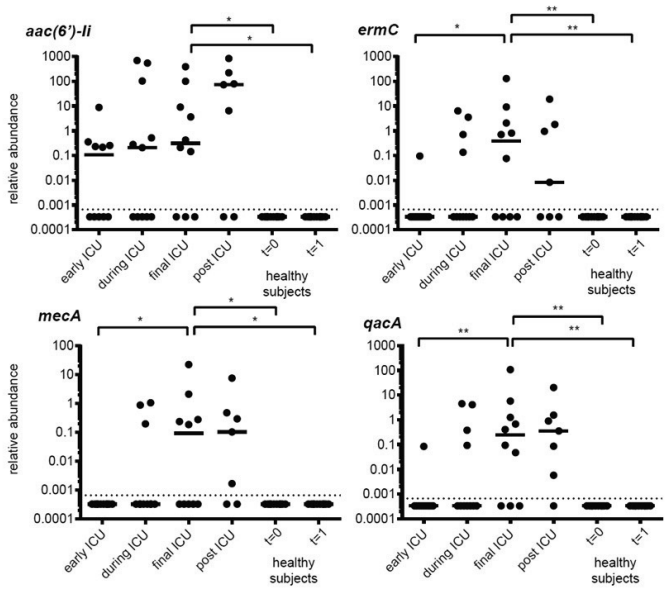

Figure 5. ARGs with increased abundance upon prolonged ICU hospitalization. ARGs that are present at higher abundance in the "final ICU" group, compared to the "early ICU" group and healthy subjects (sampled at two time points with a one year interval: t=0 and t=1). Testing for statistically significant differences between the six groups was performed by Kruskal-Wallis analysis with correction for multiple testing (FDR < 0.019). The horizontal line denotes the median value. For ARGs that fit this criterion, testing for statistical differences between "final ICU" and "early ICU" and the two groups of healthy subjects was performed using Dunn's post-hoc test (* = $p < 0.05$; ** = $p < 0.01$). The detection limit of the qPCR assay is indicated with the dashed line.

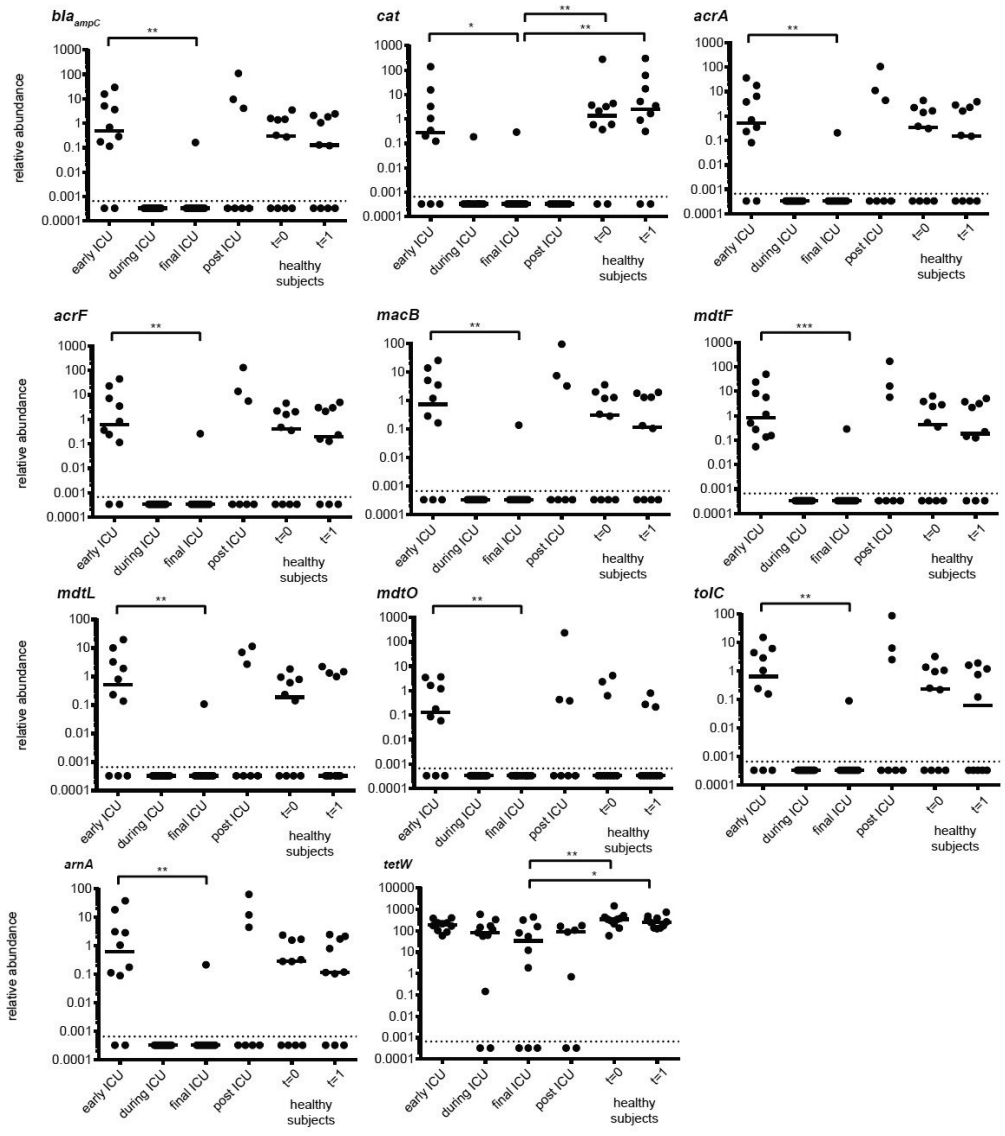

Figure 6. ARGs with decreased abundance upon prolonged ICU hospitalization. ARGs that are present at lower abundance in the "final ICU" group, compared to the "early ICU" group and healthy subjects (sampled at two time points with a one year interval: t=0 and t=1). Testing for statistically significant differences between the six groups was performed by Kruskal-Wallis analysis with correction for multiple testing (FDR < 0.019). The horizontal line denotes the median value. For ARGs that fit this criterion, testing for statistical differences between "final ICU" and "early ICU" and the two groups of healthy subjects was performed using Dunn's post-hoc test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). The detection limit of the qPCR assay is indicated with the dashed line.

Discussion

Current guidelines in the Netherlands recommend topical antibiotic decontamination in ICU patients with an expected ICU stay of two days or longer. Yet, the original claim that these interventions do not affect harmless anaerobic intestinal bacteria (van der Waaij *et al.*, 1990), has recently been questioned (Buelow *et al.*, 2014; Benus *et al.*, 2010). While culture-based studies did not demonstrate selection for antibiotic-resistant opportunistic pathogens during SDD-treatment (de Smet *et al.*, 2009; Daneman *et al.*,2013; Plantinga *et al.*, 2015), concerns remain that selection for antibiotic resistance genes occurs in the gut microbiota of patients.

The current study describes the diversity and dynamics of the gut microbiota of ICU-patients receiving SDD during ICU-stay. the gut microbiota of ICU patients was characterized by a low diversity, the increased abundance of facultatively aerobic Gram-positive bacteria (predominantly *Enterococcus*, *Granulicatella* and, in a single patient, *Staphylococcus*) and decreased abundance of anaerobic Gram-positive, butyrate-producing bacteria, particularly those of the *Clostridium* clusters IV and XIVa. These findings expand on previous findings of selection of Gram-positive cocci (Daneman *et al.*, 2013; Buelow *et al.*, 2014; van der Bij *et al.*, 2016) and depletion of *F. prausnitzii* during SDD (Benus *et al.*, 2010). In addition, we were able to demonstrate that the abundance of *E. coli* was reduced by 301-fold (median) during ICU-stay. The suppression of *E. coli* in the SDD-treated ICU patients observed here, starkly contrasts with other studies in critically ill patients not receiving SDD, in which high-level *E. coli* gut colonization is a common event (Donskey, 2006; Taur *et al.,* 2012; Zaborin *et al.*, 2014). Yet, levels of *E. coli* increased again after ICU-discharge in four of six patients, reaching levels in the gut similar to, or even surpassing, those in healthy individuals. These findings suggest that a rapid regrowth or recolonization of the intestinal tract by *E. coli*, and possibly other aerobic Gram-negative bacteria, occurs upon cessation of prophylactic antibiotic therapy. In the only prospective evaluation, SDD treatment during ICU stay was not associated with higher infection rates upon ICU discharge (de Smet *et al.,* 2009).

It, therefore, remains to be determined whether rapid post-ICU recolonization by *E. coli* increases the risk for infections with this bacterium. In addition, the reduction of butyrate-producing bacteria through SDD could possibly cause long-term gut health consequences as the production of butyrate is important for gut health and human metabolism (Canfora *et al.,* 2015).

The qPCR-based analysis of the resistome confirms previous metagenomic studies, in showing that tetracycline and aminoglycoside resistance genes and bacteroidal β-lactamases are widespread in the human intestinal microbiota (Sommer *et al*., 2009; de Vries *et al;* 2011; Forslund *et al*., 2013; Hu *et al*., 2013; Buelow *et al*., 2014). All resistance genes that increased in abundance during ICU-stay, were associated with Gram-positive bacteria. The aminoglycoside resistance gene *aac(6')-Ii* gene is a specific chromosomal marker for the nosocomial pathogen *Enterococcus faecium* (Costa *et al.,* 1993). The increased abundance of the macrolide resistance gene *ermC* may have been selected for by the use of low doses of the macrolide erythromycin, which was used as an agent to accelerate gastric emptying during ICU stay in six out of eleven patients. The *mecA* gene was only detected in ICU patients, and confers methicillin-resistance to staphylococci, including *S. aureus*. Yet, coagulase-negative staphylococci are the most frequent carriers of the *mecA* gene (Suzuki *et al*., 1992; Conlan *et al*., 2012; Becker *et al*; 2014).

Previous studies on the implementation of SDD in ICUs have not found evidence for increased rates of MRSA colonization or infection (Daneman *et al*., 2013; Plantinga *et al*., 2015). Whether increased levels of *mecA* in the gut microbiota increases the likelihood of transfer of the *mecA* gene among staphylococci, through the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*) (Wielders *et al*., 2001; Jansen *et al*., 2006), remains to be determined. Finally, the enriched *qacA* gene confers resistance to a number of disinfectants, including the biguanidine compound chlorhexidine and the quaternary ammonium compound benzalkonium chloride (Tennent *et al*.,1989; Mitchell *et al*., 1998).

Disinfectants are widely used in ICUs as cleaning and infection control agents (McDonnell *et al.*, 1999) and its use could select for *qacA* in the gut microbiota of patients. In contrast, eight ARGs that were correlated with levels of *E. coli* were eliminated during ICU stay. These findings confirm the association of these resistance genes with the *E. coli* chromosome (Blattner *et al.*, 1997). The tetracycline resistance gene *tetW* is present in anaerobic gut commensals, including the butyrate-producer *F. prausnitzii* (Scott *et al.*, 2000), and the effects of SDD on butyrate-producing anaerobes may be responsible for the lower abundance of *tetW* in the gut microbiome of ICU-hospitalized patients.

Although SDD improves survival of ICU-patients, its use remains controversial due to the perceived risk for selection of antibiotic resistance among bacteria that populate the patient gut. Based on the results from culture-independent techniques we conclude that SDD contributes to the selection for enterococci and the resistance genes associated with these bacteria. Enterococci are frequently multi-drug resistant, can cause difficult-to-treat infections and may serve as hubs for the spread of antibiotic resistance genes (Werner *et al.*, 2013). Despite the selection for enterococci during SDD, rates of enterococcal infections among ICU-patients have not increased upon introduction of SDD (de Smet *et al.*, 2009; Daneman *et al.*, 2013). We also conclude that SDD reduces the abundance of *E. coli*, and the resistance genes associated with this species, although this effect seems restricted to the duration of application of SDD. SDD is mostly used in the Netherlands, where the prevalence of multi-drug resistant bacteria in ICUs is low. In other countries, particularly in those where vancomycin-resistant enterococci, MRSA and ESBL- or carbapenemase-producing Enterobacteriaceae are more prevalent, the clinical benefits of SDD remain to be determined. Our findings demonstrate that monitoring of the resistome during ICU hospitalization by high-throughput qPCR provides more detailed information on the presence and abundance of antibiotic resistance genes, which may contribute to the prudent use of SDD in ICU patients, as it will enhance to rapidly detect and allow quantification of high-risk antibiotic resistance genes in the gut microbiota of patients during antibiotic prophylaxis.

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Supplementary data

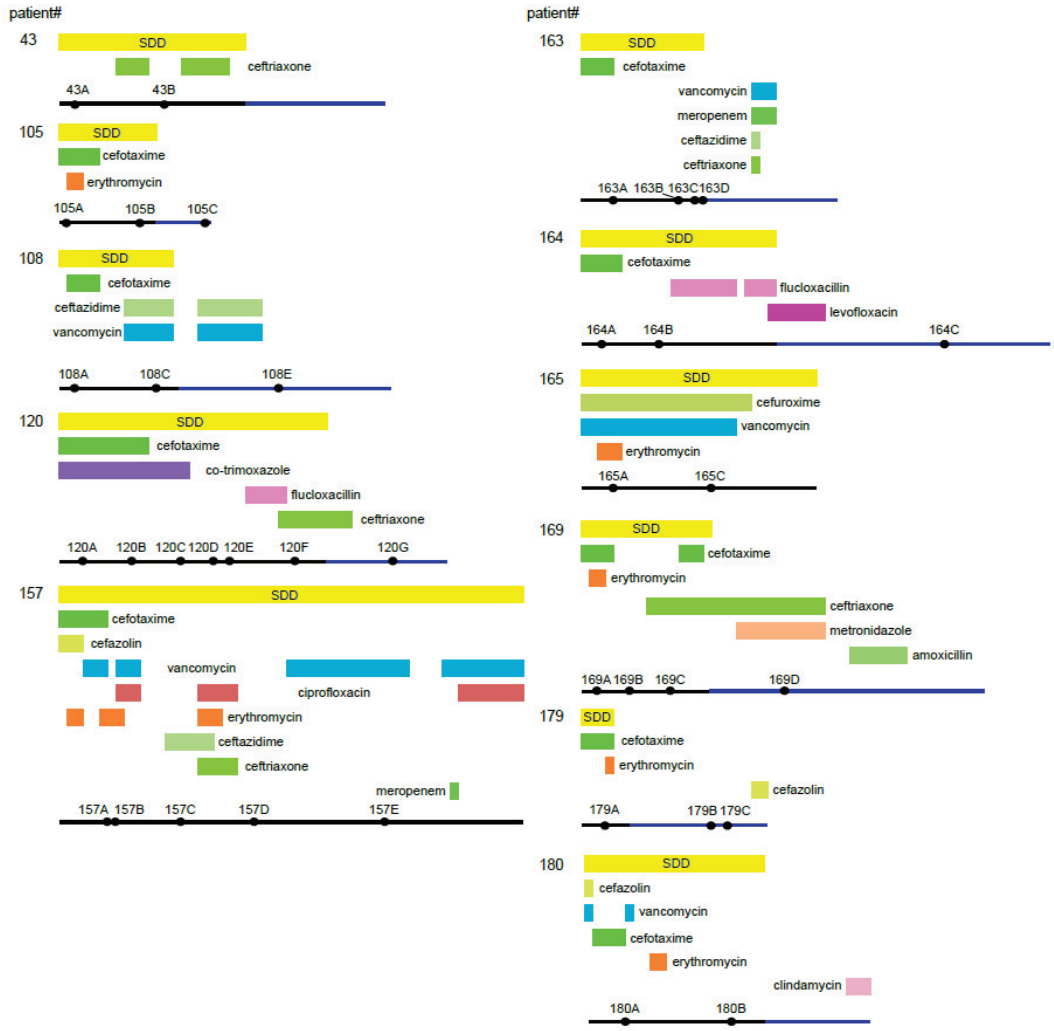

Figure S1. Patient details. The antibiotics used in treatment of patients during hospitalization and time points at fecal samples were collected are indicated. SDD indicates the administration of topical components of SDD, Black lines indicate hospitalization at the ICU, blue lines indicate hospitalization at a medium-care ward.

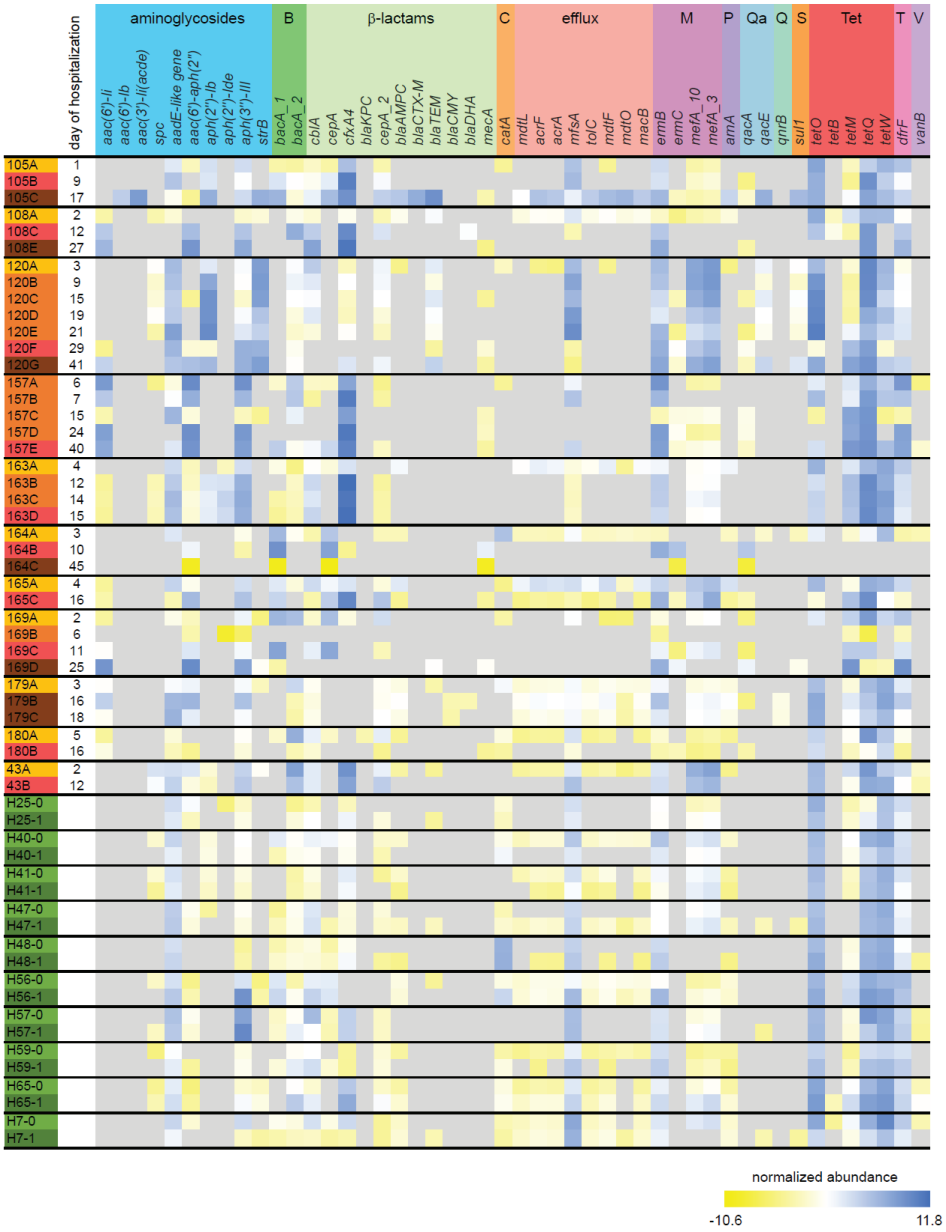

Figure S2: Resistome of hospitalized patients and healthy subjects. ARGs are grouped and color-coded according to resistance gene families (B: bacitracin, C: chloramphenicol; M: macrolides; P, polymyxins; Qa: quaternary ammonium compounds, Q: quinolones; S: sulphonamides; Tet: tetracyclines; T: trimethoprim; V: vancomycin). Abundance (log2-transformed) is visualized relative to 16S rRNA.The time points at which samples were collected are indicated and color-coded as in Fig. 1.

Table S1. Primers used in this study. Primers were developed to target targeted the indicated ARGs. Primer sequences in bold indicate ARGs which were detected in ≥1 sample.

Antimicrobial resistance	Accession	Forward primer	Reverse primer
acrB	YP_002396537	CACGGTGACACAGGTTATCG	AAGGTCAGGGTGATCTGCAC
acrF	**CAR04877.2**	**ACTGACACCGGTTGATGTG**	**GAGCAATAATCGAGGCGTTC**
tolC	**BAG78840.1**	**CTGAAAGAAGCCGAAAAAC**	**CGTCGGTAAGTGACCATCCT**
acrA	**ACI36997.1**	**GAAGGTAGCGACATCGAAG**	**CTTTCGCCAGATCACCTTTC**
aph(3')-III	**ACB90577.1**	**CCGGTATAAAGGGACCACC**	**CTTTGGAACAGGCAGCTTTC**
aph(2")-Ib	**AF207840.1**	**ATCAAATCCCTGCGGTAGT**	**CAAGGGCATCCTTTTCCTTT**
***aadE*-like gene**	**AAW34138.1**	**GCATGATTTCCTGGCTGAT**	**CCACAATTCCTCTGGGACAT**
aac(6')-aph(2")	**ABY79711.1**	**TCCAAGAGCAATAAGGGCA**	**TGCCCTCGTGTAATTCATGT**
aac(6')-Ii	**WP_002293**	**AGACAGCTCGGCAGAAGAA**	**ACCGTATTGAGGGATTGCAC**
aac(3')-Ii(acde)	**HQ246166.1**	**TGACGTATGAGATGCCGAT**	**GAGAATGCCGTTTGAATCGT**
aac(6')-Ib	**KM387722.1**	**TTGCAATGCTGAATGGAGA**	**TGGTCTATTCCGCGTACTCC**
aadA	ADW23165.1	GAACATAGCGTTGCCTTGGT	GCTGCGAGTTCCATAGCTTC
aac(6')-IIa	ACR24243.1	GAACACTACCTGCCCAGAGC	GCGACGTACGACTGAGCATA
aph(2")-I(de)	**AAC14693.1**	**CGGAGGTGGTTTTTACAGG**	**TTGCTTCGGCAGATTATTGA**
aph(3')-Ia, -Ic	CAQ58482.1	ATTCTCACCGGATTCAGTCG	ATTCCGACTCGTCCAACATC
strB	**CAJ77026.1**	**GGCGATTATAGCCGATCAA**	**CGCGACTGGAGAACATGATA**
bacA_2	**ABR38862.1**	**GAGGCATTGATCCTTGGTG**	**AAACAATGCCGAACCGATAG**
bacA_1	**CAH05846.1**	**GGCTGCGTTACTGTCGTTTT**	**GGCCAATGATAAATGCATCC**
bacA	ACL18936.1	AACTTCCCGTTCTGGTGCTA	CATAACGGGGATAGCGAGAA
bla$_{GES}$	ABG47465.1	CTGCTGCAATGACGCAGTAT	TATCTCTGAGGTCGCCAGGT
bla$_{IMP}$	AJ640197.1	GCTACCGCAGCAGAGTCTTT	CCCACCCGTTAACTTCTTCA
bla$_{VIM}$	AM183120.1	TGTCCGTGATGGTGATGAGT	TTTCAATCTCCGCGAGAAGT
bla$_{ACC}$	AJ870923.1	TTGTTACGCTACGTGCAAGC	CGATTTGAAATAGCCGGTGT
***bla*$_{DHA}$**	**AHN96243.1**	**AAAGTGCGCAAAGCCAGTA**	**AAGATTCCGCATCAAGCTGT**
bla$_{IMI}$	U50278.1	AGTCGATCCCAGCAGCTTTA	CCAAGAAACTGTGCATTCCA
bla$_{CMY}$	AF357598.1	GATCTGCTGCGTTTTGTGAA	CTACCGAGTAATGCCCTTGG
***bla*$_{AMPC}$**	**ABF06289.1**	**ACCGCTAAACAGTGGAATG**	**GCAAGTCGCTTGAGGATTTC**
cepA	**CR626927.1**	**ATGTCCTGCCCTGGTAGTT**	**CTTGCCCGTCGATAATGACT**
cepA_2	**AE016945.1**	**TGCACCAAGACGAAAGTCT**	**ACAGTGCTTCTTTGCGGAAT**
bla$_{BIC-1}$	GQ260093.1	CCATCAGCGCACAACATAGT	CCAGAACGTTTTCCAGAAGC
cblA	**AAA66962.1**	**TGCCTGCGACATCTTGATA**	**CCGTCTTCTGTTTCCGAGAG**
cfxA	**AY769933.1**	**GCGCAAATCCTCCTTTAACA**	**ACAATAACCGCCACACCAAT**
***bla*$_{CMY}$**	**AAZ99133.1**	**CGATCCGGTCACGAAATAC**	**CCTGCCGTATAGGTGGCTAA**
***bla*$_{CTX-M}$**	**ABG46354.1**	**ACTATGGCACCACCAACGA**	**GGTTGAGGCTGGGTGAAGT**

***bla*$_{TEM}$**	**NP_775035.1**	**AAGCCATACCAAACGACGA**	**TTGCCGGGAAGCTAGAGTAA**
bla$_{SHV}$	AAV83796.1	CTTTCCCATGATGAGCACCT	AGATCCTGCTGGCGATAGTG
bla$_{NDM}$	CAZ39946.1	ATATCACCGTTGGGATCGAC	TAGTGCTCAGTGTCGGCATC
bla$_{OXA}$	AAP70012.1	GTGGCATCGATTATCGGAAT	AGAGCACAACTACGCCCTGT

Antimicrobial resistance	Accession	Forward primer	Reverse primer
***bla*$_{KPC}$**	**AEL12451.1**	**TGGCTAAAGGGAAACACGA**	**TAGTCATTTGCCGTGCCATA**
cat	**ABO92401.1**	**CAATCCTCAATCGACACGA**	**GATTGTGTAGCAAGGCAGCA**
mdtL	**CAR15381.2**	**CGGACAAACCACGAGAAAA**	**GAAGGTGAGGATCACCGAA**
mdtF	**KEL93478.1**	**GGACCCGCAAAAACTCAAT**	**AGTTGACCACCGGAAATCTG**
ermF	BAD66041.1	AGCACCCGCTTTTTCCTTAT	GATCAAGAGGGGCTTTAGGG
ermB	**BAH18720.1**	**GGTTGCTCTTGCACACTCA**	**CTGTGGTATGGCGGGTAAGT**
ermG	122586.NMB0	TGCTGTCTTTTACAGGCCACT	GCATATGTTCCAGTCCCTTCA
ermC	**BAE05991.1**	**TGAAATCGGCTCAGGAAAA**	**GGTCTATTTCAATGGCAGTT**
mefA_10	**583346.CKR**	**CCTGCAAATGGCGATTATT**	**CCAAAGACCGCATAGGGTAA**
mefA_3	**286636.M6_**	**TTACCCTATGCGGTCTTTGG**	**GAACCAGCTGCTGCGATAAT**
macB	**ACR63203.1**	**GGCTGGAAGACCGTACAGA**	**GTTGGTTCATCGGCAAGAAT**
fosB	NP_372857.1	TTGAGCTTGCAGGCCTATG	GCCAATATTTAAATTCGCTGTCA
IS*S1N*	**M37395**	**GACAGAGCACCGAACTGTG**	**TGCCCTTAATCGTGGAAGAG**
IS*613*	**AB042549**	**GTGGCGGTTATTGACGACT**	**TTCAGCGTGTCCTTCTGATG**
Tn*As3*	CP000645	CTCTGTTACCTGCGCTTTCC	CCGTACTCGTTCCAGCTTTC
Tn*610*	**X53635**	**GAGAGAGCTTTTGGCATTG**	**AGAGGTAGGCTGTCGCTCTG**
IS*ecp1*	**AJ242809**	**TGAAAAGCGTGGTAATGCT**	**TCGCCCAAAATGACTTTAGC**
IS*26*	**X00011**	**ACCTTTGATGGTGGCGTAA**	**TACCGGAACAACGTGATTGA**
IS*614B*	**AY682394**	**TTTCACTGAGGGGATGGAA**	**TTGCCTTCCCATTTCTCAAC**
IS*Azs19*	NC_013860	GAACCGCTCCGAGAAAGATT	GCTCATCGCCTTTGAGAAAC
IS*SW1*	**M37396**	**TTGAACAAGACCATCGTCC**	**TCTCCATCCCCTTAATCGTG**
cfr	YP_00389602	CAAACGAAGGGCAGGTAGAA	GACCACAAGCAGCGTCAATA
mfsA	**WP_002584**	**AATATGCTCTCCGGGCTTTT**	**TTTGCACACCGTAAAATGGA**
ermA	AB047088.2	GAGGGGTTTACCGCTTCTTT	ATCGGATCAGGAAAAGGACA
mecA	**YP_184944.1**	**TCCAGGAATGCAGAAAGAC**	**GGCCAATTCCACATTGTTTC**
arnA	**CAR03684.2**	**GAAATTCACCGTCTGGTCG**	**GTGGTGCAACAGAAATCACG**
mdtO	**BAI33519.1**	**TTGTTGGCCTCTATCCAACC**	**TTAAGCGCTTGATGCATTTG**
qacA	**YP_536864**	**GACCCTTCTGGTACCCAAC**	**TCCCCATTTATCAGCAAAGG**
qacC	CAA86016.1	TGGGCGGGACTAGGTTTAG	ACGAAACTACGCCGACTATGA
acrP	AKL33057.1	CAGGCACTCCTTTCAGCTTC	GAGGCCGTGTTCAATTTGTT

chvD	CDX10534.1	ATTCTGTGGCTGGAGCAGTT	GATCCACTTCGCAGATCCAT
qacE	**NC_001735.4**	**TCGGTGTTGCTTATGCAGT**	**ATCAAGCTTTTGCCCATGAA**
qnrA	ACA43024.1	ATTTCTCACGCCAGGATTTG	ACTGCAATCCTCGAAACTGG
qnrB	**AFD54601.1**	**CGATCTGACCAATTCGGAG**	**ACGATGCCTGGTAGTTGTCC**
qnrC	ACK75961.1	GCAGAATTCAGGGGTGTGAT	AACTGCTCCAAAAGCTGCTC
qnrS	AEG74318.1	TGGAAACCTACCGTCACACA	AATCGCATCGGATAAAGGTG
spc	**AAL05549.1**	**TGACGAACGCAATGTGATT**	**TCAGCTGCCAGATCTTTTGA**
vatA	AAF24087.1	AACAGCTTCTGCAGCAATGA	CCTTGAAAGGGGACATTGAA
vatB	**AAA86871.1**	**TGGGAAAAAGCAACTCCAT**	**TTCTGACCAATCCACACATC**
aadE	CAZ55809.1	TGTGCCGCAAAGAGATACTG	TTATCCCAACCTTCCACGAC
sul1	**ADB23338.1**	**AGGCTGGTGGTTATGCACT**	**AAGAACCGCACAATCTCGTC**
tetQ	**Y08615.1**	**GCAAAGGAAGGCATACAAG**	**AAACGCTCCAAATTCACACC**
tetX	ABQ05845.1	CGGTACGCTGGATTTACACA	CATCGGAATTGCCTTTTTGT
tetW	**ACD97480.1**	**GGTGCAGTTGGAGGTTGTT**	**AAATGACGGAGGGTTCCTTT**
Antimicrobial resistance	**Accession**	**Forward primer**	**Reverse primer**
tetM	**ACO22036.1**	**TTGATGCGGGAAAAACTAC**	**TACCTCTGTCCACGCTTCCT**
tetO	**EAQ71799.1**	**GCGTCAAAGGGGAATCACT**	**CGGTATACTTCCGCCAAAAA**
tetB	**AAL09908.1**	**CAAAACTTGCCCCTAACCA**	**GCTTTCAGGGATCACAGGAG**
dfrA	BAF39170.1	AGCACGATAGTAGCCGCAGT	AAGGTTTTGGGGAAATCGTC
dfrF	**AEBU010001**	**GATTGTTGCGAGGTCAAAG**	**CGCCCCATAATAACCACATT**
vanUG	ACR77286.1	ATTTGCGAAACTCGGAAAAA	ACACCTCATTTTCGGGTACG
vanR	CAJ68489.1	TGAAGCTGTATGGGGAGAAAA	TTTCGGGTTTTTAGAAGGTTCA
vanA	ACP19236.1	GTGCGGTATTGGGAAACAGT	TGCGTTTTCAGAGCCTTTTT
vanB	**WP_0324897**	**CCTGCCTGGTTTTACATCGT**	**GCTGTCAATCAGTGCAGGAA**
vanX	NP_878017.1	CCGGTTGACGGTTATGAAGT	CAGCCAGTTCTTTTGCCTTC
int	**AAA25857.1**	**AGGATGCGAACCACTTCAT**	**GCTGTTCTTCTACGGCAAGG**
cfr_2	AJ249217.1	GCCGGAGCTTTTCCTCTACT	GGTGCCGAAAGTCAAAACAT
16S rRNA	(9)	**CAACGCGARGAACCTTACC**	**ACAACACGAGCTGACGAC**

CHAPTER 6

Mapping the diversity and colonization dynamics of antibiotic resistant bacteria in ICU patients by culture dependent and independent approaches

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In preparation

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Abstract

Patients in the intensive care unit (ICU) are generally susceptible to hospital-acquired infections due to their immunological and clinical conditions. The application of prophylactic antibiotic therapies in critically ill patients aims to reduce the incidence of infections by Gram-negative bacteria, *Staphylococcus aureus* and yeast without disrupting the anaerobic microbiota. However, the impact of the prophylactic antibiotic therapy on the commensal gut microbiota and its associated resistome remain controversial. In this study we mapped the diversity and colonization dynamics of antibiotic resistant bacteria in ICU patients receiving prophylactic antibiotic therapy by using culture dependent and independent approaches.

A total of 39 samples was collected from 11 ICU patients during and after ICU hospitalization where the patients received prophylactic antibiotic therapy. Diversity and dynamics of gut microbiota composition during the study period were evaluated by phylogenetic analysis using the Human Intestinal Tract Chip (HITChip) and cultivation under aerobic and anoxic conditions. Isolates were further characterized by antibiotic resistance phenotyping, and by detection of genes conferring resistance to macrolides, vancomycin and methicillin, in aerobic potential Gram-positive pathogens.

HITChip analysis indicated that the relative abundance of *Enterobacteriaceae* was reduced during antibiotic therapy, whereas the relative abundance of *Enterococcus* spp. increased. Moreover, the relative abundance of *Clostridium* cluster IV and XIVa, representing an important fraction of the anaerobic microbiota, was reduced during therapy. We observed three distinct patterns based on the relative abundance of *Firmicutes* and *Bacteroidetes* phyla, however, no significant association could be established with specific antibiotic treatment, hospital-acquired infection, comorbity or length of ICU stay.

A total of 130 bacterial isolates were retrieved, comprising 70 aerobes and 60 anaerobes, including 17 butyrate producing bacteria.

Seventhy-two percent (n=94) of all isolates was multidrug resistant, with resistance to tetracycline (73 out of 130 isolates) and macrolides (87 out of 130 isolates) being most frequently observed. The ESBL phenotype was detected in four *Escherichia coli* isolates, while the class C cephalosporinase (AMPc) phenotype was detected in two *Enterobacter cloacae* isolates. Antibiotic resistance genes were detected in enterococci (*erm*B and *van*C1 gene) and in staphylococci (*erm*C and the methicillin resistance encoding *ccr* cassette in non-*aureus* isolates).

In conclusion, we show that prophylactic antibiotic therapy affects the diversity and dynamics of colonization with antibiotic resistant bacteria in ICU patients, with suppression of *Enterobacteriaceae* and functionally relevant anaerobes, and increase in enterocci.

Keywords: antibiotic therapy, antibiotic resistance, commensal bacteria, colonization, gut microbiota

Introduction

The human gut microbiota constitutes a complex community composed of approximately 10^{11} - 10^{12} microbial cells per gram of content (Ley *et al.,* 2006). The principal members of this complex community are strict anaerobes followed by facultative anaerobes and aerobes (O' Sillivan, 1999). In healthy humans, the gut microbiota plays an important role in several metabolic, nutritional, physiological and immunological processes (McNeil, 1984; Maloy *et al.,* 2011). For example, the maintenance of gut homeostasis and epithelial integrity is supported by butyrate-producing bacteria that convert dietary polysaccharides to short-chain fatty acids (SCFAs) such as butyrate, acetate and propionate. Particular interest has been attributed to butyrate as the main energy source for colonocytes (Hamer *et al.,* 2008). Marcia *et al.* (2012) indicated that impaired epithelial integrity is associated with emerging diseases such as inflammatory bowel disease. During such damage, butyrate producing bacteria are generally reduced in abundance (Clemente *et al.,* 2012).

Previous studies have shown that gut microbiota composition can be affected by a range of external factors, including diet and antibiotics (Ley, 2000; Ley *et al.,* 2006). During antibiotic administration the ecological balance of the gut microbiota can be disrupted. This could lead, for example, to overgrowth of microorganisms with natural resistance, establishment of new (resistant) pathogenic bacteria, and reduction of colonization resistance (Jernberg *et al.,* 2010).

Several factors, including the target spectrum and mechanism of action of antibiotics, dosage and duration of therapy, as well as the degree of absorption of orally and parenterally administered antibiotics, influence the extent to which a given antibiotic will affect microbiota composition (Bartosh *et al.,* 2004). Furthermore, different multidrug antibiotic cocktails can differently affect the microbial community (Robinson *et al.,* 2010; Vrieze *et al.,* 2014; Reijnders *et al.,* 2016). In intensive care unit (ICU) patients, hospital-acquired infections constitute a common problem associated with high risk of morbidity, mortality and increased hospitalization costs.

Frequently, these infections are caused by multidrug resistant bacteria, which represent one of the most important problems in public health (Vincent, 2013). During ICU stay, prophylactic antibiotic therapy, and more specifically Selective Digestive Decontamination (SDD), has been implemented in order to prevent secondary colonization with Gram-negative bacteria, *Staphylococcus aureus* and yeasts, through the application of non-absorbable antimicrobials in the oropharynx and gut without disrupting the anaerobic intestinal microbiota (de Smet *et al.,* 2009). Numerous studies have been performed in order to determine the effects of SDD therapy on the dynamics of the gut microbiota, largely focusing on aerobic and facultative anaerobic, potentially pathogenic bacterial populations.

Due to the complexity of the gut microbiota, different techniques have been used to increase our knowledge of the microbial diversity in the gut and its dynamics during antibiotic treatment.

By using culture-dependent techniques, microbiologists have been able to study the effects of SDD therapy on the bacterial target groups. Recently, Oostdijk and collaborators indicated that during SDD therapy, antibiotic resistant *Enterobacteriaceae* can be eradicated from the gut (Oostdijk *et al;* 2010; Oostdijk *et al.,* 2012).

However, other groups of bacteria, mainly anaerobes, have not been explored due to the fact that cultivation techniques are laborious, time consuming, require special equipment for working under anoxic conditions, and some bacteria require specific nutrients or the presence of metabolic products from other species for growth (MacFarlane *et al.,* 1994). The anaerobic commensal microbiota represents an important reservoir of antibiotic resistance genes and plays an important role in the horizontal gene transfer to potential pathogens (Shoemarker *et al.,* 2001; Sommer *et al.,* 2009; van Schaik, 2015). Culture dependent techniques have been thought to underestimate the bacterial community size due to the fact that only a small fraction of the gut microbiota is currently considered cultivable (10%), and thus microbiota composition and the associated resistome can be determined only in a small group of microorganisms (MacFarlane *et al.,* 2004).

Interestingly, Browne *et al.* recently showed that a considerable proportion of the intestinal spore-forming bacteria can be recovered from the gut microbiota by using a single growth medium, suggesting that more than 10% of the gut bacteria are culturable (Browne *et al.,* 2016).

Advances in culture independent molecular methods have led to an increased interest in identifying both cultivable as well as uncultivable gut bacteria (Akkermans *et al.,* 2000; Vaughan *et al.*, 2000). Benus *et al.* (2010) studied the effect of SDD in comparison with standard care (SC) by using 16S ribosomal RNA (rRNA)-targeted Fluorescent In Situ Hybridization (FISH), showing that during SDD therapy the abundance of *Enterobacteriaceae* and the *Faecalibacterium prausnitzii* group was significantly reduced while the *Enterococcus* population increased compared to SC . Recently, Dubourg *et al.* (2014) implemented the use of culture dependent and independent techniques to determine the impact of antibiotics on the gut microbiota from patients treated with a broad-spectrum antibiotic cocktail. Similarly, Rettedal *et al.* (2014) demonstrated that the combination of novel cultivation conditions with high-throughput sequencing of 16S rRNA genes allowed to identify and characterize previously uncultivated species. In a previous study, by using culture independent techniques, we were able to demonstrate that ICU hospitalization and SDD therapy dramatically affected gut microbiota composition and resistome (Buelow *et al.,* 2014).

In this study, our aim was to determine the diversity and dynamics of colonization with antibiotic resistant bacteria in ICU patients receiving SDD therapy by combining cultivation-indendent community profiling using the Human Intestinal Tract Chip (HITChip) microarray platform and cultivation on a variety of culture media, and further biochemical and phenotypical characterization of aerobic and anaerobic isolates.

Materials and Methods

Sample collection

Eleven patients were included in this study after ICU admission at University Medical Center (UMC) Utrecht, The Netherlands. Selection criteria included no antibiotic administration prior to ICU admission. The protocol for this study was reviewed and approved by the institutional review board of the UMC Utrecht, The Netherlands.

During ICU stay, Selective Digestive Decontamination (SDD) was applied. This therapy consists of the administration of 1000 mg of cefotaxime intravenously four times daily for four days, an oropharyngeal paste containing polymyxin E, tobramycin and amphotericin B (each at a concentration of 2%) and administration of a 10 mL suspension containing 100 mg polymyxin E, 80 mg tobramycin and 500 mg amphotericin B via a nasogastric tube, four to eight times daily throughout ICU stay. Systemic antibiotics were applied under clinical indications.

A total of 39 fecal samples were collected upon defecation at different time points during hospitalization and stored at 4°C for 30 min to 4 h, after which the samples were split in three parts (approximately 0.5 g each) for eventual phylogenetic microarray analysis using the HITChip as described previously (Rajilic-Stojanovic *et al.,* 2009) and for cultivation of aerobes and anaerobes.

In brief, for cultivation of aerobes, fecal samples were suspended in 5 ml of oxic phosphate buffer (pH 7.0) to a final concentration of 10% (w/v) and preserved with 40% glycerol. For anaerobes, feces samples were suspended in 5 ml of 20mM anoxic phosphate buffer (pH 7.0) supplemented with 0.5 mg/ml of resazurine and 0.5g of cysteine to a final concentration of 10% (w/v). An aliquot (1ml) was transferred to an anaerobic bottle containing 4 ml of PBS and glycerol (50%). A few drops of sterile-filtered titanium citrate were added to the bottle to ensure anoxic conditions. All aliquots were transferred to -80°C.

Fecal samples were classified as follows, based on time of collection: “Initital ICU” (n=7) samples collected within 72 hours after ICU admission, “ICU stay” (n=23) samples collected during ICU stay after the initial 72h (average of 15-20 days) and “post ICU” (n=9) samples collected after ICU discharge.

A schematic representation of the different approaches used in this study is shown in **Figure 1.**

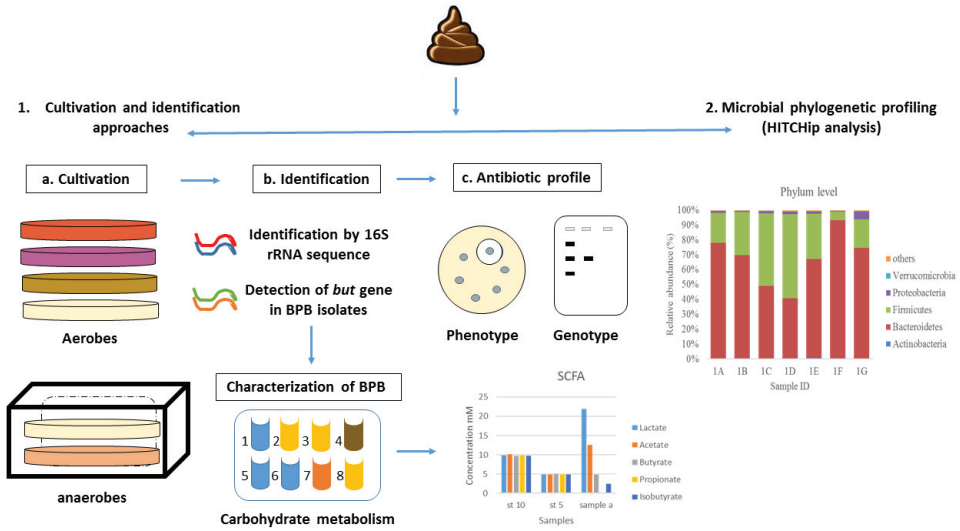


Figure 1. Schematic representation the different approaches used to study the diversity on colonization with antibiotic resistant bacteria.

Abbreviations*: BPB, Butyrate Producing Bacteria; SCFA, Short Chain Fatty Acids; HITChip, Human Intestinal Tract Chip.

Microbial phylogenetic profiling

Fecal DNA isolation and phylogenetic profiling of the gut microbiota using the HITChip was performed as described previously (Rajilic-Stojanovic *et al.,* 2009). The data was analised by using R (www.r-project.org), including the microbiome package (https://github.com/microbiome).

The dynamics of microbiota composition was studied per patient, as follow: patients were stratified based on the relative abundance of most predominant phyla observed in samples obtained during ICU stay – SDD, which led to the definition of three different groups: Group A: high relative abundance of *Bacteroidetes*, patients in this group have higher *Bacteroidetes* in more than 50% of the samples collected during ICU stay; Group B: high relative abundance of *Firmicutes*; and Group C: shift in the relative abundance of *Firmicutes* and *Bacteroidetes,* between first and second samples obtained during ICU stay. Moreover, differences in the gut microbiota composition at genus-like level based on signal probe intensities was assessed by the Wilcoxon test for unpaired data between time points (initial ICU, ICU stay and post-ICU) as implemented in the "wilcox.test" R-script (https://stat.ethz.ch/R-manual/R-patched/library/stats/html/wilcox.test.html). The diversity of the microbiota based on probe signal intensities was determined by Shannon's diversity index. Statistical differences (p-values < 0.05) were corrected for false discovery rate (FDR) by the Benjamini and Hochberg method (Benjamini *et al.,* 1995).

Cultivation of aerobic (potential pathogens) and anaerobic bacteria

An aliquot of the fecal suspension preserved in aerobic and anaerobic conditions, respectively, was diluted to a final concentration of 1% (w/v) and inoculated (10 mL) in ten different culture media: For cultivation of aerobes, the following culture media were used: Columbia Colistin Nalidixic Agar (CNA) with 5% sheep blood (staphylococci and streptococci) (Becton Dickinson, Breda, The Netherlands), Bilis Esculin Agar (BEA) (enterococci) (Oxoid B.V., Landsmeer, The Netherlands), MacConkey (enterobacteria) (Oxoid), Brain Heart Infusion Agar (BHI) (non-selective) (Oxoid). All plates were incubated in aerobic conditions at 37^{o}C for 3 days. For anaerobes we used the following culture media: Fastidious Anaerobic Agar supplemented with 5% horse blood (FAA) (Lab M Ltd., Bury, England), *Bacteroides* Supplemented BHI medium (BHIS) (BHI Agar, 5 mg haemin, 1 mg vitamin K, 5g yeast extract, 0.5g L-cysteine, resazurine 500mg / L), Reinforced Clostridial Agar (RCA) (Oxoid), *Lactobacillus* MRS Agar (MRS) (Oxoid), Peptone Yeast Glucose Agar

(PYG) (Leibniz-Institut DSMZ, Braunschweig, Germany), *Bifidobacterium* medium (DSMZ). All plates were incubated in anoxic conditions (N_2/CO_2 (80:20)) at 37°C for 5 days.

Different combinations of antibiotics were used on BHI, FAA and PYG media in order to isolate and identify the cultivable fraction of antibiotic resistant bacteria present during the specific collection time (**Table 1**). Bacterial growth was quantified by colony forming units (CFU/ml), and colonies were further characterized by macroscopic features as well as microscopically by Gram staining.

In order to isolate potential secondary fermenting bacteria, notably those involved in butyrate production, a bicarbonate-buffered anaerobic medium was used as described previously (Stams et al., 1993) supplemented with agar (15% w/v) and SDD cocktail (Polymyxin 25 µg, tobramycin 5 µg and cefotaxime 10 µg). As a carbon source, lactate (40mM), acetate (40mM), lysine (40mM) or a combination of lactate and acetate (40mM each) were added. All plates were cultured anaerobically in an athmosphere of N_2/CO_2 (80:20) at 37°C for 5 days.

Colonies were selected from the plates based on their morphology for subsequent transfer on the same medium in duplicate to obtain pure cultures and for identification and characterization of the isolates.

Table 1. List of antibiotics and concentration used per culture media

Culture media	Antibiotics	Concentration (µg/ml)
Aerobes		
Brain Heart Infusion Agar	TOB / POL	10 / 5
Anaerobes		
Fastidious Anaerobes Agar	AMP / TET / ERY	10 / 10 / 50
Peptone Yeast Glucose Agar	AMP / TET / ERY	10 / 10 / 50

Abbreviations: Ampicillin (AMP), Erythromycin (ERY), Polymyxin (POL), Tetracycline (TET), Tobramycin (TOB).

Identification of isolates

Identification of aerobic and anaerobic isolates was performed by colony PCR for the amplification of the bacterial 16S rRNA genes using the 27-F and 1492-R primers as described previously (Weisburg *et al.,* 1991). The amplified fragments were selected for partial sequence analysis of the 16S rRNA gene (~800bp) using the 1392R primer 5'- ACGGGCGGTGTGTRC -3' (GATC Biotech, Cologne, Germany). 16S rRNA sequences of all isolates were 99-100% similar to those of previously cultivable species.

The group of butyrate producing bacteria was subjected to a PCR for the detection of the butyryl-coenzyme A (CoA) CoA transferase gene (*but*) as described previously (Louis *et al.,* 2007) using *Eubacterium halli* and *Faecalibacterium prausnitzii* (Culture collection, Laboratory of Microbiology, Wageningen University, The Netherlands) as a positive control.

From all the isolates in which the *but*-gene was detected, substrate utilization was determined. In brief, a cell suspension of individual isolates was inoculated in anaerobic bicarbonate-buffered medium containing acetate and lactate as a substrate at a final concentration of 40 mM each, incubated at 37 ^{o}C for 48h. End products were determined by HPLC as described previously (van Gelder *et al.,* 2012).

Carbohydrate metabolism was determined for Gram-positive anaerobes by using API 50 CH (BioMerieux, Benelux B.V., Zaltbommel, The Netherlands).

Antimicrobial susceptibility

Aerobic isolates were tested for antimicrobial susceptibility by the disk diffusion method in Mueller Hinton Agar (MHA) (Oxoid) plates using the guidelines of the Clinical Laboratory Standard Institute (CLSI – Aerobic bacteria, 2013). For testing of antimicrobial susceptibility of aerobic Gram-positive bacteria, i.e. mainly staphylococci and enterococci, we used the following disks (Oxoid) : vancomycin (30 µg), oxacillin (1 µg), amoxicillin-clavulanic acid (20/10 µg), tetracycline (10 µg), chloramphenicol (10 µg), and ampicillin (10 µg).

Erythromycin (15 µg) and clindamycin (10 µg) were used to determine the phenotype of resistance to Macrolide Lincosamide Streptogramin B (MLS_B) by double diffusion test (Thumu *et al*., 2014). The minimal inhibitory concentration (MIC) of vancomycin was determined by E-test (Oxoid).

For the antimicrobial susceptibility in Gram-negative bacteria, i.e. mainly enterobacteria, we used: imipenem (10 µg), meropenem (10 µg), piperacillin-tazobactam (100/10 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), amoxicillin-clavulanic acid (20/10 µg), cefoxitin (30 µg), tetracycline (30 µg) and colistin (10 µg).

Anaerobic isolates were tested for antimicrobial susceptibility by ATB™ ANA EU (08) (BioMerieux) following the manufacturer's recommendations and by Agar dilution test as recommended by CLSI (CLSI – Anaerobic bacteria, 2013).

Detection of antibiotic resistance genes in Gram-positive aerobes

Staphylococcal and enterococcal isolates were tested for the presence of genes conferring resistance against vancomycin (*van*A, *van*B, and *van*C1/2) and erythromycin (*erm*A, *erm*B, *erm*C, *mef*A, *mef*E). In addition, the staphylococcal methicillin-resistance gene cassette (chromosomal *mec* type assignment genes) was tested in staphylococcal isolates by single and multiplex PCR (Depardieu et al., 2004; Zou et al., 2011; Klaassen et al., 2005; Kondo et al., 2007).

Results

We studied the diversity and colonization dynamics of antibiotic resistant bacteria in 39 fecal samples obtained from 11 ICU-hospitalized patients who received prophylactic antibiotic therapy. The number of samples collected during the study period ranged from two to seven for the different patients due to the medical conditions, constipation, prolonged stay and administration of systemic antibiotics for the control of nosocomial infections. Eight out of 11 patients developed nosocomial infections by multidrug resistant enterococci, staphylococci or enterobacteria. Characteristics of the patients included in this study are shown in **Table 2**.

Phylogenetic profiling indicated that prophylactic antibiotic therapy modified the gut microbiota composition (**Fig. 2**). The relative abundance distribution at the phylum level indicated an indivual-specific, diverse and dynamic gut microbiota composition during hospitalization in the different patients. Three different patterns were observed based on the dynamics of the gut microbiota, including Group A: High relative abundance of *Bacteroidetes* (**Fig 2a),** Group B: High relative abundance of *Firmicutes* (**Fig 2b**), Group C: shift in relative abundance between *Bacteroidetes* and *Firmicutes* during ICU stay (**Fig 2c**). The relative abundance of *Actinobacteria* and *Proteobacteria* increased slightly during ICU stay in five patients. To highlight the most significant differences at the genus level, we observed a significant increase in the relative abundance of *Enterococcus* and *Granulicatella* ($p < 0.05$) during ICU stay, whereas the relative abundance of *Enterobacteriaceae* and members of *Clostridium* clusters IV and XIVa was reduced in the tree groups.

The diversity of the microbiota calculated by Shannon's diversity index showed no significant differences between groups (group A: 5.0 ± 0.5, group B: 5.3 ± 0.6 and group C: 5.3 ± 0.4 ($p > 0.05$)). In contrast, the diversity of the microbiota observed in initial ICU samples (<72h) (5.6 ± 0.4), ICU stay (5.1 ± 0.4) and post-ICU samples (4.8 ± 0.5) showed a decrease during ICU hospitalization ($p < 0.01$).

Table 2. Characteristic of the patients included in this study.

Characteristic patients	Age (years)	Reason for ICU admission	Comorbid conditions	Length of ICU stay (days)	Additional antibiotic treatment*	Site of infection	Microorganisms isolated from the site of infections
1	52	Lung transplant	Diabetes type I Corticosteroids therapy	30	COT, FLX, CTR	Urinary tract	*E. faecium,* *S. epidermidis*
2	71	Post-operative	Cardiac disease	11	E	-	*E. coli* (ESBL)**
3	53	Trauma	Diabetes type II	14	CAZ, VAN	-	*E. cloacae**, *S. aureus***
4	74	Post-operative	Liver failure	57	CZL, VAN, E, CAZ, CPR,MER, CTR	Skin Bloodstream Cateter-related Pleural efussion	*S. epidermidis,* *S. aureus* *E. cloacae,* *E. faecium*
5	72	Heart failure	Cardiac disease	5	CZL, E	Respiratory tract	*E. coli,* *E. cloacae,* *S. maltophilia,* *C. braakii,* *P. putida*
6	48	Heart transplant	-	28	CRX, VAN, E	Cateter-related Urinary tract Respiratory tract	*E. faecalis,* *S. epidermidis*
7	61	Post-operative	Cardiac disease	22	CZL,VAN, E, CLN	Cateter-related	*S. epidermidis*
8	38	Trauma	Hypotyroidism	15	E, CTR, MTZ,AMX	Cateter-related	*E. faecalis*
9	49	Trauma	Hypertension	15	VAN, CAZ, CTR	Cateter related	*S. marcescens,* *E. faecalis* *C. striatum*
10	89	Trauma	-	23	FLX, LEV	Respiratory tract	*S. aureus,* *S. maltophilia*
11	N.A	N.A	N.A	23	CTR	N.A	N.A

Abbreviations*: COT, Cotrimoxazole; FLX, Flucloxacillin; CTR, Ceftriaxone; E, Erythromycin; CAZ, Cefatzidime; VAN, Vancomycin; CZL, Cefazolin; CPR, Ciprofloxacin; MER, Meropenem; CRX, Cefuroxime; CLN, Clindamycin; MTZ, Metronidazole; AMX, Amoxacillin; LEV,Levofloxacin, N.A., not available. ** Isolates obtained from rectal swabs samples.

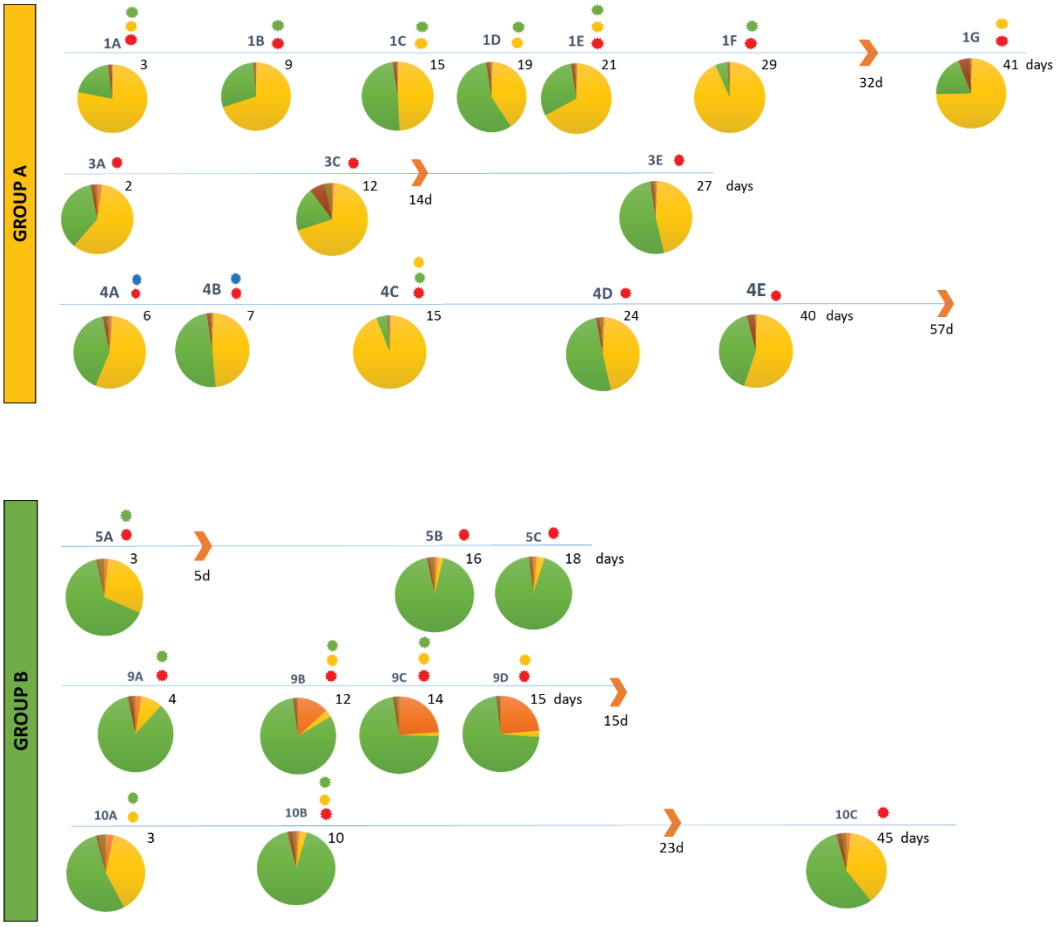


Figure 2. Relative abundance of the gut microbiota at phylum level (pie chart) per patient and per time point where samples were taken. Coloured dots indicate whether cultures were obtained for the following groups: red (Gram-positive aerobes), blue (Gram-negative aerobes), yellow (Gram-negative anaerobes), green (Gram-positive anaerobes). Orange arrows indicate the end of SDD therapy. Group A: Higher relative abundance of *Bacteroidetes* in more than 50% of samples collected during ICU stay – SDD therapy, Group B: High relative abundance of *Firmicutes* and Group C: Shift in the relative abundance of *Bacteroidetes* and *Firmicutes*.

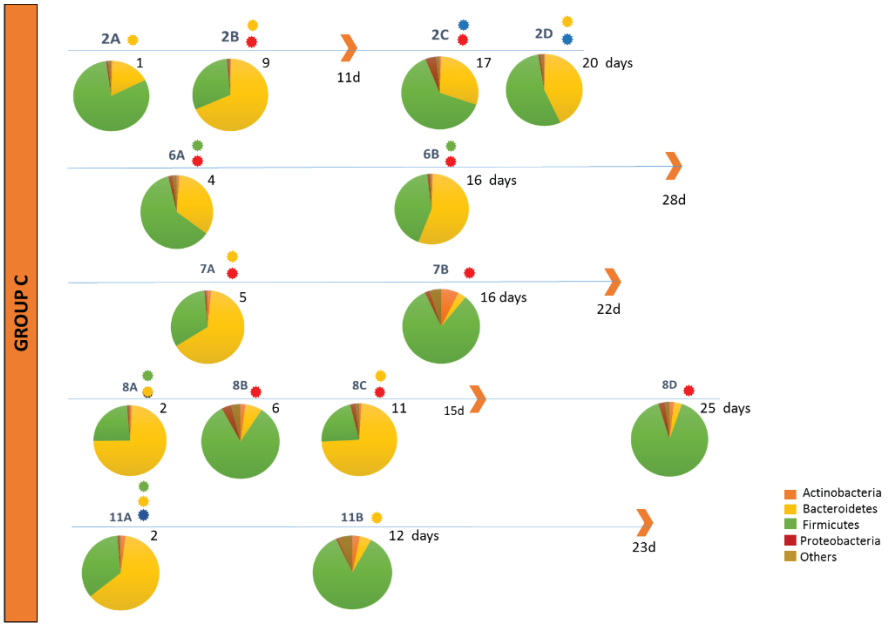


Figure 2. continued.

Microbial cultivation of aerobes and anaerobes

Overall, 130 isolates were obtained from the samples collected at initial ICU (19 isolates), ICU stay (72 isolates) and Post-ICU (39 isolates). Positive cultures for aerobes were obtained between 24-48h after incubation, while positive cultures for anaerobes were obtained mostly after 72h of incubation. A total of 70 aerobes and 60 anaerobes, including butyrate producing bacteria, were isolated on a range of different selective and non-selective culture media and further identified by 16S rRNA gene sequencing (**Table 3**).

The highest colony counts, considering all the positive cultures obtained per media per sample, expressed as CFU/ml were observed in BEA (1.79 E+ 5.0) and CNA (7.8 E+ 4.0) media for aerobes and in FAA (6.1 E+4.0), RCA (5.3 E+4.0) and BM (6.4 E+4.0) for anaerobes.

Table 3. Distribution of isolates by culture media used. Isolates were idendified based on their 16S rRNA gene sequence

Culture media	**Aerobes**	**Number of isolates**
BEA	Enterococci	48
C.N.A	Staphylococci	13
EMB -BHI	Enterobacteria	9
	Total of aerobic isolates	**70**
	Anaerobes	
RCA	*Clostridium spp*	11
MRS	*Lactobacillus lactis*	1
Bifidobacterium media	*Bifidobacterium animalis*	2
PYG	*Blautia coccoides*	1
	Eggerthella lenta	2
	Alistipes sp	2
FAA	*Anaerostipes sp*	6
BM	*Bacteroides sp*	17
	Odoribacter splancnicus	2
	Veillonella sp	1
	Parabacteroides sp	4
CP Acetate/Lactate	*Anaerostipes sp*	7
	Eubacterium limosum	1
	Ruminococcus sp	1
CP Acetate	*Anaerostipes sp*	1
CP Lactate	*Anaerostipes sp*	1
	Total of anaerobic isolates	**60**

In order to evaluate the effect of prophylactic antibiotic treatment on one of the functionally important microbial groups, potential secondary fermenting bacteria were quantified on bicarbonate-buffered anaerobic medium using either lactate, lactate and acetate or only acetate as the carbon source. Overall the total count of colonies differed between the carbon source used. Highest counts were observed when the combination of acetate/lactate was used (between 1.0 E+4.0 CFU/ml – 1.5 E+5.0 CFU/ml). When acetate and lactate were used as a single carbon source, less than 1.0 E+ 4.0 CFU/ml were obtained.

Pronounced inter-individual variation in the number and identity of aerobes and anaerobes isolated at the different time points was observed (**Table 4a,4b,4c**). A total of 61 Gram-positive and nine Gram-negative aerobic bacteria (total number of aerobes=70, comprising 13 species) were isolated in this study. From the group of Gram-positive aerobic bacteria, the most predominant genus identified in all groups was *Enterococcus* (48 isolates). A more detailed description of the enterococci, including their phenotypic and genotypic characterization, is provided in Chapter 6 of this thesis. The number of positive culture for *Staplylococcus sp* (n=13) increase in five patients during ICU stay. Co-colonization with *E. faecium* and/or *E. faecalis* and *Staphylococcus epidermidis* was observed during ICU stay in three patients. Only in the group of samples collected at post-ICU, we isolated and identified five additional *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. dispar, E. avium, E. canintestini*) and two other *Staphylococcus* species (*S. haemoliticus and S. warneri*). From the group of Gram-negative aerobes, we obtained isolates of *Escherichia coli* (n=6) and *Enterobacter cloacae* (n=3) from three patients belonging to group A and C.

We identified a total of 23 Gram-positive and 26 Gram-negative anaerobes using traditional culture media. From the group of Gram-positive anaerobes, the most predominant genus identified was *Clostridium* (11 isolates), including three different species (*C innocuum, C. aldenense and C. orbiscindens*). Members of two additional genera, *Lactococcus lactis* (n=1) and *Blautia coccoides* (n=1), were identified in samples obtained during the first 72h. Moreover, two different species of *Anaerostipes* (*A. caccae*, 4 isolates, and *A. rhamnosivorans*, 2 isolates) were identified during ICU stay. Other species identified corresponded to *Bifidobacterium animalis* (n=2) and *Eggerthella lenta* (n=2).

From the group of Gram-negative anaerobes, *Bacteroides* (17 isolates) was the most predominant genus, including five different species (*B. dorei, B. tethaiomicron, B. sp, B. fragilis and B. salyersiae*). Two different species of *Parabacteroides* (3 *P. distasoni* and 1 *P. goldsteini*), *Orodibacter splancnicus* (n=2) and two different species of *Alistipes* (*A. indistinctus* and *A. sp.*), were isolated during ICU stay. A single isolate of *Veillonella* was obtained from a post-ICU sample.

By using bicarbonate-buffered anaerobic medium supplemented with acetate, lactate or lysine used as a single carbon source and a combination of acetate and lactate, nine positive culture were obtained on acetate/lactate from five out of ten patients (37 samples in total; samples from patient 11 were not included due to limited number of samples available). In one of these five patients, an additional positive culture was obtained when lactate was used as a single carbon source. One isolate was obtained only in the presence of acetate as a carbon source. No positive culture was obtained with lysine as a single carbon source. By using 16S rRNA gene sequence analysis, we were able to identify the following bacteria: nine *Anaerostipes caccae*, one *Ruminococcus sp*, and one *Eubacterium limosum*. All isolates were found positive for PCR targeting the presence of the *but* gene that encodes butyryl CoA:acetate CoA transferase, one of the key enzymes of the butyrate producing pathways. The fermentative capacity of the isolates was tested by HPLC, with butyric acid representing 89% of the SCFA detected. The other SCFA include isobutyric acid in four out of 11 isolates. A summary of butyrate producing bacterial isolates is shown in **Table 5**.

Table 4. Number and identity of aerobes and anerobes isolated per patient, per group during ICU-hospitalization. Isolates were identified based on their 16S rRNA gene sequence, and showed in all cases 99-100% sequence identity with the 16S rRNA gene of cultured reference strains.

Group A	Initial ICU		ICU stay		Post-ICU	
Patient 1	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes
	E. faecalis [2]	*Bacteroides sp* *C. innocuum*	*E. faecalis* *S. epidermidis* [3]	*B. dorei* [2] *B. tethaiomicron* *C. innocuum* [2] *A. caccae* [4] *A. rhamnosivorans* [2] *P. distasonis*	*E. faecalis* *E. faecium* [2]	*B. dorei* *Veillonella sp*
Patient 3	*E. faecium*		*E. faecium*		*E. faecalis*	
Patient 4			*Enterobacter cloacae* [3] *E. faecium* [5] *E. faecalis* [3] *S. epidermidis*	*Bacteroides sp* *C. innocuum*		

Group B	Initial ICU		ICU stay		Post-ICU	
Patient 5	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes
	E. faecium [2]	*C. aldenense*			*E. faecium [3]* *E. avium [2]*	
Patient 9			*E. faecium [4]* *S. epidermidis*	*B. salyersiae* *Bacteroides sp* *B. dorei* *C. innocuum [2]* *P. distasoni [2]* *P. goldsteini [1]* *Bifidob. animalis* *B. tethaiomicron*		
Patient 10		*B. fragilis* *Bifidob. animalis*	*S. warneri* *S.epidermidis*	*B. fragilis* *C. innocuum*	*S. epidermidis [2]* *S. aureus* *S. haemolyticus*	

Group C	Initial ICU		ICU stay		Post-ICU	
Patient 2	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes
		A. indistinctus	*S. Epidermidis*	*Eggerthella lenta*	*Escherichia coli* [5] *E. casseliflavus* *E. canintestini* *E. dispar* *E. faecium* *E. gallinarum* *S. epidermidis*	*Eggetherlla lenta*
Patient 6			*E. faecalis* [2]	*C. innocuum* [2]		
Patient 7			*E. faecium* [4]	*B. tethaiomicron*		
Patient 8		*B. tethaiomicron* *L. lactis*	*E. faecalis* [2]		*E. faecalis* [2] *E. faecium* [5]	*B. fragilis*
Patient 11	*Escherichia coli*	*B. tethaiomicron* *C. orbiscindens* *O. splancnicus* *Blautia cocciode*		*B. tethaiomicron* *O. splancnicus* *Alistipes sp*		

Table 5. Identification of butyrate producers obtained by cultivation on bicarbonate-buffered anaerobic medium in the presence of acetate, lactate or a combination of both as the sole carbon source.

Butyrate producers/samples	Substrate utilization	(%) 16S rRNA gene identity
Patient 1 sample A	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 1 sample C	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 1 sample D	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 1 sample E	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 1 sample F	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 3 sample E	Acetate/Lactate	*Anaerostipes caccae (99)*
Patient 3 sample E	Lactate	*Anaerostipes caccae (99)*
Patient 4 sample B	Acetate/Lactate	*Ruminococcus sp (99)*
Patient 6 sample A	Acetate/Lactate	*Eubacterium limosum (99)*
Patient 5 sample B	Acetate	*Anaerostipes caccae (99)*
Patient 5 sample C	Acetate/Lactate	*Anaerostipes caccae (99)*

Antimicrobial susceptibility of aerobic isolates

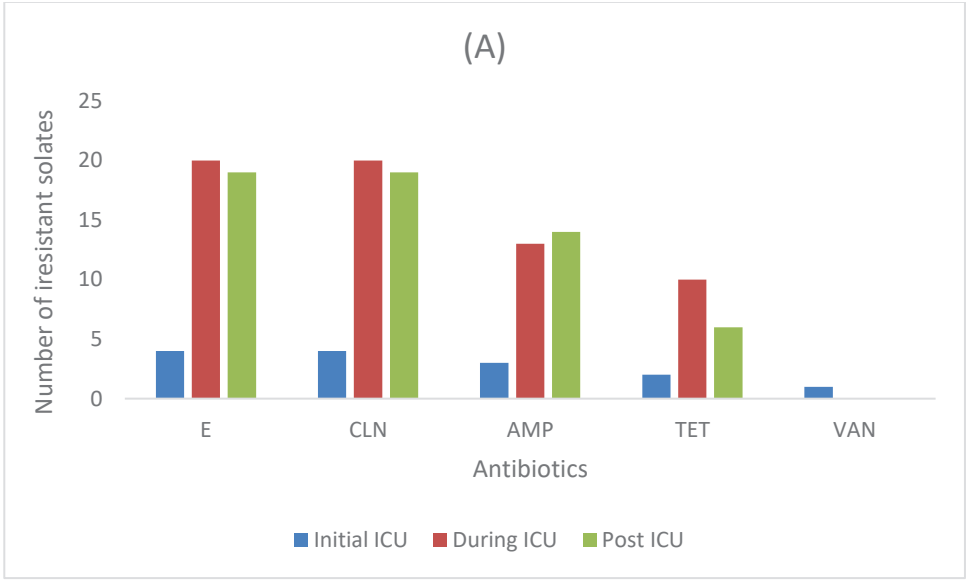
Overall, a high prevalence of resistance to erythromycin (13/13 staphylococci) was detected. The majority of these isolates displayed the constitutive erythromycin resistance phenotype (cMLSb), and no other MLSb phenotype was identified in the isolates.

The erythromycin ribosomal methylase genes *erm*C was detected in ten out of 13 staphylococci isolates. No vancomycin resistance was detected in the staphylococcal isolates.

A screening for methicillin resistance was performed by using oxacillin - penicillin disks and multiplex PCR for the *mec* gene and the *ccr* cassette. All staphylococcal isolates were resistant to oxacillin and penicillin, and the presence of the *mec*A gene was detected in 11 out of 13 isolates. Five of these carried SCC*mec* type IV (*ccr*4 – *ccr*A and *ccr*B) and SCC*mercury* (*ccr*C), three carried SSC*mec* type II (*ccr*2 – *ccr*A and *ccr*B) and SCC*mercury*, and a single *Staphylocccus epidermidis* isolate carried SSCmec types II and IV together with the SCCmercury. In two *mec*A positive isolates identified as *Staphylococcus haemoliticus* and *Staphylococcus aureus*, the *ccr* gene was not detected. An increased resistance to amoxacillin-clavulanic acid was detected in four *Staphylococcus* isolates (two. *S. epidermidis,* one *S. haemoliticus* and one *S. aureus*) during ICU stay in three patients who developed a secondary infection caused by Gram-negative bacteria.

Resistance to tetracycline was detected in three *S. epidermidis* isolates and in five *Escherichia coli* isolates, whereas resistance to chloramphenicol was detected only in two *S. epidermidis* isolates identified in a single patient during ICU stay.

Regarding the resistance profile obtained for the aerobic Gram-positive bacteria during the study period indicated that during ICU hospitalization, an increase in antibiotic resistant Gram-positive bacteria was observed as compared to the initial ICU samples (**Fig. 3a and 3b**). Among the extended spectrum beta-lactamase (ESBL) phenotype investigated in the Gram-negative isolates, the BLEE phenotype was detected in four *E. coli* isolates from one patient during the post-ICU sampling period. Two of the three *E. cloacae* isolates from one patient showed the cephalosporinase AmpC inducible and AmpC hyper-production phenotype, respectively. No resistance to carbapenem and colistin was detected among the Gram-negative aerobic isolates. The susceptibility to tobramycin and polymyxin E was tested in *Enterobacter cloacae* isolates obtained in BHI media, however, no resistance to these antibiotics was detected.



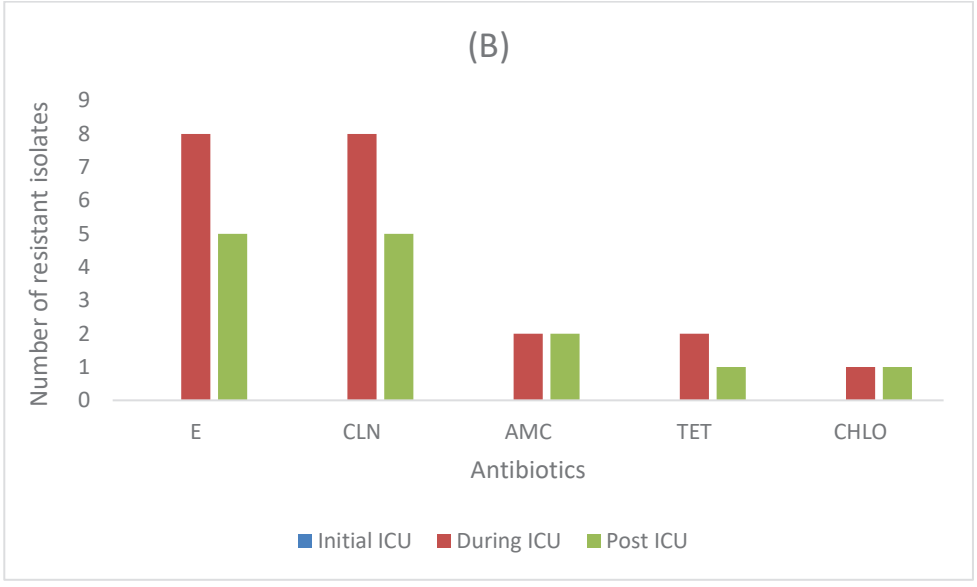


Fig 3. Resistance phenotype observed in **A**) enterococci and **B**) staphylococci during the study period.

Abbreviations: E, Erythromycin; CLN, Clindamycin; VAN, Vancomycin; AMP, Ampicillin; AMC, Amoxacillin-clavulanic acid; TET, Tetracycline; CHLO, Chloramphenicol.

Antimicrobial susceptibility of anaerobic isolates

A high prevalence of resistance to tetracycline (38 out of 60 anaerobic isolates) was observed, with 15 Gram-positive isolates including *Blautia coccoides* (n=1), *Bifidobacterium animalis* (n=2), *Lactobacillus lactis* (n=1), *Anaerostipes* (n=6) and *Clostridium innocuum* (n=5), and 23 Gram-negative isolates including 16 out of 17 *Bacteroides* isolates, *Odoribacter splancnicus* (n=2), *Alistipes indistinctus* (n=2), *Veillonella sp*. (n=1) and *Parabacteroides distasonis* (n=2). Besides tetracycline, also resistance to erythromycin and the lincosamide clindamycin was highly prevalent among anaerobic isolates, including 12 Gram-positive isolates: *Anaerostipes caccae* (n=4), *Clostridium innocuum* (n=5), *Blautia coccoides* (n=1) and *Bifidobacterium animalis* (n=2), and three Gram-negative isolates, including *Veillonella* sp. and *Alistipes indistinctus* isolates. No resistance to vancomycin was observed in *Anaerostipes* or *Clostridium i*solates.Resistance to metronidazole was found only in *Bacteroides tethaiomicron* (n=3) isolates. From the beta-lactams class of antibiotics, resistance to ampicillin was observed *in Anaerostipes caccae* (n=4), and *Odoribacter splancnicus* (n=2) isolates, whereas carbapenems (meropenem and imipenem) resistance was observed in two *Bacteroides dorei* isolates, two *Parabacteroides* isolates (*P. distasonis* and *P. goldsteini*), and in a single *B. tethaiomicron* isolate during and after ICU stay. Resistance to cefotaxime was limited (8%), and observed in four isolates identified as *Bacteroides tethaiomicron* (n=2), *Bifidobacterium animalis* (n=1) and *Parabacteroides distasonis* (n=1).

For the group of butyrate producing bacteria detected by cultivation on bicarbonate-buffered anaerobic medium supplemented with lactate and acetate as a single carbon source, only one out of 11 isolates were resistant to ampicillin (*Anaerostipes caccae*).

Discussion

During ICU hospitalization, patients are exposed to a selective pressure of antibiotic treatment, parenteral nutrition and use of drugs for example to accelerate gastric motility. These factors, together with the host physiological stress, can contribute to the disruption of the ecological balance of the gut microbiota with, as a consequence, reduced microbial diversity, changes in microbial composition, and selection of resistance genes in the remaining community (Zaborin *et al*., 2014). In spite of the fact that prophylactic antibiotic therapies such as SDD have been shown to reduce the morbidity and mortality in ICU patients, the impact of such therapies on colonization with antibiotic resistant bacteria, and especially regarding anaerobes, remains poorly characterized (Ochoa-Ardila *et al.,* 2011). Therefore, we followed the dynamics and diversity of the gut microbiota of eleven ICU hospitalized patients under SDD therapy using HITChip phylogenetic analysis and cultivation in aerobic and anoxic conditions.

HITChip phylogenetic analysis revealed three different patterns at the phylum level in the studied patients, allowing for stratification based on the relative abundance of *Bacteroidetes* and *Firmicutes*. However, no association of any of the three groups (A, predominant *Bacteroidetes*; B, predominant *Firmicutes*; C, shifting) with specific antibiotic treatment, hospital-acquired infection, comorbity or length of ICU stay could be detected. In addition, no statistical differences were observed between groups based on the diversity of microbiota composition. It has been previously shown that the gut microbiota is particular per individual and that internal and external factors influence the composition (Ley *et al*., 2006). In this study we showed pronounced dynamics of gut microbiota diversity and composition during ICU hospitalization. We can, however, not exclude that in addition to the SDD therapy, other factors, including application of additional antibiotics for the control of nosocomial infections, also affected the gut microbiota. Previously, Dubourg and collaborators (2014) showed, that during antibiotic treatment, the total number of bacteria was not affected systematically, but that especially prolonged treatment with broad spectrum antibiotics can affect microbial composition.

At the genus level, the relative abundance of *Enterobacteriaceae* was decreased, in line with the goal of the SDD protocol, and low rate of infection by members of this family were found during the study period. In contrast, the relative abundance of enterococci increased, confirming that these are not targeted by the therapy. Furthermore, during ICU stay, also a reduction in the relative abundance of *Clostridium* clusters XV and XIVa was observed. Members of these bacterial groups play important roles in maintaining colonization resistance and represent the main source for butyrate production, which promotes the growth of colonocytes and contributes to mucosal stability (Pride *et al*., 2002). Our results confirm that SDD therapy has an impact on the composition of the anaerobic gut microbiota as previously reported (Benus *et al.,* 2010).

By using traditional cultivation-based approaches, we were able to capture a broad range of taxonomic groups, which allowed us to determine the antibiotic resistance phenotype of individual isolates directly, and link this information with the HITChip phylogenetic analysis.

For the group of aerobic bacteria that in many cases represent potential pathogens, a high preavelence of antibiotic resistant-enterococci was detected in all the patients throughout the study. A more detailed analysis of the resistance phenotype, resistance genes and virulence factors present in these isolates is provided in Chapter 6 of this thesis. Besides enterococci, staphylococcal isolates carrying methicillin-resistance genes were detected during and after ICU stay in the three patient groups, and similar to enterococci, an association with staphylococcal nosocomial isolates could not be established. Neither vancomycin resistant enterococci (VRE) nor methicillin resistant *Staphylococcus aureus* (MRSA) were found in this study, in line with their low prevalence in ICU patients receiving SDD therapy as previously described (Daneman *et al.,* 2013). On the other hand, low prevalence of antibiotic resistant *Enterobacteriaceae* was found. Two particular cases of faecal carriage of antibiotic resistant *Enterobacteriaceae* are represented by patient 2 and 3.

Patient 2 carried extended-spectrum betalactamase (ESBL)-producing *Enterobacteriaceae* during and after SDD without developing nosocomial infection, whereas patient 3 carried a cephalosporin resistant *E. cloacae* at the beginning of the ICU stay, and developed a bloodstream and pleural infection during ICU stay by a cephalosporin resistant *E. cloacae*. Previous studies indicated that the prevalence of ESBL and cephalosporin resistance in Gram-negative bacteria decreased during SDD therapy (Oostdijk *et al.,* 2012; Camus *et al.,* 2016).

For the group of Gram-positive anaerobes, members of *Clostridium* and *Anaerostipes* were most often retrieved. From the group of *Clostridium* isolates, *C. innoccum* and *C. aldenense* have been infrequently associated with human infections (Crum-Ciaflone *et al*., 2009; William *et al.,* 2010), whereas *C. orbiscindens* is known for the ability of cleaving flavonoids compounds, which have beneficial effects on human health based on a variety of properties (Shoefer *et al*., 2003). All of these isolates were obtained only at the initial ICU time point and in the first sample obtained after 72h of SDD therapy initiation. Among these isolates, only *C. innoccum* isolates were resistant to tetracycline, macrolides, lincosamides and carbapenems, in line with what has been described previously (Stark *et al.,* 1993). Two different species of *Anaerostipes*, *A. caccae* and *A. rhamnosivorans,* were isolated from samples obtained from a single patient during ICU stay. Both species have been previously recognized as members of the butyrate producing bacteria present in the gut microbiota (Schwiertz *et al.,* 2002; Bui *et al.,* 2014). Both species were resistant to tetracycline, while only *A. caccae* isolates were also resistant to macrolides, lincosamides and ampicillin. Resistance to tetracycline is the only resistance phenotype reported for *Anaerostipes caccae* (Antibiotic Resistance Genes Database (ARDB;http://ardb.cbcb.umd.edu/cgi/search.cgi?db=L&field=ni&term=ZP_02419744). To the best of our knowledge, this is the first report that describes this resistance phenotype in *A. caccae*. Furthermore, by using bicarbonate-buffered anaerobic medium, nine additional *A. caccae* isolates were obtained from the same patient in addition to another two patients. From one of them, another ampicillin resistant *A.caccae* isolate was detected.

Considering that in this particular culture medium, the SDD cocktail was used, only a selective group of Gram-positive anaerobic bacteria was able to grow.

Our data showed that six of the eleven patients carried enterococci and *Clostridium* with the same resistance phenotype (macrolide and tetracycline resistance), suggesting that a transfer of resistance genes between Gram-positive aerobes and anaerobes might occur as previously indicated (Salyers *et al.,* 2004). Antibiotic resistance in bacteria used as probiotics has been previously reported (Gueimonde *et al.,* 2013). In this study we isolated representatives of two bacterial genera of which strains are often marketed as probiotics, i.e. *Bifidobacterium* and *Lactobacillus*. All isolates were found to be resistant to tetracycline and macrolides. Since efflux pumps are involved in resistance to both groups of antibiotics as a common mechanism, and since both genera are frequently used as a probiotics, particular attention is required with respect to the antibiotic resistance of probiotics strains.

For the group of Gram-negative anaerobes, *Bacteroides* and *Parabacteroides* constituted the most predominant genera. Resistance to tetracycline was detected in 94% of the *Bacteroides* isolates (16 out of 17) and 50% of *Parabacteroides* isolates (2 out of 4). Multidrug resistance was detected only in two species of *Bacteroides* (*B. fragilis* and *B. dorei*), and in one *Parabacteroides distasonis* isolate. In fact, a trend towards increased resistance of these species to carbapenems and cephalosporins such as cefoxitin in Europe has been reported recently (Nagy *et al*., 2011; Trevino *et al.,* 2012). Resistance to macrolides and lincosamides was detected only in isolates identified as *Veillonella* and *Alistipes indistinctus*, while resistance to metronidazole was found in *B. tethaiomicron* isolates. Veloo and van Winkelhoff recently studied the antibiotic susceptibility profile of anaerobic pathogens in the Netherlands by E-test and MIC determination and observed an increase in the prevalence of resistance to clindamycin in *B. fragilis* while no resistance to metronidazole were detected (Veelo *et al.,* 2015).

It has to be acknowledged that the study reported here was constrained by a number of limitations: a) the limited numbers of patients, b) the heterogeneous set of samples obtained per patient due to constipation, administration of opioids and clinical conditions, c) absence of a control group, since the majority of ICUs in The Netherlands uses the SDD protocol for the control of infection in ICU patients, d) the unavoidable use of systemic antibiotics, e) the lack of control for cross-transmission and re-colonization, and f) the absence of biotyping for clinical isolates.

To conclude, in this study we observed that the diversity and dynamics of the gut microbiota composition was affected during SDD therapy. Molecular analysis indicated that the relative abundance of *Enterobacteriaceae* was reduced during SDD therapy, whereas enterococci were significantly increased. In addition, SDD therapy seemed to negatively affect the anaerobic gut microbiota. Furthermore, cultivation on a range of complementary media yielded a diverse and dynamic range of aerobic and anaerobic bacteria, including butyrate producing bacteria. To this end, we observed an increased prevalence of antibiotic resistance in Gram-positive bacteria, and mainly among enterococci, and the suppression of resistance within enterobacteria. The variety of taxonomic groups obtained by anaerobic cultivation supports the idea that these groups of microorganisms act as reservoir for the accumulation of antibiotic resistance genes that can be acquired by and/or transferred to other commensal bacteria and pathogens.

In general, high prevalence of resistance to tetracycline, macrolides and lincosamides was detected in this group of isolates, however, it should be considered that antibiotic resistance patterns can vary between species, hospital, country and antibiotic administration and that only a selective group of antibiotics was tested in all the isolates. Future analysis on antibiotic resistance patterns in anaerobes, identification of the resistance genes and monitoring the antibiotic resistance in commensals and potential pathogen isolates could contribute to a more quantitative estimation of the spread of antibiotic resistance.

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CHAPTER 7

Dynamics of *Enterococcus* colonization in intensive care unit hospitalized patients receiving prophylactic antibiotic therapies

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Abstract

Enterococci have emerged as important opportunistic pathogens in intensive care units (ICUs). In this study, the dynamics of *Enterococcus* spp. colonization in ICU hospitalized patients receiving prophylactic antibiotic therapies was investigated. In total 48 *Enterococcus* spp. strains were isolated and characterized from 11 patients at different time points during and after ICU hospitalization, including *E. faecalis* (n=17), *E. faecium* (n=26), *E. gallinarum* (n=1), *E. dispar* (n=1), *E. avium* (n=2) and *E. canintestini* (n=1). Multi locus sequence typing revealed a high prevalence of ST 6 in *E. faecalis* isolates (59%) and ST 117 in *E. faecium* (46%). Also a new sequence type, ST 589, was identified, representing four *E. faecalis* isolates.

Furthermore, the antibiotic resistance phenotyping and the presence of vancomycin and macrolide resistance as well as virulence factor-encoding genes (*asa*1, *esp-fm, esp-fs*, *hyl* and *cyl*) was investigated in all *Enterococcus* strains. Fourty-five out of 48 isolates displayed the cMLSb phenotype, and 34 of them harboured the *ermB* gene. Vancomycin resistance was detected only in a single strain (*E. gallinarum*), encoded by the *vanC1* gene. Furthermore, 31 (65%) and 23 (48%) of the isolates were resistant to ampicillin and tetracycline, respectively. The most prevalent virulence genes were *asa*1 in *E. faecalis* (65%) and *esp* (*esp-fm* (69%), *esp-fs* (59%)).

Our results show that multiple *Enterococcus* species carrying several antibiotic resistance and virulence genes, occurred simultaneously in five individual patients. Furthermore, simultaneous presence and/or replacement of *E. faecium* sequence types was observed, further reinforcing the importance of enterococci as a potential cause of nosocomial infections in critically ill patients.

Introduction

The genus *Enterococcus* encompasses indigenous commensal bacteria reported from the human and animal gut as well as the oral cavity and vagina in humans, where they have adapted to a nutrient-rich, oxygen-depleted and ecologically complex environment (Kayaoglu and Ørstavik , 2004).

In the human gut, the genus *Enterococcus* can constitute up to 1% of the total bacterial microbiota in healthy individuals, with *Enterococcus faecium* and *Enterococcus faecalis* as most common species (Sghir *et al.,* 2000). In contrast to their commensal role, over the past decades *E. faecium* and *E. faecalis* have also emerged as agents of nosocomial infections such as endocarditis, bacteraemia, meningitis, wound and urinary tract infections (Klein, 2003; Dwormiczek *et al.,* 2012). In addition, other enterococcal species including *Enterococcus durans*, *Enterococcus avium*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus raffinosus*, and *Enterococcus hira*e have sporadically been associated with infections in humans (Klein, 2003).

Most of the *E. faecium* and *E. faecalis* infections are opportunistic and are increasingly difficult to treat due to high rates of resistance to β-lactams, aminoglycosides and vancomycin, which are mostly associated with *E. faecium* strains (Cattaneo *et al.,* 2000; Huycke *et al.*, 1998).

In addition, both *E. faecium* and *E. faecalis* can carry a variety of genes that contribute to virulence in the immunocompromised patient. For *E. faecalis* these include e.g. genes encoding aggregation substance (*asa1*) (Hallgren *et al.,* 2009), cytolysin (*cyl*) (Jett *et al.,* 1994), enterococcal surface protein (*esp-fs*) (VanKerckhoven *et al.*, 2004) and haemolysin (*hly*) (Libertin *et al.,* 1992), whereas for *E. faecium* genes associated with virulence encode, among others, a putative hyalorunidase (*hyl*) (Fisher and Phillips, 2009) and enterococcal surface protein (*esp-fm*) (Hendrickx *et al.*, 2012).

Modes of action include i) colonization of a specific niche such as enterococcal adherence to renal tubular cells and neutrophils (Guzman *et al.,* 1989; Joyanes *et al.,* 2000), ii) evasion or inhibition of the immune response by e.g., destruction of red blood cells and secretion of toxins that affect the host defence systems (Kreft *et al.,* 1992; Olmestd *et al.,* 1994), iii) biofilm formation (Top *et al.,* 2013), or iv) obtaining nutrients from the host (Vergis *et al*., 2002). Similar to resistance genes, virulence genes are also frequently encoded on mobile elements and are therefore thought to disseminate frequently via intra- and interspecies horizontal gene transfer within the genus *Enterococcus* (Laverde *et al.,* 2011; Coburn *et al.,* 2007).

Intestinal commensal enterococci in healthy humans rarely harbour genetic elements that contribute to antibiotic resistance or confer virulence, and for decades enterococci have been used as probiotics both in humans and farm animals (Mundy *et al.,* 2000; Arias *et al*., 2012). In contrast, *Enterococcus* isolates derived from clinical and animal sources frequently carry virulence factors and in several cases have been associated with high levels of antibiotic resistance (Eaton and Gasson, 2001; Franz *et al.,* 2001). The genomic diversity of *E. faecalis* and *E. faecium* isolates encountered in hospitals is of particular interest. Studies using Multi Locus Sequence Typing (MLST) have shown that there is a remarkable difference in the population structure between *E. faecalis* and *E. faecium* (Palmer *et al.,* 2014). In *E. faecium*, high-risk clonal-complexes exist, which exhibit high levels of antibiotic resistance and are significantly associated with clinical infections in hospitalized patients (Leavis *et al.,* 2006; Willems *et al*., 2012; Lebraton *et al.,* 2013).

Patients in an intensive care unit (ICU) are at a high risk for developing nosocomial infections with multi-drug resistant bacteria due to impaired health and often strong selective antibiotic pressure (Streit *et al.,* 2004). Several studies have shown that the exposure of patients to broad-spectrum antibiotics, combined with prolonged hospital stay, can result in colonization by multi-drug resistant enterococci leading to nosocomial transmission and infection (Austin *et al.,* 1999; Carmeli *et al.,* 2002).

The prophylactic therapies Selective Oropharyngeal Decontamination (SOD) and Selective Digestive Decontamination (SDD) aim to prevent secondary infection with potential pathogens in ICU hospitalized patients and decrease mortality in these patients, compared to standard care (de Smet *et al.,* 2009). While SOD and SDD can efficiently suppress gut colonization by Gram-negative bacteria, an increase of enterococci during SDD therapy was observed when compared to other regimens (van der Bij *et al.,* 2016).

This reflects the fact that enterococci were not considered a target during the introduction of SDD in the ICU, and it has been demonstrated that, for example, *E. faecalis* colonization increases during similar usage of topical antibiotics (Bonten *et al.*, 1995).

Previous studies on the effect of colonization by enterococci during SOD and SDD therapies have only addressed the presence or absence of enterococci (de Smet *et al.,* 2009). Therefore, we decided to investigate the dynamics of *Enterococcus* colonizing ICU hospitalized patients receiving SOD and SDD therapy and to evaluate in more detail the genetic relatedness of *E. faecalis* and *E. faecium* isolates, using MLST and Bayesian analysis of the population structure (BAPS). Furthermore, we determined carriage of genes encoding antimicrobial resistance and virulence determinants in this population.

Materials and Methods

Patients, bacterial culture conditions and initial characterization

Enterococci were isolated from 28 out of 40 faecal samples obtained from 11 hospitalized patients: two patients received SOD (patients 100 and 101) and nine received SDD (patients 1 - 9). The SOD and SDD protocols were reviewed and approved by the institutional review board of the University Medical Center Utrecht (Utrecht, The Netherlands). The SOD protocol consisted of an oral application of 0.5 g of a paste containing 2% tobramycin 2% polymyxin E and 2% amphotericin B, given four times daily. The SDD protocol comprised the application of oral antibiotics identical to the SOD regime. In addition, a suspension containing 80 mg tobramycin, 100 mg polymyxin E and 500 mg amphotericin B was administered through a gastric tube four times daily, and cefotaxime (4x 1000 mg) was given intravenously for the first four days after ICU admission. The isolates were obtained from faecal samples taken during hospitalization and classified according to the collection time: Intitial ICU (samples taken during the first 72h at ICU, n=5), during ICU (samples taken after the first 72h at ICU; individual patients stayed in the ICU for up to 40 days, n=27) and post-ICU (samples taken after ICU discharge – ward / SDD-SOD discontinuation, n=8).

In order to isolate enterococci from faecal samples, we used Bile-Esculin Agar (BEA) (Oxoid B.V., Landsmeer, The Netherlands). Typical colonies were selected for phenotypic and biochemical characterization by standard methods (Winn *et al.,* 2006). Haemolysis was determined by cultivation on Blood Agar supplemented with 5% sheep blood (Oxoid) after incubation at 37^{o}C for 24 hours.

DNA isolation was performed using the protocol for Gram-positive bacteria of the QIAamp® DNA Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands). DNA was used for the identification of the isolates and detection of antibiotic resistance and virulence genes by Polymerase Chain Reaction (PCR) as described below.

Identification and classification of isolates

The complete bacterial 16S ribosomal RNA (rRNA) gene was amplified from genomic DNA using T7prom-Bact-27-F and Uni-1492-R primers as described previously (Rajilic-Stojanovic *et al.,* 2009). The amplified fragments were selected for partial sequence analysis of the 16S rRNA gene (~800bp) using the 16S-1392R primer 5'-ACGGGCGGTGTGTRC -3' (GATC Biotech, Cologne, Germany).

Partial 16S rRNA gene sequences obtained in this study were deposited at GenBank under accession numbers KX577731, KX577732, KX577733, KX577734.

Antimicrobial susceptibility

Vancomycin resistance of enterococci was tested on Mueller-Hinton Agar (MHA) (Oxoid) containing 6 μg/ml vancomycin. Colonies were tested by E-test (Biomerieux) to determine the minimal inhibitory concentration (MIC) of vancomycin, following CLSI guidelines (CLSI, 2013). Resistance to macrolides and lincosamides, more specifically to erythromycin and clindamycin, was tested by using a double disk diffusion test (Thumu and Halami, 2014), in order to determine the Macrolide Lincosamide Streptogramin B phenotype (MLS_B). In brief, isolates were grown on MHA in the presence of an erythromycin (15μg) disk and one containing the lincosamide clindamycin (10 μg), separated by 20mm. As erythromycin would act as an inducing agent, isolates carrying erythromycin resistance genes will grow in the presence of clindamycin. A D-shaped inhibition zone around the clindamycin disk indicates an inducible MLS_B phenotype ($iMLS_B$). Resistance to both antibiotics, i.e. lack of any inhibition zone, indicates a constitutive MLS_B phenotype ($cMLS_B$). Isolates carrying the *mef* gene will show resistance to erythromycin and sensitivity to clindamycin with a circular zone of inhibition around clindamycin indicating the M phenotype (Thumu and Halami, 2014). In addition, the disk diffusion method was used to test for susceptibility to ampicillin (10 μg) and tetracycline (30 μg) (CLSI, 2013).

Detection of antibiotic resistance- and virulence factor-encoding genes

Antibiotic resistance genes were detected using a multiplex PCR for the vancomycin-resistance genes *vanA, vanB*, and *vanC* (*vanC1 – vanC2/vanC3*) (Depardieu *et al*., 2004), and a single PCR for *ermA, ermB, ermC* and *mefA/mefE* genes (Zou *et al.,* 2011). PCR products of *mefA* and *mefE* genes were discriminated by BamHI restriction analysis, as only *mefA* carries a single restriction site, giving rise to fragments of 284bp and 64bp as described previously (Klaassen and Moutin, 2005). Genes coding for virulence factors, i.e. enterococcal surface protein (*esp-fm, esp-fs*), aggregation substance (*asa*1), cytolysin (*cyl*B) and hyalorunidase (*hyl*), were selected for detection by PCR as described previously (Hallgren *et al.,* 2009; Vankerkhoven *et al.,* 2004). *E. faecalis* ATCC29212, *E. faecium* (E5) and *E. faecalis* (E507) (Department of Medical Microbiology, Utrecht Medical Centre, UMC, The Netherlands) and *E. gallinarum* HSIEG1 (van den Bogert *et al.,* 2013) (Laboratory of Microbiology, Wageningen University, The Netherlands) were used as positive controls for the detection of antibiotic resistance and virulence factor encoding genes. Amplicons were visualized by agarose gel electrophoresis.

Clonal relatedness and analysis of population structure

In order to establish the clonal relationship of *Enterococcus* isolates, we applied the MLST schemes proposed by Ruiz-Garbajosa *et al*. 2006 and Homan *et al*. 2002 for *E. faecalis* and *E. faecium*, respectively. Sequences were compared with published alleles, and sequence types (STs) were assigned using the MLST database (http://pubmlst.org/efaecium/ and http://pubmlst.org/efaecalis/). BAPS groups were determined as previously described (Willems *et al.,* 2012).

Results

Diversity of intestinal *Enterococcus* isolates from intensive care patients

A total of 48 isolates was obtained from 40 fecal samples of 11 ICU hospitalized patients and classified to the enterococcal species level by 16S rRNA gene sequencing. The most commonly found species were *E. faecium* (26 isolates) and *E. faecalis* (17 isolates). Other enterococcal species identified included *E. avium* (2 isolates), *E. canintestini* (1 isolate), E. *gallinarum* (1 isolate) and *E. dispar* (1 isolate), all of which were isolated only during the post-ICU phase (**Figure 1, Supplementary Table S1**). From five of the 11 patients, faecal samples could be collected during the first 72h after ICU admission, three of which were colonised with *E. faecalis* (n=2) and *E. faecium* (n=3). The same strains were also found in samples taken later from these patients during ICU stay and post-ICU. During ICU stay (i.e. from day 4 until the end of SOD - SDD therapies), isolates of *E. facalis* (n=13) and *E. faecium* (n=15) were retrieved from nine patients. Throughout post-ICU, *E. faecalis* isolates (n=2) were identified in only two patients, while *E. faecium* isolates (n=8) were identified in four patients. In addition, five isolates belonging to other species were obtained after SDD therapy in two of the patients.

Six patients receiving SDD developed nosocomial enterococcal infections during ICU stay (**Figure 1**), including one pleural infection caused by *E. faecium*, six urinary tract infections (two episodes in a single patient) caused by *E. faecalis* (five cases) and *E. faecium* (one case), and one central line catheter associated infection caused by *E. faecalis* (two episodes in a single patient). Unfortunately, however, these isolates were not available for further analysis.

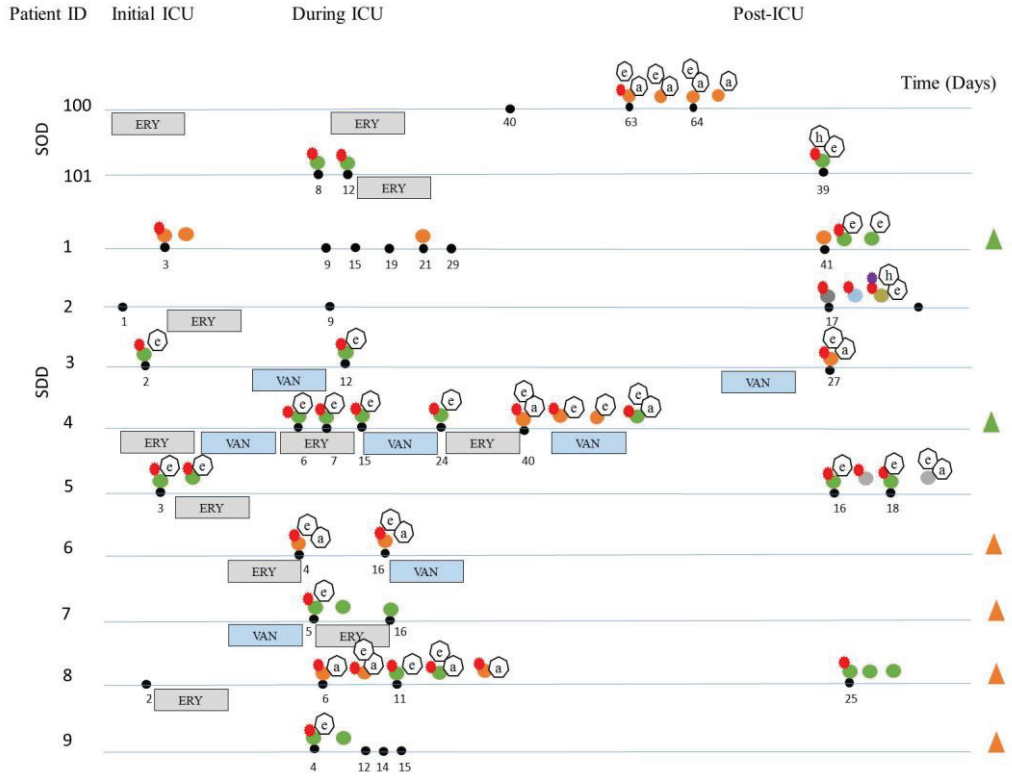

Figure 1. Overview of the dynamics of colonization by *Enterococcus* species and carriage of antibiotic resistance and virulence genes during and after ICU hospitalization. The black dots indicate days where fecal samples were taken during hospitalization. The different species isolated are indicated by differently coloured dots: orange (*E. faecalis*), green (*E. faecium*), dark grey (*E. canintestini*), blue *(E. dispar*), brown (*E. gallinarum*) and light grey (*E. avium*). Isolates not connected to a black dot were obtained from the sample closest to the left. The presence of antibiotic resistance genes is indicated by red (*erm*B) and purple dots (*van*C1). Virulence factors are shown in heptagonal shapes (a-*asa*1), (e-*esp-fm* and *esp-fs*), (h-*hyl*). Patients that developed nosocomial infections during ICU stay with *E. faecalis* and *E. faecium* are indicated by green (*E. faecium*) and orange (*E. faecalis*) triangles. Grey and blue boxes indicate systemic antibiotics given under clinical indications (ERY= erythromycin, VAN = vancomycin).

Antimicrobial susceptibility

Vancomycin susceptibility testing showed that a single isolate of *E. gallinarum* obtained during the post-ICU phase was resistant to vancomycin (MIC 16 µg/ml). All other isolates were susceptible to vancomycin (MIC 1.5-2 µg/ml). Forty-five out of 48 enterococcal isolates were resistant to both erythromycin and clindamycin (constitutive phenotype – $cMLS_B$). No other erythromycin – clindamycin phenotype was detected.

Ampicillin resistance was detected in 31 out of 48 isolates (24 *E. faecium*, 6 *E. faecalis* and 1 *E. avium*). The highest prevalence of resistant strains was found amongst *E. faecium*, the majority of them being obtained from the group of patients that received SDD therapy.

Ampicillin resistant isolates were obtained from samples taken during and after ICU stay and in two patients during the first 72h after ICU admission. Resistance to tetracycline was detected in 23 out of 48 isolates (11 *E. faecalis*, 9 *E. faecium*, 1 *E. dispar, 1 E. avium* and *1 E. canintestini*), the majority of which was obtained during ICU stay and in one patient during the first 72h after admission. A complete overview of resistance phenotypes is given in **Table S1.**

Detection of antibiotic resistance- and virulence factor-encoding genes

Because 45 out of 48 enterococcal isolates displayed the $cMLS_B$ phenotype, we assayed these for the presence of the *ermA*, *ermB* and *ermC* genes, which encode macrolide-lincosamide-streptogramin (MLS) resistance. PCR-based detection of antibiotic resistance genes revealed the presence of the *ermB* gene in 34 out of 45 erythromycin-resistant isolates that were obtained during the entire study period. No other MLS_B resistance genes were detected. From the group of vancomycin resistance genes tested, only the *vanC1* gene was identified in the single *E. gallinarum* isolate that was also found resistant (**Figure 1, Table S1**).

Three out of the four targeted genes encoding enterococcal virulence factors were detected. The *asa1* gene was frequently present in *E. faecalis* isolates (n=11), whereas the *esp* gene was more often found in *E. faecium* isolates (n=18), including three isolates obtained during the first 72h after ICU admission. The *esp* gene was also present in one *E. avium* and one *E. gallinarum* isolate.

Finally, the *hyl* gene was detected post-ICU in a single isolate of *E. faecium* and *E. gallinarum*. The *cylB* gene was not detected in any of the isolates (**Figure 1**).

We detected the presence of more than two virulence factor genes and/or virulence factor and antibiotic resistance genes in 27 out of 48 individual isolates during the entire study period. Among these 27 isolates, *E. faecium* isolates (n=17) were associated with the presence of *ermB* and *esp* genes and high levels of resistance to ampicillin, whereas *E. faecalis* isolates (n=8) were more frequently associated with the presence of *asa* and *ermB* genes and low levels of ampicillin resistance. The other two isolates included one *E. faecalis* isolate associated with the presence of *esp* and *ermB* genes and one *E. gallinarum* carrying *esp, hyl*, *ermB* and *vanC1* genes.

Clonal relatedness and analysis of population structure.

Using MLST, we established the clonal relationship of all *E. faecium* and *E. faecalis* isolates obtained in this study. In total, we identified seven different STs among the *E. faecium* isolates (**Figure 2, Table 1, Table S1**). Analysis of their population structure using BAPS revealed that these STs belonged to four BAPS (sub) groups, which were previously associated with hospitalized patients (Willems *et al.,* 2012). The majority of the STs belonged to BAPS group 2.1a (19 isolates), and 16 of them were susceptible to tetracycline and resistant to ampicillin (ST117 n=12, ST78 n=3 and ST730 n=1). Other sub-groups observed included BAPS 3.2 (2 isolates), BAPS 1.2 (2 isolates) as well as BAPS 3.3a2 (3 isolates). In five patients, we identified two or more different STs in the same patient during hospitalization (**Figure 2**).

Among the *E. faecalis* isolates, we identified three STs (ST6, ST81 and ST16), which were previously detected among hospitalized patients (27), as well as a new ST (ST589), represented by four isolates (**Figure 2 and Table 1**). All isolates belonging to ST589 were susceptible to ampicillin but resistant to tetracycline, and were obtained from a single patient from samples taken throughout the study. Three out of these four ST589 isolates showed the cMLSb resistance phenotype, and carried the *ermB* gene. From the group of *E. faecalis* isolates belonging to ST6 (n=10), seven carried *ermB, asa* and *esp* genes and were susceptible to ampicillin, while the other three isolates carried *asa* and *esp* genes and displayed resistance to ampicillin. BAPS cluster analysis subdivided the *E. faecalis* isolates into BAPS groups 1 (11 isolates) and 3 (2 isolates) (**Table 1**). In contrast to the situation in the *E. faecium* isolates, we neither detected simultaneous presence of *E. faecalis* STs nor clonal replacement over time within individual patients.

Table 1. Sequence type (ST) and BAPS analysis of *Enterococcus faecalis* and *Enterococcus faecium* isolates.

	BAPS group	**BAPS subgroup**	**ST number**	**Number of isolates**
***E. faecalis* (n=17)**	1		6	10
	3		81	1
	1		16	2
			589	4
***E. faecium* (n=26)**	3	3.2	271	2
	3	3.3a2	17	3
	2	2.1a	78	3
	2	2.1a	117	12
	2	2.1a	730	4
	1	1.2	361	1
	1	1.2	60	1

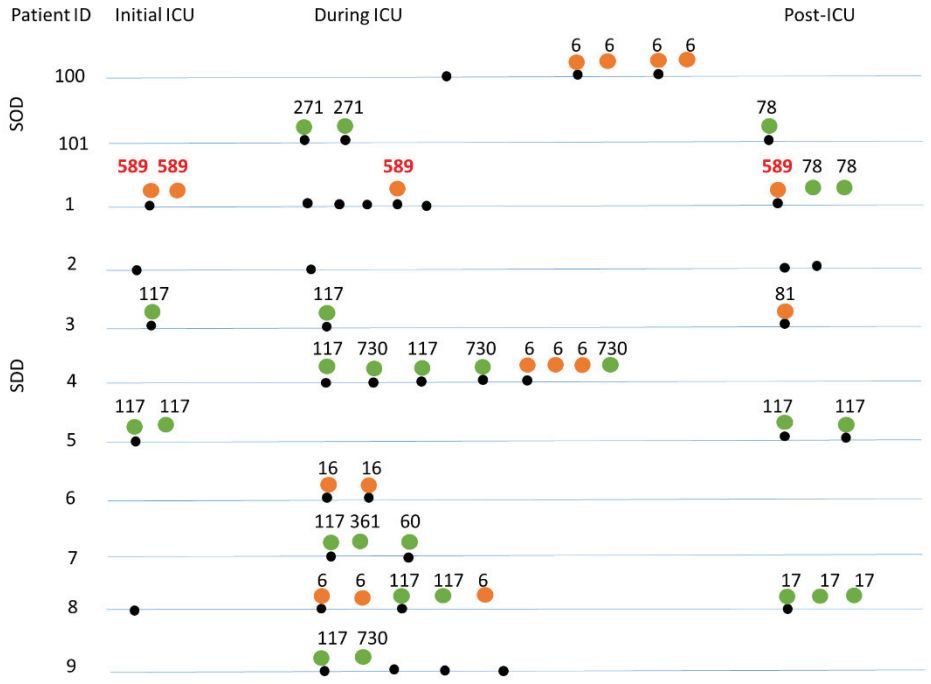

Figure 2. Sequence types (ST) identified per sample, per patient during and after SOD-SDD therapy. The differently coloured dots indicate the species: orange (*E. faecalis*), green (*E. faecium*). Numbers indicate the sequence types. The new ST (589) detected in a single patient is indicated in red. Black dots indicate the time point (days) where samples were taken during hospitalization. Isolates not connected to a black dot were obtained from the sample closest to the left.

Discussion

In the present study we analysed the dynamics of colonization by *Enterococcus* species isolated from faecal samples of ICU patients receiving prophylactic SOD or SDD therapy. We observed a pool of diverse enterococcal species and sequence types that harboured virulence and antibiotic resistance genes, with the majority of isolates carrying at least two virulence factor- (n=11) and/or virulence factor- and antibiotic resistance genes (n=26). During ICU hospitalization and concomitant SOD or SDD, we observed dynamic patterns of colonization by enterococcal species, probably due to prolonged hospital stay and selective antibiotic pressure.

The most prevalent species in both groups of patients were *E. faecium* and *E. faecalis,* both previously identified as important human pathogens associated with nosocomial infections (Schaberg *et al.,* 1991). In three patients, these two species were detected in samples collected during the first 72h, which could suggest that these patients were colonized with the recovered strains before ICU admission. This is in line with previous studies, as recently reviewed by Guzman Prieto and co-authors, showing that enterococci are present in healthy humans as well as in the environment, and that the acquisition of resistance genes and mobile elements rapidly increases and facilitates the colonization and subsequent infection in hospitalized patients (Guzman Prieto *et al.,* 2016). Other identified enterococcal species included *E. avium*, *E. canintestini, E. dispar* and *E. gallinarum*, albeit only during post-ICU. One possible explanation could be that due to the suspension of the antibiotic selective pressure during post-ICU, other strains not belonging to *E. faecalis* and *E.faecium* were isolated. From these species, *E. gallinarum* and *E. avium* have been identified in fecal samples of animals and healthy humans (Layton *et al.,* 2010; Silva *et al.,* 2011), while *E. dispar* and *E. canintestini* have only been identified in human and canine fecal samples, repectively (Collins *et al.,* 1991; Naser *et al.,* 2005). *E. avium, E. dispar* and *E. gallinarum* have been infrequently linked to a human enterococcal infections (Tan *et al.,* 2010; Varun *et al.,* 2016).

We were furthermore able to identify more than one enterococcal species per sample in five out of 11 patients. This highlights the importance of analysing multiple colonies per culture to adequately sample the diversity of the enterococcal population.

In our study, we observed a low prevalence of vancomycin resistance among *E. faecalis* and *E. faecium* isolates. This is in line with the previously reported prevalence (<1% for both *E. faecium* and *E. faecalis*) of vancomycin-resistance among enterococci in clinical infections in the Netherlands, as showed in the European Antimicrobial Resistance Surveillance System (EARSS) (ecdc.europa.eu/en/activities/surveillance/EARS-Net). The only vancomycin-resistant isolate was identified as a strain of *E. gallinarum* (vancomycin MIC of 16 µg/ml), which carried the *vanC1* gene that is naturally present in this species (Toye *et al.*, 1997). Practically all isolates (94%) were resistant to macrolides and displayed the cMLSb phenotype. In our study, the presence of the *ermB* gene was detected in 76% (34 of 45 strains) of the erythromycin-resistant isolates. Similarly, Schmitz *et al.* (2000) found that the *ermB* gene (93%, n= 70) was most often detected in a set of 75 clinical isolates of *E. faecium*, followed by the *ermA* gene (4%, n= 3) (Schmitz *et al.*, 2000). Hence, our results confirmed that *ermB* is the most frequent resistance gene among erythromycin-resistant enterococci. From the cMLSb phenotype isolates obtained in our study, which were negative for the *ermB* gene, also the *ermC* gene, as well as the *mefA* and *mefE* genes encoding an efflux mechanism, could not be detected.

Colonization by ampicillin-resistant *Enterococcus* (ARE) is frequently associated with previous exposure to selective antibiotics, and ampicillin resistance is a specific trait for nosocomial isolates (de Regt *et al.,* 2012). In our study we found a high prevalence of ARE, being notorious during ICU stay especially among *E. faecium* isolates. Resistance to tetracycline was detected in 48% of all 48 isolates (n=23) and predominantly in *E. faecalis* isolates (n=11), which is in accordance with previous studies (Templer *et al.,* 2008).

All isolates were further screened for the presence of selected virulence genes. The *esp* gene was the most prevalent virulence determinant detected in throughout the study period. Similar results were found by Billstrom *et. al.* (2008) and Sharifi *et. al.* (2013), where the *esp* gene was detected in more than 50% of *E. faecium* isolates from hospitalized patients. The *asa1* gene was detected in 14 isolates, mainly during ICU stay, including two *E. faecium*, one *E. avium*, and 11 *E. faecalis* isolates, in line with the prevalence of this gene previously reported by Hallgren *et. al.* (Hallgren *et al.,* 2009). Next, we detected the presence of the *hyl* gene in one *E. faecium* and one *E. gallinarum* isolate only post-ICU. It should be noted, however, the *hyl* gene has been identified not only in *E. faecium* and *E. faecalis*, but also in *E. casseliflavus*, *E. mundtii* and *E. durans* isolated from food-stuffs (Trivedi *et al.*, 2011), showing that the *hyl* gene can be present in a variety of *Enterococcus* spp. We cannot exclude that isolates obtained here contain other virulence genes that were not targeted in the present study.

Finally, the clonal relationship and population structure (BAPs groups) found in *E. faecium* and *E. faecalis* isolates indicated that the majority of our *E. faecium* isolates (85%) clustered in subgroups 2.1a and 3.3a2, representing separate hospital lineages that belong to clade A1, which contains most nosocomial *E. faecium* isolates (Willems *et al.,* 2012). Most *E. faecalis* isolates (71%) clustered in BAPs group 1, of which the majority belonged to ST 6 that was previously found in both hospitalized and non-hospitalized patients (Willems et al., 2012; Tedim *et al.,* 2015). In three patients, ST 589 (*E. faecalis*) and ST 117 (*E. faecium*) were detected during the first 72h, which were continuously present in all the isolates identified during the study period in those patients.

Unfortunately, we were not able to identify the ST of the clinical isolates responsible for the nosocomial infections. It is also not known whether these infections were due to translocation from the gut. In our study we described a high diversity of *Enterococcus* spp., including the recovery of multiple species and STs from individual patients.

During ICU stay, we observed the simultaneous presence of sequence types and clonal replacement over time among *E. faecium* isolates, whereas this was not the case for *E. faecalis*. Furthermore, we detected the simultaneous presence of more than two virulence factors and/or virulence factor and antibiotic resistance genes in *E. faecalis*, *E. faecium, E. gallinarum* and *E. avium* isolates. The prevalence of *Enterococcus* in ICU hospitalized patients, combined with the carriage of antibiotic resistance and virulence genes, described in this study, underline the importance of this group of organisms as a potential cause of nosocomial infections in critically ill patients.

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Supplementary data:

Table S1. Characteristic of the *Enterococcus* isolates colonizing ICU patients

Initial ICU/Patient ID	Identification	MIC Van (µg/ml)	Macrolide phenotype	Resistance gene	Virulence factor	MLST
Patient 1	*E. faecalis*	2	Susceptible	N.A	no detected	589
	E. faecalis	2	cMLSb	*erm* B	no detected	589
Patient 3	*E. faecium*	0.5	cMLSb	*erm* B	*esp*	117
Patient 5	*E. faecium*	0.75	cMLSb	*erm* B	*esp*	117
	E. faecium	0.75	cMLSb	*erm* B	*esp*	117

ICU stay/ Patient ID	Identification	MIC Van (µg/ml)	Macrolide phenotype	Resistance gene	Virulence factor	MLST
Patient 101	*E. faecalis*	1.5	cMLSb	*erm* B	*asa,esp*	6
	E. faecalis	1.5	cMLSb	no detected	*asa,esp*	6
	E. faecalis	1.5	cMLSb	no detected	*asa,esp*	6
	E. faecalis	1.5	cMLSb	no detected	*asa*	6
Patient 100	*E. faecium*	1.5	cMLSb	*ermB*	no detected	271
	E. faecium	1	cMLSb	*ermB*	no detected	271
Patient 1	*E. faecalis*	2	cMLSb	no detected	no detected	589
Patient 3	*E. faecium*	0.5	cMLSb	*ermB*	*esp*	117
Patient 4	*E. faecium*	1	cMLSb	*ermB*	*esp*	117
	E. faecium	1	cMLSb	*ermB*	*esp*	730
	E. faecium	1	cMLSb	*ermB*	*esp*	117
	E. faecium	1	cMLSb	*ermB*	*esp*	730
	E. faecium	1	cMLSb	*ermB*	*esp,asa*	730
	E. faecalis	1	cMLSb	*ermB*	*esp,asa*	6
	E. faecalis	1	cMLSb	*ermB*	*esp*	6
	E. faecalis	1	cMLSb	no detected	*esp*	6
Patient 6	*E. faecalis*	1	cMLSb	*ermB*	*esp,asa*	16
	E. faecalis	1.5	cMLSb	*erm* B	*esp, asa*	16
Patient 7	*E. faecium*	0.5	Susceptible	N.A	no detected	60
	E. faecium	1	cMLSb	*erm* B	*esp*	117
	E. faecium	1	cMLSb	no detected	no detected	361
Patient 8	*E. faecalis*	1	cMLSb	*ermB*	*asa*	6
	E. faecalis	1	cMLSb	*ermB*	*asa,esp*	6
	E. faecalis	1	cMLSb	*ermB*	*asa*	6
	E. faecium	1	cMLSb	*ermB*	*esp*	117
	E. faecium	1	cMLSb	*ermB*	*esp,asa*	117
Patient 9	*E. faecium*	0.75	cMLSb	no detected	no detected	117
	E. faecium	0.75	cMLSb	*erm* B	*esp*	730

Post ICU/ Patient ID	Identification	MIC Van (µg/ml)	Macrolide phenotype	Resistance gene	Virulence factor	MLST
Patient 100	*E. faecium*	1	cMLSb	*erm* B	*esp,hyl*	78
Patient 1	*E. faecium*	2	cMLSb	no detected	*esp*	78
	E. faecium	2	cMLSb	*erm* B	*esp*	78
	E. faecalis	2	cMLSb	no detected	no detected	589
Patient 2	*E. canintestini*	0.5	cMLSb	*erm* B	no detected	
	E. dispar	0.5	cMLSb	*erm* B	no detected	
	E. gallinarum	16	cMLSb	*erm* B, *van* C1	*esp, hyl*	
Patient 3	*E. faecalis*	0.5	cMLSb	*erm* B	*asa, esp*	81
Patient 5	*E. faecium*	0.75	cMLSb	*erm* B	*esp*	117
	E. faecium	0.75	cMLSb	*erm* B	*esp*	117
	E. avium	0.5	cMLSb	*erm* B	no detected	
	E. avium	0.5	Susceptible	N.A	*esp, asa*	
Patient 8	*E. faecium*	1	cMLSb	no detected	no detected	117
	E. faecium	1	cMLSb	no detected	no detected	78
	E. faecium	1	cMLSb	*erm* B	no detected	17

CHAPTER 8

High throughput cultivation-based screening on the MicroDish platform allows targeted isolation of antibiotic resistant human gut bacteria

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Abstract

The emergence of bacterial pathogens that are resistant to clinical antibiotics poses an increasing risk to human health. The most important reservoir from which bacterial pathogens can acquire resistance is the human gut microbiota. However, to date, a large fraction of the gut microbiota remains uncultivated and has been little-studied with respect to its reservoir-function. Here, our aim was to isolate yet uncultivated resistant gut bacteria by a targeted approach. Therefore, faecal samples from 20 intensive care patients who had received prophylactic antibiotic treatment (selective digestive decontamination [SDD], i.e. tobramycin, polymyxin E, amphotericin B and cefotaxime) were inoculated anaerobically on MicroDish porous aluminium oxide chip (PAO Chip) placed on top of poor and rich agar media, (including media supplemented with the SDD antibiotics). Biomass growing on the chips was analysed by 16S ribosomal RNA gene amplicon sequencing, showing large inter-individual differences in bacterial cultivability, and enrichment of a range of taxonomically diverse operational taxonomic units [OTUs]. Furthermore, growth of *Ruminococcaceae* (2 OTUs), *Enterobacteriaceae* (6 OTUs) and *Lachnospiraceae* (4 OTUs) was significantly inhibited by the SDD antibiotics. Strains belonging to 16 OTUs were candidates for cultivation up to pure culture as they shared ≤95% sequence identity with the closest type strain and had a relative abundance of ≥2%. Six of these OTUs were detected on media containing SDD antibiotics, and considered as prime candidates to be studied with regards to antibiotic resistance. One of these six OTUs was obtained in pure culture using targeted isolation. This novel strain, which was initially classified as member of the *Ruminococcaceae*, was later found to share 99% nucleotide identity with the recently published *Sellimonas intestinalis* BR72^T. In conclusion, we showed that high-throughput screening of growth communities can guide targeted isolation of bacteria that may serve as reservoirs of antibiotic resistance.

Keywords: antibiotic resistance / gut microbiota / bacteria / anaerobic cultivation / MicroDish

Introduction

The emergence of bacterial pathogens that are resistant to most clinical antibiotics is an increasing threat to public health. A common route through which pathogens can acquire resistance is by genetic exchange with human-associated bacteria, and especially the gut microbiota. Indeed, it has been shown that the commensal gut microbiota harbours diverse resistance genes (Forslund *et al.,* 2013; Hu *et al.,* 2013), and that these genes can be acquired by (opportunistic) pathogens (van Schaik, 2015). Horizontal gene transfer (HGT) is considered the main mechanism by which resistance genes are disseminated, and it has been shown that HGT events occur exceedingly more often in the gut microbiota than in other environments with complex bacterial communities (Smillie *et al.*, 2011). Novel resistance determinants are typically described once bacteria are obtained in pure culture.

On the other hand, resistance genes carried by yet uncultivated gut bacteria appear to be largely uncovered, which is reinforced by the observation that functional metagenomics studies of human gut microbiota consistently yield novel resistance genes (Sommer *et al.*, 2009; Cheng *et al.*, 2012). This "black box" of little-studied uncultivated bacteria has been estimated to constitute 40-70% of gut microbes (Sommer, 2005; Kim *et al.*, 2011). Even though application of culture-independent methods (e.g. functional metagenomics) has provided us with useful insights into uncultivated bacteria, their cultivation will be essential to comprehensively study the antibiotic resistance phenotype, and the potential roles and mechanisms of these bacteria in antibiotic resistance dissemination. Nowadays, to isolate members of yet uncultivated taxa, innovative culturing techniques that apply high-throughput screening and/or better simulate the natural environment of these bacteria are increasingly being used. Recent methodical advances include cultivation inside chambers placed in the native environment (Ferrari *et al.,* 2005; Bollmann *et al.*, 2007), the use of custom-designed media (Tripp *et al.*, 2008), application of multi-well micro culture chips (Ingham *et al.,* 2007), high-throughput identification of isolates (Pfleiderer *et al.*, 2013), and microfluidic cultivation (Ma *et al.,* 2014).

Furthermore, a recent study by Rettedal and co-authors combined high-throughput sequencing with selective cultivation conditions, allowing to cultivate previously uncultured species from the human gut by a targeted approach (Rettedal *et al.*, 2014). To this end, the authors used, among other criteria, the "most wanted" list of microbial taxa that has recently been introduced in order to guide efforts towards the cultivation of human gut bacteria (Fodor *et al.*, 2012).

In short, the most wanted list contains human-associated bacterial taxa of which the genome has not yet been sequenced, not considering whether members of these taxa from other environments might already have been sequenced. High priority most wanted taxa were defined as those of which the 16S rRNA centroid read shared less than 90% identity with either the GOLD-Human or Human Microbiome Project (HMP) strains, and which were detected in at least 20% of samples from any body habitat analysed. Medium priority taxa are those that share between 90% and 98% identity with the same habitat prevalence threshold.

Antibiotics are generally administered upon detection of an infection. In addition, in most Dutch hospitals, patients who are admitted to the intensive care unit (ICU) receive prophylactic antibiotic therapies, of which selective decontamination of the digestive tract (SDD) is currently the most common treatment. SDD combines the application of tobramycin, polymyxin E and amphotericin B in the oropharynx and gastrointestinal tract with a short systemic administration of a third-generation cephalosporin. This therapy aims to eradicate potential pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* and yeast, while maintaining the anaerobic members of the microbiota (de Smet *et al.,* 2012). SDD therapy has been shown to decrease infections and mortality of ICU patients (de Jonge *et al.*, 2003; de Smet *et al.*, 2009). Although a meta-analysis showed that SDD therapy resulted in a decrease in resistance carriage with respect to cultivable bacteria (Daneman *et al.*, 2013), a recent case study (Buelow *et al.*, 2014) and a more extensive follow-up with 13 ICU patients (Buelow, 2015) indicated that prophylactic therapy may in fact increase resistance carriage among mostly uncultivated anaerobic gut residents.

It was speculated that the expanded resistome, i.e. the collection of all resistance genes in a bacterial community (Wright, 2007), might thereby increase the risk of future pathogens becoming resistant. Indeed, the risk that pathogens develop antibiotic resistance is a major concern that has prohibited wide implementation of prophylactic therapies (Daneman *et al.,* 2013). In view of the above, it is clear that the role of uncultivated anaerobic bacteria in the emergence of resistance pathogens merits deeper investigation.

In this study, we aimed to identify and isolate potential reservoir strains for antibiotic resistance in the anaerobic microbiota of the human gut. Therefore, faecal material from 20 Dutch ICU patients was inoculated on poor and rich agar media under anoxic conditions. These media were prepared without antibiotics, or supplemented with the antibiotics that the patients received as part of their SDD therapy. Bacteria were cultivated on the MicroDish porous aluminium oxide (PAO Chip) that facilitates efficient parallel processing of a large number of samples, and reduces potential inhibition of bacterial growth by agar (Ingham *et al*., 2007).

Chips that were placed on top of different media solidified with agar were inoculated with faecal suspensions, and bacterial biomass was investigated by 16S ribosomal RNA (rRNA) gene amplicon sequencing, based on which growth patterns were analysed and target species were identified for cultivation up to pure culture. Accordingly, we isolated a close relative of *Sellimonas intestinalis* BR72^T that grew on top of the PAO Chip on agar media containing tobramycin, cefotaxime and polymyxin E, and that could serve as an antibiotic resistance reservoir.

Materials and Methods

Sample collection

Faecal samples were collected from 20 patients no later than five days after admission to the ICU at Utrecht Medical Center, Utrecht, Netherlands. During this period, the patients received SDD therapy. The SDD protocol was reviewed and approved by the institutional review board of the University Medical Center Utrecht. Faecal samples were collected upon defecation and stored at 4°C for 30 min to 4 h, after which an aliquot of the sample (approximately 0.5 g) was suspended in 5 ml of anaerobic PBS (pH 7.0). Subsequently, 1 ml of the suspension was transferred to an anaerobic bottle containing 4 ml of PBS, 25% (v/v) glycerol, 0.5 g resazurin and 0.5 g cysteine. To preserve anaerobic conditions, a few drops of titanium citrate (100 mM) were added to the bottle before storage at -80°C.

Cultivation conditions

A high throughput cultivation technique using the MicroDish PAO Chip (MicroDish, Utrecht, Netherlands) was applied. Faecal bacteria were cultured on ethanol-sterilized PAO Chip on top of two different media: (i) GIFU anaerobic agar media (GAM) (Hyserve, Uffing, Germany), and (ii) bicarbonate-buffered anaerobic media (referred to in the text as CP medium) (Stams *et al.*, 1993) supplemented with 1.5% (w/v) agar and 1% (v/v) faecal supernatant. The faecal supernatant was prepared from a pool of faecal samples obtained from three healthy volunteers who had not received antibiotics for at least six months. In brief, equal amounts of faecal sample from the three volunteers were added to anaerobic PBS (pH 7.0) to a final concentration of 25 % (w/v). Subsequently, the mixture was centrifuged at 14,000 rpm for 30 min, after which the supernatant was transferred to an anaerobic bottle (N_2/CO_2 – 80:20, v/v) and autoclaved. All samples were cultivated on agar media both in the presence and in the absence of the SDD cocktail of antibiotics (25 µg/ml tobramycin, 5 µg/ml polymyxin E and 10 µg/ml cefotaxime; the antifungal drug amphotericin B was not included in this study).

An aliquot (5 µl) of faecal suspension was applied per PAO Chip. Inocula consisted of undiluted and 100-fold diluted cryopreserved faecal suspension. In addition, 10-fold sample dilutions were included for three patients (designated 210, 131 and 148) in order to study the effect of dilution on bacterial growth. PAO Chips on top of GAM agar and CP agar were harvested two and three days after inoculation under anoxic conditions at 37 °C, respectively. Upon harvesting, PAO Chips with bacterial growth were placed in an Eppendorf tube containing 1 ml of anaerobic PBS (pH 7.0). The tube was vortexed for 30 s to dissociate the cells from the PAO Chip.Subsequently, the suspension was split into two fractions; one fraction was used for DNA extraction whereas the other fraction was added to an anaerobic bottle containing glycerol (final concentration: 25-30%) in PBS, and stored at -80 °C. Biological duplicates were analysed for each growth community.

DNA extraction and 16S rRNA gene amplicon sequencing

Barcoded 16S rRNA gene amplicon sequencing was used to investigate the bacterial composition of faecal samples and of the growth communities on the PAO Chips. Primers used for 16S rRNA amplicon sequencing are described in **Supplementary Table S1**. The cells in these samples were lysed and (cellular) debris was removed with an adapted bead beating protocol (Salonen *et al.,* 2010). In case of cryo-preserved faecal material, 500 µl of sample was added to a screw-cap tube that already contained 0.5 g of 0.1 mm zirconium beads (Biospec Products, Bartlesville, United States) and three 5 mm glass beads (Biospec Products). Subsequently, 300 µl STAR buffer (Roche, Basel, Switzerland) was added, after which the contents of the tube were homogenized in the Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) at 5.5 ms (3 rounds of 1 min). The sample was then incubated at 95 °C at 100 rpm for 15 min. Particles were spun down at 4 °C at >10,000 g for 5 min, and subsequently the supernatant was transferred to a fresh tube for DNA extraction. The DNA yield was improved by another two iterations of beat-beating that started with re-suspending the pellet in 300 µl STAR buffer. In case of bacteria suspended in PBS (i.e. the growth communities), 150 µl of sample was processed by identical methods except at a smaller scale.

Therefore, 0.25 g of 0.1 mm zirconium beads and three 2.5 mm glass beads were added to the screw-cap tube, and STAR buffer was used in portions of 150 µl. Following the bead beating protocol, DNA was extracted from 250 µl of the combined supernatants using the Maxwell 16 Tissue LEV total RNA purification kit starting from the post lysis step (Promega, Madison, United States). 16S rRNA gene amplification, which also attached the barcodes, was done with a 2-step PCR protocol (Tian *et al.*, 2016). The product from the second PCR step was analysed on a 1% agarose gel and purified using the CleanPCR Kit (GC Biotech, Alphen aan den Rijn, Netherlands) according to manufacturers' instructions. The DNA concentration was measured by Qubit® 2.0 (Thermo Fisher Scientific). Subsequently the sample was included in a pool that in total contained 48 equimolar mixed samples. The pool of samples, which constituted a library, was sent for Illumina paired end MiSeq sequencing (2 x 300 bp) at GATC Biotech (Constance, Germany). In total, eight libraries were sent MiSeq for sequencing. Technical replicates at the level of 16S rRNA amplicon sequencing were analysed for the original faecal samples.

Processing of 16S rRNA gene amplicon data, statistical analyses and detection of most wanted and novel species

The 16S rRNA gene amplicon data were analysed using the NG-tax pipeline (Ramiro-Garcia *et al.*, 2016). In short, NG-tax initially defines operational taxonomic units (OTUs) as clusters of 16S RNA gene amplicons that share 100% nucleotide identity. Subsequently, the OTUs are expanded by including 16S RNA gene amplicons with one nucleotide mismatch. OTUs at <0.1% relative abundance are discarded.

The quality of the sequencing was analysed by including a mock community sample in each library (**Supplementary Table S2**). The output OTU table and centroid OTU sequences were used as input for detection of most wanted (Fodor *et al.*, 2012) and novel species. For statistical analysis we used a rarefied OTU table with 2,500 reads per sample, where samples with <2,500 reads were excluded.

Pearson correlation coefficients between bacterial communities in inocula (i.e. faecal material of ICU patients) and their respective growth communities were calculated based on OTU-level data using IBM SPSS statistics 23.0.0.2. Shannon diversity, richness and phylogenetic diversity whole tree metrics of bacterial communities were calculated using QIIME (Caporaso *et al.,* 2010). The two-tailed t-test without assuming equal variance was used to investigate if Shannon, richness and whole-tree phylogenetic diversity values of bacterial communities differed significantly when grouped according to experimental variables (e.g. growth medium or supplementation of the medium with antibiotics). The t-test used averaged values for biological and technical replicates.

The QIIME script *compare_taxa_summaries.py* was used to calculate Pearson correlation coefficients of OTU-level taxa between mock communities and their theoretical composition. Canonical Correspondence Analysis (CCA) as implemented in Canoco 5 (Smilauer *et al*., 2014) was used to investigate, which variables could best explain the variation in bacterial composition between bacterial communities. Linear mixed-effect models were fitted by the R package "lmerTest" (https://CRAN.R-project.org/package=lmerTest) (R development core team, 2010) in order to analyse how media type and addition of antibiotics affected bacterial composition. As input an adapted OTU table was used in which values were log1p transformed to meet normality assumptions.

Furthermore, OTUs were removed from the table if they were detected in <5 samples or by <50 reads across all samples. Parameter-specific p-values were obtained by using the Satterthwaite approximation. P-values were corrected for multiple testing by the function p-.adjust in the package "stats", using methods "Bonferroni" and "BH". Bray-Curtis dissimilarity hierarchical clustering was performed using R package 'Vegan' based on OTU-level relative abundance data of bacterial communities.

In order to investigate the presence of most wanted taxa (Fodor *et al.,* 2012) and novel species, the representative reads of the OTUs were compared by Blastn (Altschul *et al.,* 1990) to the V1-V3 sequence data of the most wanted OTUs, and to the 16S rRNA genes in the SILVA database of type strains (Quast *et al.*, 2013), respectively. A custom Perl script was used to parse the BLAST results for the best hits (bitscore sorted). Furthermore, the script tabulated the relative abundance of the OTUs and their distribution across all samples.

Targeted cultivation

Based on analysis of the 16S rRNA gene sequence data of the bacterial growth communities, OTUs were selected for targeted isolation. Therefore, the original faecal inoculum and the enriched growth fractions that contained the target OTU were re-plated under identical conditions, i.e. on PAO Chips placed on the same media. A dilution series was inoculated to yield single colonies. Per PAO Chip, three colonies per unique colony morphology were transferred to a fresh PAO Chips. Subsequently, the 16S rRNA gene was amplified using the 27F and 1492R primers (Jiang *et al.*, 2006), and the PCR products were Sanger sequenced at GATC Biotech (Cologne, Germany) using the 907R primer (Schauer *et al.*, 2000). The 16S rRNA gene sequences were compared by BLASTn to those in the NCBI ribosomal 16S RNA sequences database for species identification. Only for target species the near full-length 16S rRNA gene was then Sanger sequenced using the 27F and 1492R primers.

Results

Bacterial growth on PAO Chips

As inoculum, faecal samples were used from 20 patients that at the time of sampling had received SDD treatment for no longer than five days. Three 10-fold serial dilutions of the samples, starting at undiluted, were inoculated in duplicate on PAO Chips on top of GAM and CP agar media, either with or without supplementation of the SDD antibiotic cocktail. Agar media inoculated at the lowest dilution of faecal material and in absence of antibiotics always yielded confluent growth on GAM media, whereas on CP media confluent growth was observed on 34 of 40 PAO Chips (**Supplementary Table S3**). In general, less biomass grew on media if the faecal material was inoculated at a higher dilution, if the media contained the SDD cocktail of antibiotics, and if the faecal material was inoculated on CP media. Growth on most PAO Chips (371 of 480) was confluent as opposed to colonies that could be visually distinguished (**Figure 1A**).

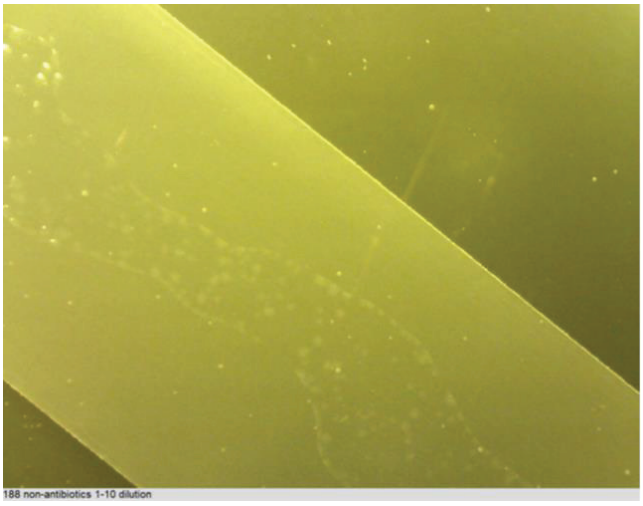

Figure 1A. Close-up pothograph of microbial growth on a PAO Chip that was placed on top of CP agar. The PAO Chip was inoculated with 10X diluted cryo-preserved faecal sample from patient 188. The area that was inoculated is visualized as a smear in which individual colonies can be distinguished. The white dots in the picture represent air bubbles in the agar medium.

Faecal inocula (in duplicate) and a selection of PAO Chips with bacterial growth, including chips inoculated with undiluted and 100-fold diluted faecal suspensions, as well as with 10-fold diluted suspensions for three samples, were analysed by 16S rRNA gene amplicon sequencing, which amounted to a total of 324 samples. The NG-tax pipeline was used to process the sequencing data of our samples as well as mock communities with known composition that were added to each library (Ramiro-Garcia *et al.,* 2016). The average Pearson correlation value of OTU-level taxa between the included mock communities and their theoretical composition was 0.82 (min-max 0.77-0.88), supporting the reliability of the applied approach (data not shown). Samples with 0 reads (n=4) assigned were removed from all further analysis yielding 319 samples with an average read depth of 40,999 ± 49,592 reads (**Supplementary Table S4**), and 3,832 assigned OTUs.

Comparison of bacterial growth communities

Bacterial diversity

Averaged across all faecal samples, the most abundant bacterial phyla were *Firmicutes* (61.0% ± 24.0), *Bacteroidetes* (34.0% ± 25.0), *Proteobacteria* (2.6% ± 6.4), *Actinobacteria* (1.6% ± 5.0) and *Verrucomicrobia* (0.5% ± 1.2) (**Figure 2A**). The corresponding growth communities on GAM and CP media were dominated by *Firmicutes* and *Bacteroidetes*, together comprising on average >80% of the bacterial community. On GAM agar without antibiotics the relative abundance of *Proteobacteria* on average constituted 5.1% ± 17.0 of the communities whereas on GAM agar with the SDD cocktail (GAM-SDD), was 0.03% ± 0.1. Similarly, on CP-SDD media the *Proteobacteria* had reduced relative abundance (a decrease from 13.6% ± 27.7 to 9.8% ± 2.9) as compared to CP media without antibiotics. Notably, *Cyanobacteria* were not detected in the faecal samples or on GAM media, but they were detected on CP media averaging 0.5% ± 6.4 relative abundance.

As expected, the average Shannon diversity of faecal samples was significantly higher than that of growth communities grouped by medium, addition of antibiotics or dilution (two-tailed t-test, p = <0.01 for all comparisons) (**Figure 2B**). Lower Shannon diversity values were also observed in growth communities inoculated with more diluted faecal sample. However, this difference was only significant between communities on CP-SDD agar that were inoculated with undiluted and 100-fold diluted faecal sample (two-tailed t-test, p = 0.01). The addition of the SDD antibiotics significantly reduced the Shannon diversity on GAM media (p = <0.01) but not on CP media.

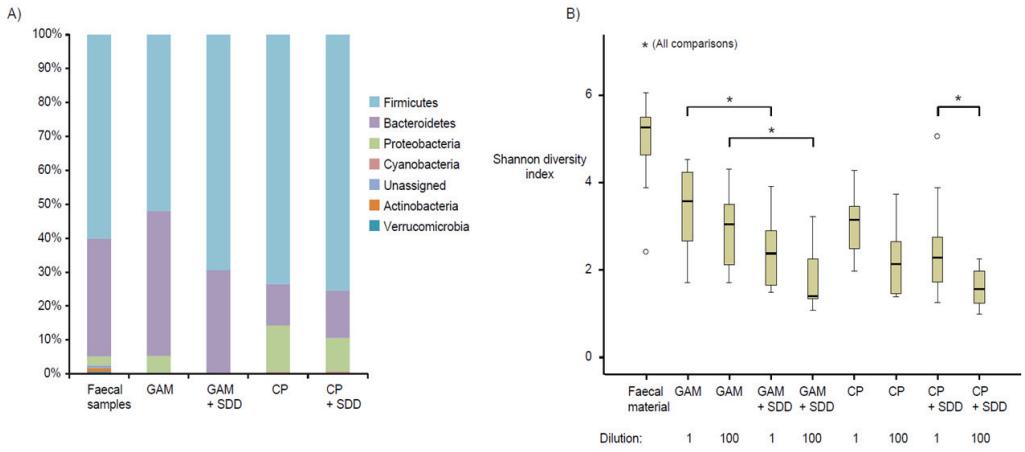

Figure 2. A) Bacterial phyla that were detected in the faecal samples of 20 intensive care patients and in their corresponding growth communities on GAM and CP agar media. Growth on these media was further subdivided based on the addition of the SDD antibiotics. Phyla with a relative abundance <0.5% are not shown. The relative abundance values are based on the combined reads of all samples in the different experimental groups. **B)** Boxplots depicting the distribution of Shannon diversity values of bacterial communities in the different experimental groups. Asterisks indicate that Shannon values of bacterial communities in experimental groups were significantly different (p = <0.05) based on the two-tailed t-test. Medium (i.e. GAM vs. CP) did not significantly affect Shannon values of bacterial growth communities. Shannon values of faecal samples were in all cases higher than those for growth communities, irrespective of medium, dilution and addition of antibiotics (p = <0.01 for all comparisons).

Differences in OTU richness and whole-tree phylogenetic diversity between the sample groups followed the same trends as differences in Shannon diversity i.e. higher values were obtained for faecal samples and lower values were obtained if media were inoculated with more diluted faecal material or included antibiotics (**Figure S1**). However, surprisingly, a higher dilution of the faecal inoculum did not affect whole-tree phylogenetic diversity on CP media without antibiotics (p = 0.81). Canonical correspondence analysis (CCA) of OTU-level data from all bacterial communities (faecal inoculates and cultivable fractions) indicated that cultivation medium and presence/absence of antibiotics could explain in total 3.7% of the variation in bacterial composition (p = 0.002) (**Figure 3**). However, bacterial growth on CP agar was not found to be significantly affected by the addition of the SDD cocktail of antibiotics. The dilution factor of faecal inocula was also evaluated as explanatory variable but was found to not affect bacterial composition.

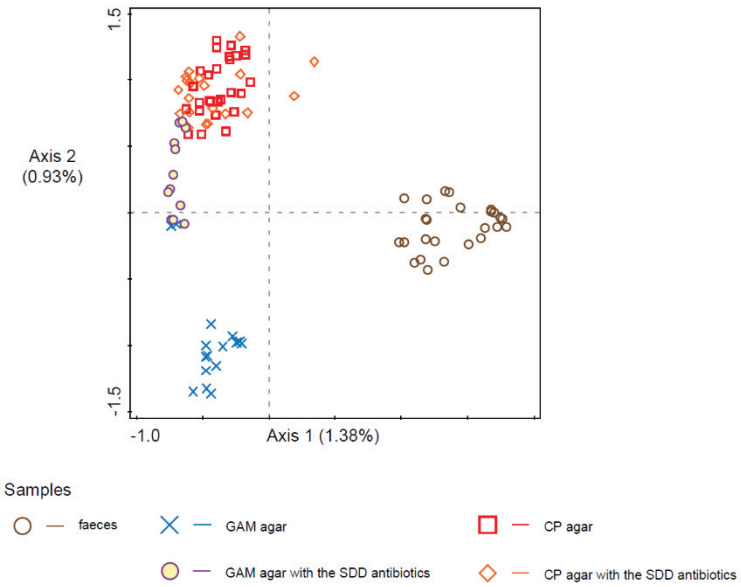

Figure 3. Canonical correspondence analysis (CCA) of OTU-level data was used to investigate to what extent growth conditions can explain the variation in the composition of bacterial communities. Included in the analyses were the faecal samples (inocula) as well as their corresponding growth communities on GAM agar and CP agar. Growth on agar media was further distinguished based on the addition of the SDD antibiotics.

Inter-individual differences in communities

While bacterial communities grouped by experimental variables (i.e. medium or addition of antibiotics) showed significant differences, we were also interested in potential differences between patients. Hierarchical clustering of OTU-level data using Bray-Curtis dissimilarity indicated high dissimilarity between individual faecal samples with mutual dissimilarity values being >0.8 in all but two cases (**Figure 4**).

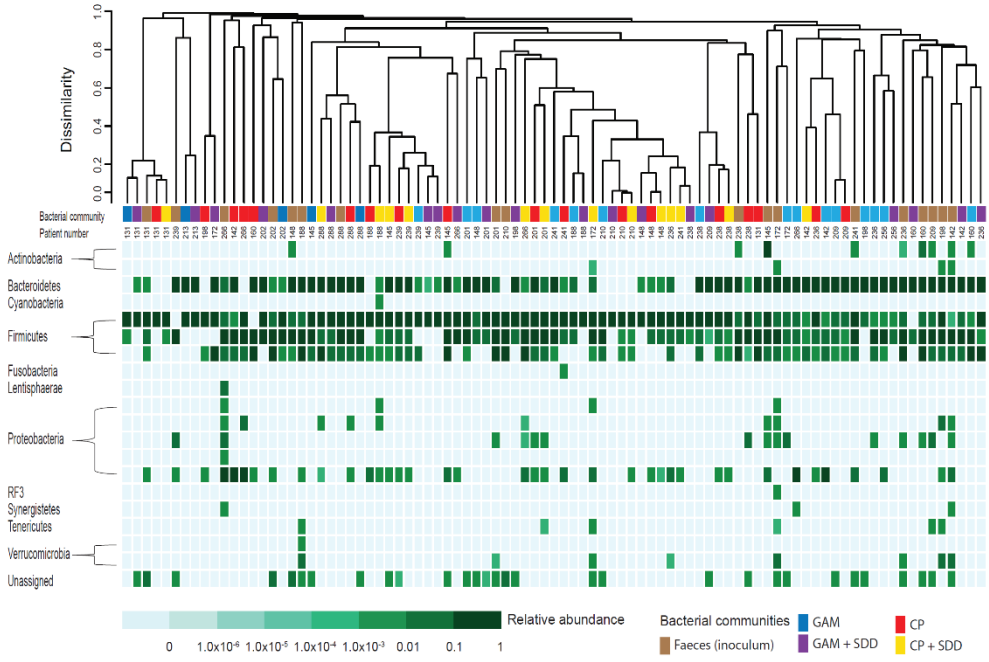

Figure. 4. Hierarchical clustering using Bray-Curtis dissimilarity based on 16S rRNA gene amplicons generated from faecal samples of intensive care unit patients and corresponding biomass retrieved from PAO chips on GAM and CP agar. Growth on these media was further distinguished based on the addition of the SDD antibiotics. Hierarchical clustering was performed at the OTU-level. The heatmap corresponds to relative abundance values of class-level phylogenetic groups. The dissimilarity tree shows that bacterial dissimilarity between faecal samples is high.

Growth communities derived from only 9 of 20 patients all clustered together indicating that other factors besides inoculum influenced bacterial growth. For example, growth communities of patient 131 all clustered together with mutual dissimilarity values <0.25 whereas for growth communities of patient 236 dissimilarity values exceeded 0.8. Moderate clustering by medium and presence/absence of antibiotics confirmed that cultivation conditions affected growth as was shown before by CCA. We also evaluated by Pearson correlation to what extent bacterial growth communities resembled the faecal samples from which they were derived (Figure S2).

For different individuals the average Pearson correlations ranged from 0.02 to 0.99, indicating that there were large inter-individual differences in bacterial cultivability. No trends were discovered between the applied medium, antibiotics and/or dilution of faecal inoculum, and how well the growth communities resembled the faecal samples.

Effects of media composition and antibiotics on bacterial growth

In the following, we aimed to identify OTUs that were enriched as a result of specific cultivation conditions. We fitted linear mixed-effect models on OTU-level data so that also differences between individual patients could be taken into account (**Supplementary Table S5**). A total of 35 OTUs were significantly enriched under the different cultivation conditions (**Table 1**). Considering Bonferroni-corrected p-values, a total of seven OTUs belonging to the families *Bacteroidaceae* (5 OTUs), *Staphylococcaceae* (1 OTU) and *Enterococcaceae* (1 OTU) were present in significantly higher abundance on GAM media as compared to the respective faecal samples. In contrast, on CP media, OTUs belonging to the families *Halomonadaceae* (2 OTUs), *Lachnospiraceae* (6 OTUs), *Ruminococcaceae* (1 OTU), *Streptococcaceae* (1 OTU), *Enterococcaceae* (3 OTUs), *Porphyromonadaceae* (2 OTUs), and *Oxalobacteraceae* (1 OTU) were enriched.

A significantly lower abundance of *Ruminococcaceae* spp (1 OTU), *Enterobacteriaceae* spp (6 OTUs) and *Lachnospiraceae* spp. (4 OTUs) was detected on media supplemented with the SDD antibiotics in comparison to media without antibiotics, indicating that the antibiotics inhibited the growth of these bacteria.

Table 1. Linear mixed-effect models of OTU-level composition data of bacterial growth were applied to investigate which OTUs varied in abundance as a results of cultivation conditions. This table lists the taxonomic affiliations of OTUs that were found to be enriched at Bonferroni corrected p-values of <0.05. Bacterial communities were cultivated on GAM agar and CP agar, both in the presence and absence of the SDD antibiotics.

		Taxonomy	
Enriched on	**No. of OTUs**	**Family**	**Genus**
GAM agar	5	*Bacteroidaceae*	*Bacteroides*
	1	*Staphylococcaceae*	*Staphylococcus*
	1	*Enterococcaceae*	*Enterococcus*
CP agar	2	*Halomonadaceae*	*Halomonas*
	6	*Lachnospiraceae*	Unspecified
	1	*Ruminococcaceae*	Unspecified
	1	*Streptococcaceae*	*Streptococcus*
	3	*Enterococcaceae*	*Enterococcus*
	2	*Porphyromonadaceae*	*Parabacteroides*
	1	*Oxalobacteraceae*	*Undibacterium*
Media (CP and GAM) without antibiotics	1	*Ruminococcaceae*	Unspecified
	6	*Enterobacteriaceae*	*Escherichia-Shigella*
	4	*Lachnospiraceae*	Unspecified
CP agar without SDD cocktail of antibiotics	1	*Ruminococcaceae*	Unspecified

Growth of novel species

We further aimed to investigate whether novel species or members of taxa on the most wanted list were present in the cultivable fraction of the faecal samples. Eleven high priority most wanted OTUs were detected in the growth communities; however, none of these OTUs was present at a relative abundance of >0.8% (**Supplementary Table S6**). Furthermore, three medium priorities most wanted OTUs (OTUs 236, 172 and 288) were detected in the cultivable fraction with 1-3% relative abundance. Nevertheless, members of medium priority OTUs were not considered candidates for isolation because they shared 100% identity with strains that were previously isolated. Comparison of OTUs with the SILVA database of type strains yielded 16 OTUs with >2% relative abundance of which the OTU representative read shared <95% identity with the 16S rRNA gene sequence of the closest type strain (**Table 2**).

Table 2. Faecal samples of 20 patients were inoculated on PAO chips on top of GAM and CP agar media, and growth was analysed by 16S rRNA gene amplicon sequencing. This table shows OTUs of which the representative read shares <95% identity with the closest 16S rRNA gene sequence in the SILVA database of type strains. In addition, the relative abundance of these OTUs was ≥2% on at least one PAO chip.

OTU ID	No. samples	GAM/ CP medium	SDD/ NAB	Highest rel.ab. (%)	Detected in inoculum?	Closest type strain	Acc. number	% identity
3088	30	GAM/CP	SDD/NAB	49.8	yes/no	*Ruminococcus torques*	L76604	93.4
322	15	CP	NAB	23.9	yes/no	*Hydrogenoanaerobacterium saccharovorans*	EU158190	89.3
3797	2	CP	NAB	13.2	no	*Thalassiosira pseudonana* (chloroplast)	EF067921	85.9
2642	4	GAM	NAB	7.4	yes	*Bacteroides ovatus*	EU136682	94.7
2024	2	CP	SDD	5.6	yes	*Oscillibacter ruminantium*	JF750939	91.1
2026	3	CP	SDD	5.5	yes	*Oscillibacter ruminantium*	JF750939	91.1
2082	1	GAM	SDD	3.9	no	*Coprobacter fastidiosus*	JN703378	94.7
2724	1	GAM	SDD	3.8	no	*Bacteroides faecis*	GQ496624	96.7
2985	2	CP	NAB	3.6	no	*Clostridium clostridioforme*	M59089	96
3067	4	GAM	NAB	3.0	yes	*Ruminococcus torques*	L76604	93
3103	4	GAM	NAB	2.8	yes	*Ruminococcus torques*	L76604	93
2252	3	GAM	SDD/NAB	2.6	no	*Bacteroides nordii*	EU136693	95
3375	2	GAM	NAB	2.2	yes	*Coprococcus comes*	EF031542	97
2884	2	GAM	NAB	2.2	no	*Clostridium bolteae*	AJ508452	96
3070	5	GAM	NAB	2.2	yes/no	*Ruminococcus torques*	L76604	93
2893	2	GAM	NAB	2.0	no	*Clostridium bolteae*	AJ508452	96

Therefore, these OTUs were considered to i) potentially represent novel species, and ii) to be sufficiently abundant for isolation by colony picking. Among these 16 OTUs, OTUs 3088, 322, 3797, 2642 and 2024 were considered prime candidates for targeted isolation based on their relative abundance on the PAO Chips (>5%) and novelty. OTU3088 shared 93.4% identity with the closest type strain, that is, *Ruminococcus torques*, and was present on GAM-SDD agar at 49.8% relative abundance (**Supplementary Table S6**). OTU3088 was also detected on GAM, CP and CP-SDD agar media, albeit at a lower relative abundance. We detected three additional OTUs (OTUs 3067, 3103 and 3070) at >2% relative abundance of which the closest type strain was also *Ruminococcus torques*. Since these three OTUs were always detected in samples that contained OTU3088, and since their representative reads shared high nucleotide identity with OTU3088 (>99%), we considered that they may be derived from the same bacterial strains.

The best hits in the SILVA type strain database of strains OTU322 and OTU3797 were *Hydrogenoanaerobacterium saccharovorans* and the chloroplast of the diatom *Thalassiosira pseudonana*, respectively, and both were detected at >10% relative abundance (OTU322, max. 23.9%; OTU3797, max. 13.2%). Both OTU322 and OTU3797 were only detected at >1% relative abundance on CP agar media in the absence of the SDD antibiotics. The representative read of OTU2642 shared 94.7% nucleotide identity with the 16S rRNA gene of *Bacteroides ovatus*. OTU2642 was only detected on GAM media at a maximum of 7.4% relative abundance. Finally, the closest type strain of OTU2024 was *Oscillibacter ruminantium*, and it was detected at >1% relative abundance exclusively on CP-SDD media.

Targeted cultivation

To provide proof of concept, we aimed to isolate strains corresponding to OTUs 3088 and 2024 as they were considered prime candidates for isolation based on novelty. Furthermore, the fact that these OTUs grew on media containing the SDD antibiotics suggested they may be antibiotic resistance reservoir species.

We prepared dilution series of the growth fractions in which these OTUs were most enriched and subsequently inoculated the diluted samples under the exact conditions that previously yielded enrichment of the target OTUs. This experiment, however, did not yield isolation of the target OTUs. Therefore, the protocol was repeated using the original faecal samples as inocula instead of the enriched growth fractions. By this method we isolated a member of OTU3088, which was demonstrated by the fact that the representative read of OTU3088 shared 100% identity with the 16S rRNA gene Sanger read of our isolate (**Figure 1B**).

BLASTn of the 16S rRNA gene read of the isolate against the NCBI ribosomal 16S RNA sequences database showed that closely related strains (98-99% nucleotide identity) have recently been isolated in four other laboratories. One closely related strain that was isolated from human faeces was recently published as a novel species named *Sellimonas intestinalis* BR72^T (Seo *et al.*, 2015).

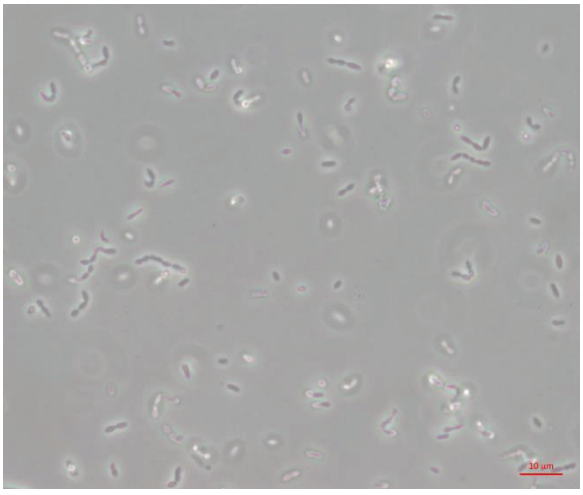

Figure 1B. A light microscopy picture of the strain corresponding to OTU3088 that was isolated by a targeted approach. The strain shares 99% 16S rRNA gene identity with *Sellimonas intestinalis* BR72^T

Discussion

In this investigation, we studied the cultivability of anaerobic human faecal bacteria in order to isolate strains that can serve as reservoirs of antibiotic resistance. Therefore, bacterial growth communities on GAM and CP agar media derived from faeces of 20 ICU patients receiving SDD therapy were studied. We applied the MicroDish PAO Chip to reduce potential toxicity of agar and to facilitate efficient parallel processing of a large number of samples. We also applied media supplemented with the SDD antibiotics that the patients received upon arrival on the ICU.

A first selection of bacteria was made by comparing the cultivated species with the strains on the most wanted list that comprises human-associated bacteria of which the genome has not yet been sequenced and that are grouped into priority classes based on novelty (Fodor *et al.,* 2012). We did not detect high priority taxa at >1% relative abundance in the growth communities. Medium priority taxa were detected at 1-3% relative abundance but they shared 100% 16S rRNA gene sequence identity with strains for which the genome has been already sequenced. Therefore, due to the low relative abundance and/or high similarity to previously genome-sequenced bacteria, the detected medium and high priority taxa were not further considered prime candidates for isolation. However, comparison of the OTU centroid reads with the strains in the SILVA type strain database yielded 16 OTUs with <95% nucleotide identity and >2% relative abundance.

Based on their novelty, members of these OTUs were considered candidates for isolation. Although their prevalence in the gut microbiota is expected to be <20%, i.e. they are not on the most wanted list, understanding the biology of such populations might be highly relevant in a more personalized approach, where individual-specific microbiota signatures are considered key to success (Raes, 2016). Our results show that novel human-associated bacteria can still be cultivated using conventional methods.

The extent to which novel bacteria can still be isolated by conventional methods was shown recently by Browne and co-authors in an experiment in which they isolated ~4,000 pure culture bacterial strains from faeces of six human individuals. The authors found that these isolates comprised as much as 96% of the bacterial abundance at the genus level and 90% of the bacterial abundance at the species level based on average relative abundance across faecal samples of six individuals (Browne *et al.,* 2016).

Almost all of the 16 OTUs detected in the growth communities represented novel species belonged to the *Firmicutes* (11 OTUs) and *Bacteroidetes* (4 OTUs), and these phyla were also detected in highest relative abundance in the faecal samples. Notably, a single OTU was detected on CP agar at a maximum relative abundance of 13.2% that shared 85.9% nucleotide identity with the best hit in the SILVA type strain database, namely the chloroplast of *Thalassiosira pseudonana*.

Chloroplasts are thought to have originated from cyanobacteria (Falcon *et al.*, 2010), and this finding might suggest the growth of a eukaryote capable of photosynthesis. OTU3088, which is a novel OTU belonging to the *Firmicutes*, was detected in samples of nine different patients. Furthermore, on one PAO Chip OTU3088 was detected in the presence of the SDD antibiotics at a relative abundance of 49.8%. Therefore, it was selected for isolation, which was achieved on GAM-SDD media (**Figure 1B**). After isolation, the 16S rRNA gene of the isolate turned out to share 99% nucleotide identity with the recently published *Sellimonas intestinalis* (Seo *et al.,* 2015), and high identity (98-99%) with three other strains recently isolated from human gut microbiota (accessions KT156811, LN828944 and AY960564).

Therefore, even though a close relative of our isolate has been recently described, these results demonstrate that high-throughput cultivation-based screening can be used to isolate novel antibiotic resistant bacteria by a targeted approach. The strain in question is a candidate to be further analysed as resistance reservoir (e.g. by genome sequencing).

Besides OTU3088, five other novel OTUs were found to grow in the presence of the SDD antibiotic cocktail, and as such are additional candidates for isolation and characterization (**Table 2**). However, it should be noted that antibiotics may be broken down by adjacent bacteria, and that therefore these bacteria may themselves be not resistant. For example, cefotaxime may be degraded through the secretion of a β-lactamase (Deak *et al.*, 1998; Buchschmidt *et al.,* 1992). Bacterial isolation in general can also be hampered by the dependence on microbe-microbe interactions or host-microbe interactions (Pham *et al.*, 2012). Out of the 16 novel OTUs detected on agar media, only OTU (OTU3088) was detected on both GAM and CP media at >2% relative abundance. This indicates that the number of target species for isolation might be increased by including different media.

We also investigated bacterial growth not pertinent to the isolation of novel species. We showed that the composition of bacterial growth communities was significantly impacted by medium and by supplementation of media with antibiotics (**Figure 3**). The cocktail used in SDD therapy contains antibiotics that are predominantly active against Gram-negative bacteria and fungi (Al Naiemi *et al.*, 2006), and was designed in order to eradicate potentially pathogenic bacteria from the gut without harming the anaerobic microbiota (de Smet *et al.*, 2012; Buelow, 2015). Indeed, we found that six OTUs belonging to the genus *Escherichia*, one of the SDD-target taxa, grew in significantly lower relative abundance on media containing the SDD cocktail. However, we also found that five OTUs belonging to the families *Ruminococcaceae* and *Lachnospiraceae* grew to a significantly lower relative abundance in the presence of the SDD antibiotics.

Members from these families are Gram-positive and lack aerobic respiration (Wolf *et al.*, 2004), and are therefore collaterally affected by the application of the SDD antibiotics that aim to lower the risk of infection with Gram-negative aerobic opportunistic pathogens (17).

Remarkably, we found that the correlation of bacterial composition of the faecal communities and the growth communities varied extensively between patients. We cannot exclude that this may have resulted from differences in viability of cryo-preserved faecal samples, but it might also be related with the individual-specific composition of the gut microbiota.

In conclusion, we have shown that high-throughput screening of growth communities for bacterial resistance can guide targeted isolation of potential reservoir species. The fact that a member of one novel antibiotic-resistant OTU (OTU3088) was successfully isolated demonstrates the viability of the approach. Follow-up isolation and characterization will be required to analyse the role of previously uncultivated species in the dissemination of resistance genes in the gut microbiota, including the transfer to potential pathogens.

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Supplementary data

Supplementary Table S5. Linear mixed-effect models of OTU-level composition data of bacterial growth were applied to investigate which OTUs varied in abundance as a results of cultivation conditions. This table lists the taxonomic affiliations of OTUs that were found to be enriched at FDR-corrected p-values of >0.05. Bacterial communities were cultivated on GAM agar and CP agar, both in the presence and absence of the SDD cocktail of antibiotics.

Supplementary table S6. Faecal samples of 20 patients were inoculated on PAO chips in duplo. The bacterial growth communities were analysed by 16S rRNA gene amplicon.

Data available: http://fungen.wur.nl/supplementary-information_bello-gonzalez/

Supplementary Table S1. Primers that were used for 16S rRNA gene amplicon sequencing.

Primers PCR step 1 (5' → 3')	**Primer Name**	**Barcode**	**Unitag**	**Degenerated primer**
	Unitag1-27F-DegS		GAGCCGTAGCCAGTCTGC	GTTYGATYMTGGCTCAG
	Unitag2-338R-I		GCCGTGACCGTGACATCG	GCWGCCTCCCGTAGGAGT
	Unitag2-338R-II		GCCGTGACCGTGACATCG	GCWGCCACCCGTAGGTGT
Primers PCR step 2 (5' → 3')	Miseq-46_TGCCTCTC_Unitag1	TGCCTCTC	GAGCCGTAGCCAGTCTGC	
	Miseq-46_TGCCTCTC_Unitag2	TGCCTCTC	GCCGTGACCGTGACATCG	

Supplementary Table S2. A mock community was included in every library that was sent for 16S rRNAgene amplicon sequencing. This table shows the OTU-level phyla that were presentin the mock community.

#OTU taxonomic affiliation	Relative abundance
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__*Bifidobacterium*	0.025913966
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Corynebacteriales;f__Nocardiaceae;g__*Rhodococcus*	0.996691
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__*Bacteroides*	0.149503648
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__g	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__*Parabacteroides*	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__g	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__*Prevotella*	0.000996691
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__*Alistipes*	9.96691
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__g	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__f;g__g	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__*Bacillus*	0.049834549
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__*Granulicatella*	0.024917275
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__*Enterococcus*	0.024917275
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__*Lactobacillus*	0.074751824
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__*Lactococcus*	0.024917275
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__*Streptococcus*	0.049834549
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__*Clostridium*	0.024917275
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Anaerostipes*	0.024917275
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Blautia*	0.996691
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Incertae_Sedis*	0.074851493
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Pseudobutyrivibrio*	0.024917275
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__*Incertae_Sedis*	0.024917275
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__*Faecalibacterium*	0.000996691
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__*Incertae_Sedis*	0.024917275
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__*Veillonella*	0.024917275
k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;f__Fusobacteriaceae;g__*Fusobacterium*	0.024917275
k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;f__f;g__g	0
k__Bacteria;p__Lentisphaerae;c__Lentisphaeria;o__Victivallales;f__Victivallaceae;g__*Victivallis*	0.024917275
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Citrobacter*	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Enterobacter*	0.049834549
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Escherichia-Shigella*	0.049834549
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Serratia*	0.024917275
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__g	0.049834549
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__f;g__g	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__*Pseudomonas*	0.049834549
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__*Akkermansia*	0.024917275
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Micrococcales;f__Micrococcaceae;g__*Micrococcus*	9.96691
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Dorea*	0.024917275
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Klebsiella*	0.024917275

Supplementary Table S3. Faecal samples of 20 patients were inoculated on PAO chips in duplo. This table gives information about the growth observed on each chip at the time that the growth communities were harvested. Communities on GAM agar were harvested after 48 h and those on CP agar after 72 h. C stands for confluent growth.

GAM			
Sample ID	10-0	10-1	10-2
241 (1)	c	c	c
241 (2)	c	c	c
188 (1)	c	c	c
188 (2)	c	c	c
202 (1)	c	c	17
202 (2)	c	c	10
145 (1)	c	c	c
145 (2)	c	c	c
256 (1)	c	c	c
256 (2)	c	c	c
238 (1)	c	c	c
238 (2)	c	c	c
239 (1)	c	c	c
239 (2)	c	c	c
142 (1)	c	c	c
142 (2)	c	c	c
201 (1)	c	c	c
201 (2)	c	c	c
288 (1)	c	c	c
288 (2)	c	c	c
213 (1)	c	c	c
213 (2)	c	c	c
160 (1)	c	c	c
160 (2)	c	c	c
210 (1)	c	c	c
210 (2)	c	c	c
131 (1)	c	c	c
131 (2)	c	c	c
148 (1)	c	c	c
148 (2)	c	c	c
172 (1)	c	c	c
172 (2)	c	c	c
209 (1)	c	c	c
209 (2)	c	c	c
198 (1)	c	~110	20
198 (2)	c	~120	27
236 (1)	c	c	c
236 (2)	c	c	c
266 (1)	c	c	c
266 (2)	c	c	c

GAM + SDD			
Sample ID	10-0	10-1	10-2
241 (1)	c	c	c
241 (2)	c	c	c
188 (1)	c	c	16
188 (2)	c	c	12
202 (1)	33	3	-
202 (2)	8	3	-
145 (1)	c	c	34
145 (2)	c	c	40
256 (1)	c	1	-
256 (2)	c	-	-
238 (1)	c	c	c
238 (2)	c	c	c
239 (1)	c	c	c
239 (2)	c	c	c
142 (1)	c	c	6
142 (2)	c	35	3
201 (1)	c	c	c
201 (2)	c	c	c
288 (1)	c	c	c
288 (2)	c	c	c
213 (1)	c	c	c
213 (2)	c	c	c
160 (1)	42	1	-
160 (2)	55	-	-
210 (1)	c	c	c
210 (2)	c	c	c
131 (1)	c	c	c
131 (2)	c	c	c
148 (1)	c	c	c
148 (2)	c	c	c
172 (1)	c	c	c
172 (2)	c	c	c
209 (1)	-	-	-
209 (2)	-	-	-
198 (1)	5	1	-
198 (2)	12	-	-
236 (1)	c	c	c
236 (2)	c	c	c
266 (1)	c	~140	41
266 (2)	c	~110	52

CP					CP + SDD			
Sample ID	10-0	10-1	10-2		Sample ID	10-0	10-1	10-2
241 (1)	c	c	c		241 (1)	c	c	c
241 (2)	c	c	c		241 (2)	c	c	c
188 (1)	c	c	50		188 (1)	c	c	10
188 (2)	c	c	50		188 (2)	c	c	10
202 (1)	-	30	2		202 (1)	-	8	-
202 (2)	-	30	2		202 (2)	-	8	-
145 (1)	c	c	58		145 (1)	c	c	40
145 (2)	c	c	58		145 (2)	c	c	40
256 (1)	-	-	-		256 (1)	-	-	-
256 (2)	-	-	-		256 (2)	-	-	-
238 (1)	c	c	c		238 (1)	c	c	c
238 (2)	c	c	c		238 (2)	c	c	c
239 (1)	c	c	c		239 (1)	c	c	c
239 (2)	c	c	c		239 (2)	c	c	c
142 (1)	c	c	c		142 (1)	c	c	c
142 (2)	c	c	c		142 (2)	c	c	c
201 (1)	c	c	c		201 (1)	c	c	c
201 (2)	c	c	c		201 (2)	c	c	c
288 (1)	c	c	c		288 (1)	c	c	c
288 (2)	c	c	c		288 (2)	c	c	c
213 (1)	-	-	-		213 (1)	-	-	-
213 (2)	-	-	-		213 (2)	-	-	-
160 (1)	c	c	c		160 (1)	c	4	-
160 (2)	c	c	c		160 (2)	c	4	-
210 (1)	c	c	c		210 (1)	c	c	c
210 (2)	c	c	c		210 (2)	c	c	c
131 (1)	c	c	c		131 (1)	c	c	c
131 (2)	c	c	c		131 (2)	c	c	c
148 (1)	c	c	c		148 (1)	c	c	c
148 (2)	c	c	c		148 (2)	c	c	c
172 (1)	c	c	c		172 (1)	c	c	c
172 (2)	c	c	c		172 (2)	c	c	c
209 (1)	c	4	-		209 (1)	c	2	-
209 (2)	c	4	-		209 (2)	c	2	-
198 (1)	c	c	58		198 (1)	c	c	2
198 (2)	c	c	58		198 (2)	c	c	2
236 (1)	c	c	c		236 (1)	c	c	c
236 (2)	c	c	c		236 (2)	c	c	c
266 (1)	c	c	c		266 (1)	c	2	-
266 (2)	c	c	c		266 (2)	c	2	-

Supplementary Table S4. Faecal samples of 20 patients were inoculated on PAO chips on top of agar media in duplo. This table gives information about the number of reads that were obtained for each sample by 16S rRNA gene amplicon sequencing (MiSEQ). Communities on GAM agar were harvested after 48 h and those on CP agar after 72 h.

			GAM			GAM + SDD			CP			CP + SDD		
Patient	Faecal samples	Sample ID	10-0	10-1	10-2	10-0	10-1	10-2	10-0	10-1	10-2	10-0	10-1	10-2
241	2	241 (1)	12075		34574	14019		51838	8920			2012		22929
	(54813, 14032)	241 (2)	222887		63202	114244		41963	15668			15377		2617
188	2	188 (1)	16486	3819	9616	3905	27528	980	159914		17067	4412		24330
	(95917, 237616)	188 (2)	19136	6996	2831	9355	14529	7742	60259		14121	9225		11636
202	2	202 (1)	1387	8723	15794	30244								
	(1272, 56304)	202 (2)	13225	4195	1821	11016								
145	1	145 (1)	47949		12446	32533	29082	77004	55035		6088	14799		2697
	(2678)	145 (2)	132419		56373	45249	25892	25948	6464		1079	2314		7897
256		256 (1)	25098		46535	205207								
		256 (2)	10994		48081	12837								
238	2	238 (1)	21396		62244	19894		90769	310326			86633		36188
	(87486, 43467)	238 (2)	68745		149739	104929		19713	96942			62351		
239	2	239 (1)	2538		6648	18311		15827	86178		12585	5507		12258
	(42032, 52258)	239 (2)	15092		32535	16853		17431	4121		2747	3538		35720
142	2	142 (1)	30912		40247	28811	136283	8223	61010		206341	89583		9045
	(100391, 74306)	142 (2)	50838		78848	131025	131544	12246	121780		371078	4088		14184
201	2	201 (1)	87727		61353	49327		10454	44113			0		121045
	(16813, 21971)	201 (2)	39260		34525	41148		4162	210211			5673		10168
288	2	288 (1)	43265		42504	44114		8780	7381			5458		
	(18841, 40696)	288 (2)	3356		48352	30409		3281	16008			40471		
213		213 (1)	86702		2	6432		67109						
		213 (2)	16757		21710	135068		17415						
160	2	160 (1)	19197		63636	74931			84155					
	67824, 87621)	160 (2)	75250		154135	93536			43297					
210	2	210 (1)	22409	20505	4503	57428	991	66212	49021	8852	7281	37091	4311	211
	(36981, 6641)	210 (2)	12019	697	11316	35206	13100	6099	4139	9539	0	1291	8427	18301
131	2	131 (1)	5848	4289	4471	15	164	1849	3854	10348	11217	12467	10094	2729
	(25992, 102860)	131 (2)	36390	1	6623	14130	1222	440	474	25538	5263	86479	2449	72669
148	2	148 (1)	22169	47133	129203	682	2120	8040	37399	6279		28368	4253	45832
	(9397,33138)	148 (2)	36925	117595	9585	71259	29283	45741	2949	67716		143264	1191	1415
172	2	172 (1)	15894	10167	15425	68358		153978	46485		60002	6502		45755
	(68479, 2765)	172 (2)	171442		157845	49916		72298	37319		58173	1166		45113
209	2	209 (1)	60367		13846				29706					
	(73128, 60618)	209 (2)	50252		17047				48309					
198	2	198 (1)	49604	24055	8720	50169			49310					
	(50210, 40964)	198 (2)	33006	50400	43972	45197			30126					
236	2	236 (1)	83989		23360	170058		0	33954		32425	7147		6775
	(0, 31850)	236 (2)	22966		88299	24917		14299	21234		58080	8512		7889
266	2	266 (1)	62787		34698	11696	0	1284	98639		80814	11777		
	(15506, 6309)	266 (2)	32979		10456	6008	2107	6814	155180		105253	10872		

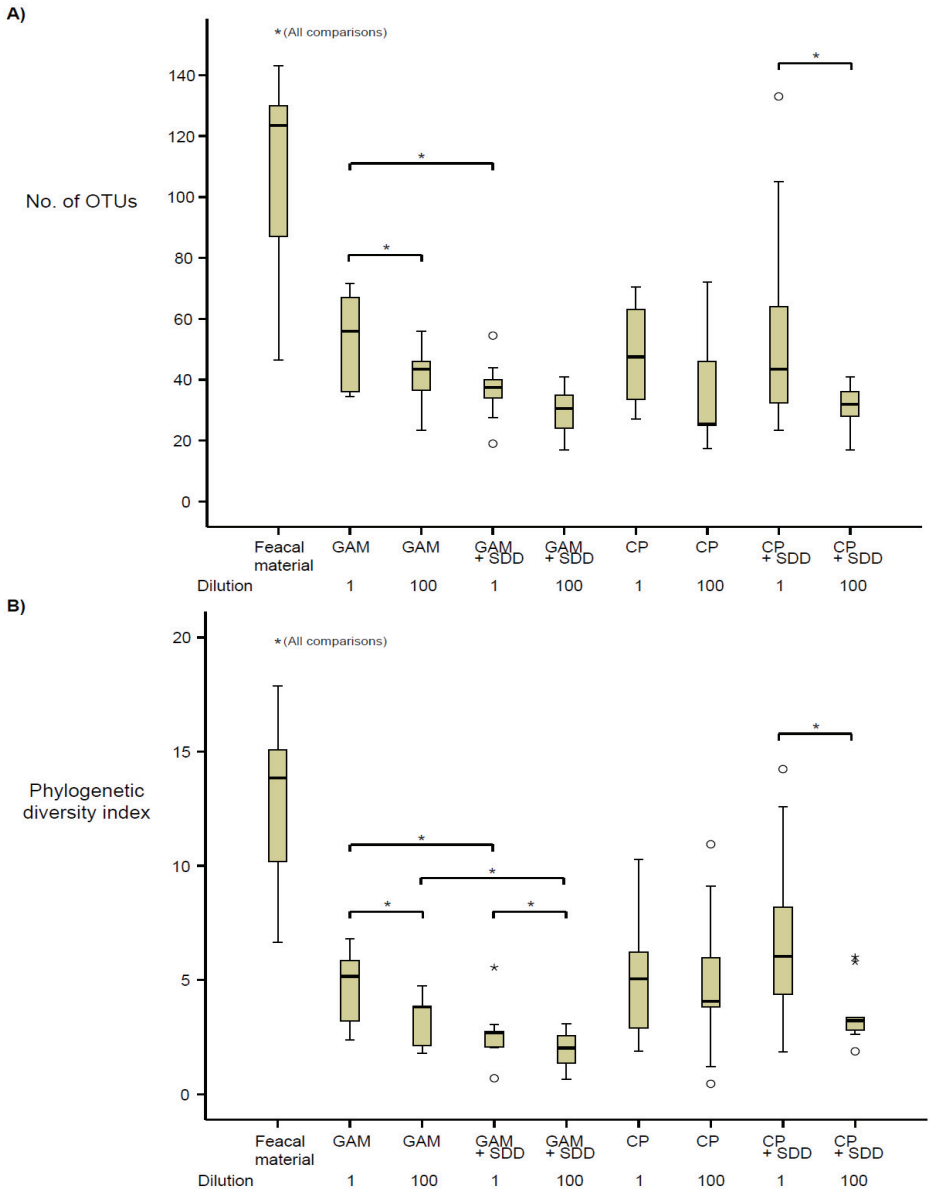

Figure S1. Boxplots depicting the distribution of no. of OTUs (panel A) and phylogenetic diversity values **(Panel B)** of bacterial communities in different experimental groups. The experimental groups are the faecal inocula and their corresponding growth communities on GAM and CP agar media. Growth on these media was further subdivided based on the addition of the SDD antibiotics. Asterisks indicate that these richness and diversity values of bacterial communities in experimental groups were significantly different ($p = <0.05$) based on the two-tailed t-test

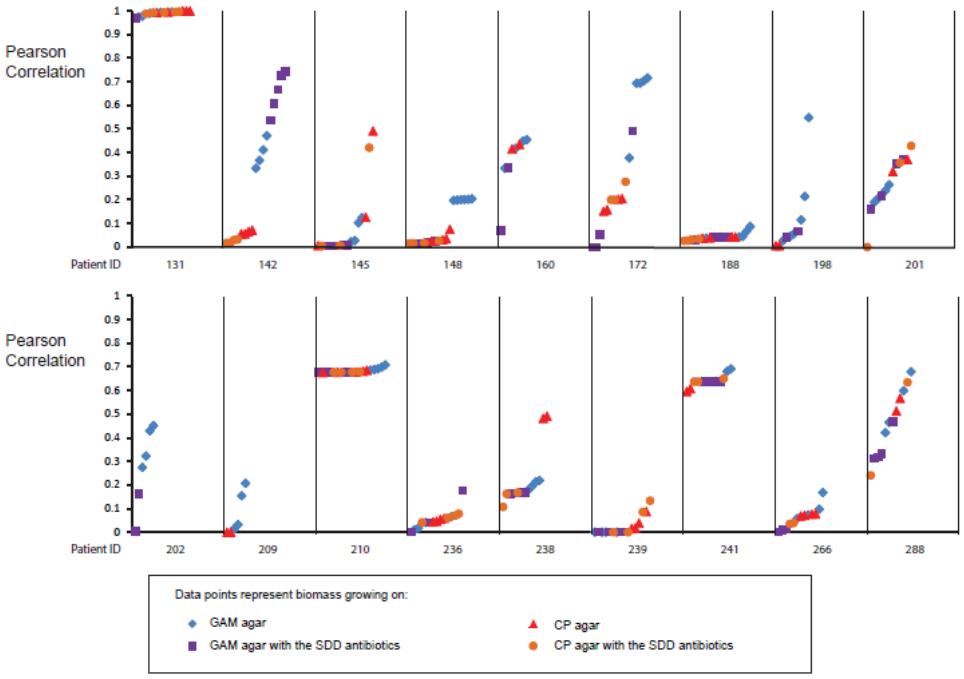

Figure S2. The bacterial communities in the faecal samples of 18 intensive care patients were compared to the corresponding growth communities on GAM agar and CP agar by calculating the Pearson correlation coefficients of OTU-level taxa. Growth on these media was further distinguished based on the addition of the SDD antibiotics. Per patient the Pearson coefficients are sorted from lower to higher values.

CHAPTER 9

Discussion

Outlook and Future perspective

DISCUSSION

Antibiotics represent one of the most powerful tools for the treatment of human infectious diseases caused by bacteria. They are also used in agriculture, where the influence of antibiotics in the environment and association to human health is still unclear (Schmieder and Edwards, 2012). Unfortunately, the extensive use of antibiotics is frequently associated with a negative consequence: the development of antibiotic resistance in microbes, which generates an enormous problem in the health care system (Hancock, 2007; Roca *et al.,* 2015; Barlam *et al.,* 2016). This situation affects especially critically ill patients, and is associated with an increase of morbidity, mortality and health care cost; in addition to its contribution to the failure of antibiotic therapies (Vincent *et al.,* 2011).

The dissemination of antibiotic resistant bacteria and resistance genes is influenced by different factors including, among others, the selective pressure of antibiotics (Jernberg *et al.,* 2010). The majority of the currently known resistance genes have been identified from clinical and veterinary bacterial isolates by using culture dependent techniques. This focus has led to an underestimation of the vast number of uncultured bacteria and the importance of other environments that can serve as a potential reservoir of antibiotic resistance genes (Seveno *et al*., 2002). It has been established that commensal bacteria serve as a reservoir of antibiotic resistance genes, which in turn are frequently located on mobile elements and can be transferred from commensal to pathogenic bacteria (Marshall *et al*., 2009). In fact, many antibiotic resistance genes present in human commensal bacteria are highly homologous and share similar genetic features with resistance genes found in pathogens (Shoemaker *et al.,* 2001). In contrast, in soil, bacterial communities were shown to harbour a distinct resistome compared to that associated with pathogens, suggesting that antibiotic resistance genes present in soil bacteria do not transfer between species (Forsberg *et al.,* 2014).

The increasing number of publications using culture independent approaches on putative environmental reservoirs of resistance genes, including soil, water, food and gut microbiota is improving our knowledge of the ecological context in which these resistance genes are present. Also, it adds to our understanding about the mechanisms underlying their spread and distribution in different environments (Schmieder and Edwards, 2012).

In the gut a complex microbial community exists, which is adapted to its particular niche, and associated with several nutritional, metabolic, immunological and physiological functions (Backhed *et al.,* 2005). Because of its role in the host, diversity and dynamics of the gut microbiota have been intensely studied in the last decades (Sekirov *et al.,* 2010).

Antibiotic therapy has since been shown to disturb the ecological balance of the gut microbiota, providing a perfect scenario to exchange resistance traits with other members of the gut, including potential pathogens (Perez-Cobas *et al.,* 2013). The common consequence is the emergence of antibiotic resistance in close proximity to the human host, resulting in actual health impact.

The work presented in this thesis aimed to enhance our understanding of the ecological context of antibiotic resistance and subsistence. Moreover, the diversity and colonization dynamics of the gut microbiota and associated resistome was studied by using a variety of high throughput techniques and metagenomics sequencing approaches in combination with traditional and high throughput cultivation techniques (Porous aluminium oxide – PAO Chips) to identify and characterize the potential reservoir of antibiotic resistance.

Subsistence phenotype: an ecological context

The majority of the antibiotics used in clinical settings is derived from antibiotic producing bacteria present in the environment such as Actinomycetes. Several members of the bacterial community present in the environment have been identified as reservoirs of antibiotic resistance genes (Riesenfeld *et al.,* 2004; D'Costa *et al.*, 2006). In addition to resistance, recent studies showed that few bacterial species present in soil, seawater and gut microbiota of humans, farm and zoo animals are able to use antibiotics as a sole carbon source, known as the subsistence phenotype (Barnhill *et al.,* 2011, Dantas *et al.,* 2008; Dopazo *et al.,* 1988; Xin *et al.,* 2012, **chapter 2** of this thesis). In **chapter 2**, different approaches were implemented to study the genetic determinants involved in this phenotype. The results provided insights into the mechanisms, and we concluded that a) the presence of a common aminoglycoside resistance gene (aminoglycoside 3' phosphotransferase II (APH (3') II) is needed for the bacteria to display the phenotype, b) by using both higher and lower concentrations of the aminoglycoside to mimic the concentrations used for the control of infectious and those present in the environment, the subsistence phenotype was still displayed, and c) glycosyl hydrolases appeared to play a key role in the subsistence phenotype, since the presence of a specific inhibitor (Deoxynohirimycin – DNJ) hampered the bacteria to grow on antibiotics as compared with growth obtained by using glucose as a growth substrate. However, a discrepancy between the subsistence phenotype and measurable antibiotic degradation was observed since no antibiotic degradation could be detected.

Previously, Walsh *et al* (2013) tried to reproduce and verify the hypothesis that soil bacteria are capable to subsist on antibiotics that was brought forwards by Dantas *et al* (2008). Similar to our results, Walsh and coworkers found that soil bacteria were not able to degrade antibiotics and as a consequence, it is unlikely to be used as a carbon source.

Alternatively, Walsh *et al* (2013) proposed that bacteria possibly use a well characterized resistance mechanism that has not been previously linked to antibiotic subsistence.

Nevertheless, future studies on antibiotic degradation should allow researchers to elucidate the mechanisms and genes associated with the subsistence phenotype. These studies could include the metabolic pathways and role of enzymes involved in antibiotic degradation, and could in addition address genetic elements involved in the regulation of carbon utilization. Laboratory evolution experiments will be required in order to assess the genomic adaptation of bacteria towards the subsistence phenotype. Moreover, a deep analysis on the ecological context in which the subsistence phenotype occurs would provide insight into the microorganisms capable to catabolize antibiotics and their environmental niche, including the genetic elements that could participate through enzyme inactivation and their capacity to transfer to other members of the microbial community. Until now, the group of bacteria that have been described to display the subsistence phenotype are phylogenetically diverse and closely related to pathogens associated to human infectious diseases. Therefore, it will be important to define the relationship between antibiotic resistance and antibiotic subsistence phenotype.

Diversity and dynamics of the gut microbiota in non-human primates

Several studies using high resolution 16S rRNA gene targeted phylogenetic analyses and high-throughput sequencing efforts indicated that great apes, including humans, have a particular gut microbiota composition, similar to the most closely related species (Ochman *et al*; 2010). Such evolutionary conservation could hint at functional relevance of the microbiota, and perhaps adaptive alterations of the hosts, for example regarding diet. In **chapter 3**, the applicability of the Human Intestinal Tract Chip (HITChip) for non-human primates was assessed by studying the gut microbiota composition of chimpanzee, gorilla and marmoset, and comparing them with faecal samples obtained from healthy humans.

The results indicate that the HITChip provides a robust alternative to study the microbiota composition of chimpanzees and gorillas. For marmoset samples, however, only low signal intensities were observed, suggestive of limited applicability. In addition, two human enterotypes were detected in the chimpanzee and gorilla samples, reinforcing previous observations that enterotypes are not exclusive to humans but can also be encountered in non-human primates (Moeller *et al.,* 2012). A strong correlation was obtained when the results were compared with a dataset obtained by 454 pyrosequencing from the same animal species. Previous studies showed that phylogenetic microarray and pyrosequencing analysis methods are strongly correlated, both methods allowing to determine in-depth the phylogenetic profile of gut microbial communities (Claesson *et al.,* 2009). Future applications of this technique could include the study of microbiota composition of wild and captive non-human primates and the effects of external factors such as diet and antibiotic administration.

Antibiotic therapy and the human gut microbiota

Hospital acquired-infections are a common complication in hospitalized patients, especially those associated with prolonged stay and caused by antibiotic resistant bacteria (Vincent *et al*., 2011). In the ICU, the application of antibiotic prophylactic therapies aims to prevent secondary infections by potential pathogens that could be already present in the body or acquired during ICU stay (Houben *et al.,* 2014). Previous studies have shown that antibiotic prophylactic therapies reduce the incidence of ventilator-associated pneumonia, decrease the morbidity and mortality and improve the patient outcomes (Pileggi *et al.,* 2011). However, the diversity and colonization dynamics of gut microbiota in ICU hospitalized patients has been poorly characterized, and the rate of colonization with antibiotic resistant bacteria during and after the application of prophylactic antibiotic therapies in critically ill patients is still controversial.

In order to assess the diversity and dynamics of the gut microbiome and resistome in ICU hospitalized patients, a high throughput phylogenetic microarray platform (HITChip) and functional metagenomics approaches were implemented to determine the dynamics of the gut microbiota composition during hospital stay in a single patient (**Chapter 4**). The results indicated that the gut microbiota composition was highly dynamic, with fluctuations in the relative abundance of *Bacteroidetes, Clostridium* cluster XIVa and IV during SDD therapy, increases of *Bacilli* after therapy discontinuation, and notorious changes after hospital discharge (high relative abundance of *Firmicutes* dominated by *Clostridium* cluster XIVa). Functional metagenomics analysis indicated that the aminoglycoside resistance genes (*aph (2'')-Ib* and an *aadE*-like gene) were the two most dominant genes that increased in abundance during ICU stay. The *aph (2'')-Ib* gene was associated with mobile elements and was harboured by a strain from the genus *Subdoligranulum* that includes members of the group of anaerobic commensal microbiota. Our results suggested that these genetic mobile elements can be mobilized and/or have been acquired through horizontal gene transfer, as reported previously for members of the *Firmicutes* (Jones *et al.,* 2010), which could contribute to the risk of transfer of antibiotic resistance genes from commensals to potential pathogens. It cannot be excluded that the increase of aminoglycoside resistance genes may also be an effect of the SDD therapy since tobramycin is used as part as the cocktail administrated to the patients.

Recently, Oostdjik *et al.,* reported in a randomized clinical trial an increase of aminoglycoside resistant Gram-negative bacteria during SDD therapy (Oostdjik *et al.,* 2014). Based on these observations, a control of the use of aminoglycoside should be considered during therapy, especially for the group of aerobic Gram-negative bacteria.

In clinical settings, metagenomics is not implemented as a routine procedure for the analysis, identification and quantification of antibiotic resistance genes due to high cost and time constraints.

To this end, the recent technological advances in high throughput quantitative PCR approaches allow for the identification and quantification of multiple antibiotic resistance genes and has been used as an alternative to metagenomics sequencing due to lower costs and faster turn-around times. In **chapter 5**, an extended study of the gut microbiota and resistome was performed by using HITChip and nanolitre scale high throughput PCR.

The study included faecal samples from eleven ICU-hospitalized patients receiving SDD therapy and a control group comprising healthy individuals. The results of this study reinforced that SDD therapy disturbs the ecological balance of the gut microbiota as an uncontrollable secondary effect and decreases the diversity of the gut microbiota as compared to healthy individuals. In healthy adult individuals, *Clostridium* cluster IV represents the predominant group of gut bacteria, and is considered to play a beneficial role in gut homeostasis (Louis *et al*., 2009; Machiels *et al.,* 2014). In ICU patients we firstly observed a decrease in the abundance of *Enterobacteriaceae* as well as undetectable levels of associated resistance genes, most probably as a consequence of colistin administration during the therapy. This can be seen as a beneficial effect of the therapy, however, it remains unclear if a recolonization post SDD occurs. Secondly, the relative abundance of enterococci was increased, and it is tempting to speculate that this might be related to the decrease in the population of enterobacteria as described previously (Brandl *et al.,* 2008) and a decrease in the relative abundance of *Clostridium* cluster IV and XIVa as reported previously (Benus *et al*., 2010; Daneman *et al.,* 2013), which could have a sustained effect on the homeostasis of the gut microbiota. Thirdly, the presence of genes conferring resistance to beta-lactams, tetracycline and aminoglycosides associated with commensal bacteria has been described as a protective effect against colonization with antibiotic resistant Gram-positive bacteria (Stiefel *et al.,* 2014), whereas an increase of antibiotic resistance genes associated with Gram-positive bacteria during ICU stay highlight the importance to carefully examine the applicability of SDD therapy in countries with high prevalence of antibiotic resistant bacteria.

Likewise, future studies using e.g. prebiotics and probiotics as a strategy to restore the gut microbiota are needed, especially because the reduction of the microbial diversity could facilitate the overgrowth by antibiotic resistant-potential pathogens. So far, all these methods allow to only identify the resistance genes without being able to identify the bacterial host. In fact, only culture dependent techniques are commonly used to determine the prevalence of colonization with antibiotic resistant bacteria in ICU hospitalized patients as a part of the surveillance control, focusing mainly on aerobic bacteria (Daneman *et al.,* 2013). As a consequence, the ecological perturbation induced by SDD therapy is underestimated or even neglected since the anaerobic commensal bacterial community, an important reservoir of antibiotic resistance genes, is not taken into consideration.

Previous studies have shown that different antibiotic treatments affect the gut microbiota (Robinson and Young, 2010). Observed effects are related not only to the antibiotic class and structure, but also to the pool of resistance genes present in the microbial community, since the dynamics of the resistome is affected by the antibiotic target resistance and by the surviving community (Perez-Cobas *et al.,* 2013). In addition to the antibiotic class and structure, Zhang *et al.,* showed that oral administration of antibiotics led to increases of antibiotic resistance genes in the gut, while the effect of intravenous antibiotic administration was less pronounced. Nevertheless, both effects can be more or less pronounced depending on the administered antibiotic dose and the route of excretion (Zhang *et al.,* 2013). It has been reported that selection for resistance in bacteria could occur at lethal or non-lethal antibiotic concentrations, which in the latter case could increase the rates of mutations and enrich the pool of antibiotic resistant bacteria (Anderson and Hugues, 2012). During SDD therapy, a cocktail of antibiotics is administered through the oral cavity as well as intravenously, and under such conditions it has been demonstrated for instance that the microbial diversity is altered and resistance genes can be selected for in the surviving populations (Zaborin *et al.,* 2014).

The remaining microbiota could include potential pathogens with the capacity to overgrow and survive, in addition to commensal anaerobic bacteria that could serve as a reservoir of antibiotic resistance genes.

Despite efforts to control infections in ICU patients, a better understanding of the antibiotic resistant bacteria colonizing the gut microbiota is needed.

In order to expand our knowledge on the ecological perturbation induced by SDD therapy, an extended study of the diversity and colonization dynamics of the gut microbiota was performed in eleven ICU patients receiving SDD therapy (same patients as chapter 5) by using traditional microbial cultivation approaches combined with HITChip analysis (**Chapter 6**). A range of culture media and selective culture conditions allowed to detected a variety of taxonomic groups, including the three most common potential aerobic pathogens associated with hospital-acquired infections, namely enterobacteria, staphylococci and enterococci, with enterococci being the most predominant genus identified, and several members of the commensal anaerobic microbiota including butyrate producing bacteria. The diversity and colonization dynamics of the gut microbiota in these patients was supported by the phylogenetic analysis, which indicated that SDD therapy could have a replacement effect on the bacterial community since a suppression of *Enterobacteriaceae* and a concomitant increase of the *Enterococcus* population was observed. In addition, the relative abundance of *Clostridium* clusters XIVa and IV was reduced during therapy. Similar results were obtained by Benus *et al.* (2010) by using 16S rRNA-targeted Fluorescent In Situ Hybridization (FISH), suggesting that the *Enterococcus* population needs to be considered during the application of this therapy in countries with high prevalence of enterococcal acquired-infections.

Using traditional cultivation techniques helps not only to isolate a variety of taxonomic groups but also provides an opportunity to map the antibiotic phenotypes of these isolates and determine the colonization dynamics with antibiotic resistant bacteria during ICU-hospitalization. However, since the majority of the patients received additional systemic antibiotic treatment for the control of infections, the

exact effect of SDD therapy on the gut microbiota composition in ICU patients remains unclear.

However, based on the antibiotic phenotypes of the majority of the isolates, the antibiotic classes of macrolides and tetracyclines may be the main contributors to the antibiotic resistance profile observed. Previous studied showed that erythromycin and tetracycline antibiotic resistance genes can be acquired by conjugative plasmids and conjugative transposons and transfer between Gram-positive and Gram-negative bacteria (Salyers *et al.,* 2004; Gupta *el al.,* 2003; Wang *et al.,* 2003). Therefore, future studies that focus on the genetic elements present in the isolates could help to understand the dynamics of the resistance genes present during antibiotic treatment. However, it should be noted that merely mapping the presence of mobile elements will not disclose the extent or directionality of gene transfer.

It has been indicated that an emergence of polymyxin resistance in Gram-negative bacteria could occur after the introduction of SDD therapy, especially in patients that carry Gram-negative bacteria that are resistant to tobramycin (Halaby *et al*., 2013; Oostidjk *et al.,* 2013). In the study presented in this thesis, a low rate of antibiotic resistance to tobramycin and polymyxin seems to persist in Dutch ICUs as was previously reported (Wittekamp *et al.,* 2015). Moreover, during SDD therapy an association with the emergence of ESBLs has been described as a result of the use of cephalosporins as part of the antibiotic cocktail (Al Naiemi *et al*., 2006). However, the results obtained in that study indicated that SDD therapy is still a useful therapy in the control of Gram-negative ESBL bacilli.

It is still unknown whether the antibiotic concentration present in the gut during SDD therapy in patients with constipations make a pre-selection of antibiotic resistant bacteria or increase the lateral transfer of resistance genes from one bacteria to another. Moreover, considering that the endogenous anaerobic microbiota is altered, contributing to an altered (reduced) colonization resistance, these results suggest that a re-definition of the concept of selective decontamination,

i.e. “SDD therapy does not affect the anaerobic gut microbiota” (van der Waaij *et al.,* 1990), needs to be considered.

The work presented here has several limitations that need to be taken into consideration for the full interpretation of the results obtained. These include, for example, the small number of patients and the inherent limited statistical power. Furthermore, the classification of the samples by groups based on ICU stay days was established arbitrarily, mainly, because the absence of equal distribution of the samples obtained, clinical conditions, administration of opioids and altered gut motility that did not allow to obtain faecal samples in the first 24-48 hours of hospitalization for all the patients. In addition, it is often unavoidable that patients in ICUs are exposed to invasive procedures or receive additional antibiotic treatment, which likely affect the results especially because of the antibiotic selective pressure present in the gut and use of broad spectrum antibiotics to control infectious diseases. Moreover, it was not possible to include a clinical control group as the majority of the ICUs in Netherlands nowadays use SDD therapy. Finally, the long-term perturbation induced by antibiotic treatment could not be established and future studies need to be performed in order to answer this and many other questions regarding to the ecological perturbation induced by the administration of antibiotics cocktails which leads to collateral damage of the commensal microbiota and therefore potentially human health.

So far, the data obtained in **chapters 4, 5 and 6** suggest that the diversity of the microbial community is reduced during SDD therapy and resistance genes can be selected for in the remaining community members as a strategy to survive the environmental conditions, limitation of nutrients and antibiotic pressure. In **chapter 7,** a characterization of *Enterococcus* colonization dynamics in ICU hospitalized patients receiving SOD and SDD therapy was investigated. Overall, the results showed a pool of diverse enterococcal species colonizing individual ICU patients during prophylactic therapies, being *E. faecalis* and *E. faecium* as the most dominant species identified.

It has been previously suggested that SDD therapy could increase *Enterococcus* colonization in ICU patients (Humphreys *et al.,* 1992).

In the study presented in this thesis, an increase in the clonal diversity and clonal replacement was observed for *E. faecium* isolates, whereas a narrow clonal diversity was observed in *E. faecalis* isolates, including a new sequence type. Furthermore, we detected the simultaneous presence of more than two virulence factors and/or virulence factor and antibiotic resistance genes. Recently, Muruzábal-Lecumberri *et al.* (2015) showed a high prevalence of *E. faecalis* isolated from ICU patients receiving SDD therapy and that those isolates were associated with multidrug resistance and virulence genes.

The ability of enterococci to adapt to different environmental conditions facilitates their colonization and subsequent infection in hospitalized patients (Guzman Prieto *et al.,* 2016). Further studies are needed to investigate the cellular and molecular interaction that promotes colonization and the resulting enterococcal infections. Even if the percentage of enterococcal infections and antibiotic resistance is low in the Netherlands, more research could be focusing on determining the prevalence of *Enterococcus* especially in critically ill patients receiving SDD or SOD therapy and in future strategies to prevent and control the spread of antibiotic resistant strains.

Microbial culture chip targeting members of the most wanted list

The recently reported “most wanted” taxa list from the Human Microbiome Project (HMP) suggested that several members have been poorly studied due to the difficulties to cultivate them. Moreover, the National Institute of Health (NIH) started to actively support the development of novel cultivation techniques in order to isolate and characterize these microorganisms and study their role in human health and disease (Fodor *et al.,* 2012). In the last years, few studies have been performed in order to cultivate the currently uncultivable fraction of the human gut

microbiota by using a combination of culturomics and high-throughput sequencing techniques (Goodman *et al*., 2011; Lagier *et al.,* 2012; Rettedal *et al*., 2014).

More recently, Lau *et al*. (2016) showed that by using culture-enriched molecular profiling, the majority of the bacteria present in faecal samples can be cultivable. The combination of culture dependent and independent techniques has been used to determine the effects on antibiotic treatment in the gut microbiota, focusing in the anaerobic microbiota as a potential reservoir for antibiotic resistance genes (Rashid *et al.,* 2015). In **chapter 8**, we aimed to isolate previously uncultured resistant anaerobic bacteria from faecal samples of 20 patients receiving SDD therapy by using a high throughput cultivation approach (PAO-Chips) and 16S rRNA gene amplicon sequencing. The results of this study indicated that the PAO Chip is a promising tool that allows to isolate several members of the most wanted taxa.

Moreover, the use of rich and poor media and the addition of antibiotics to the media provide useful information regarding the conditions in which specific bacteria are able to grow as demonstrated by the relative abundance of *Cyanobacteria* obtained by using CP media. Nevertheless, the implementation of the PAO chip to obtain pure cultures using targeted isolation remains challenging, and future studies in these directions could help to optimize such techniques. Furthermore, the use of antibiotics in the media and the implementation of PAO Chips as a support for the bacterial growth will allow to study the syntrophic interaction between bacterial species. This study showed that high-throughput screening of growth communities for bacterial resistance can guide targeted isolation of potential reservoir species, providing useful information on the diversity of the gut microbiota and its antibiotic resistance phenotype that cannot be derived by using culture independent techniques solely. Future studies including antibiotic resistance phenotyping and further genetic and physiological characterization of the isolates could contribute to understand the spread of antibiotic resistance genes and the possible transfer to other members of the gut microbiota.

Outlook and future perspectives

Subsistence phenotype

The antibiotic resistance phenotype and antibiotic resistance genes have evolved before the use of antibiotics as therapeutics. Moreover, antibiotics and antibiotic resistance genes seem to play multiples roles in the environment (Sengupta *et al.*, 2013). In **chapter 2**, the subsistence phenotype of bacteria present in the gut microbiota of healthy humans and zoo animals was investigated. Although antibiotic degradation was not detected, the results obtained provide an insight into the genetic background involved in the antibiotic subsistence phenotype. Future studies could focus on the metabolic pathways of antibiotic degradation. The information generated could fill the gap of knowledge regarding the relationship between antibiotic resistance and antibiotic subsistence and the ecological context in which the phenotype is displayed naturally. Of particular interest is to study if bacteria display the antibiotic subsistence phenotype at low concentration and whether these concentrations could contribute to the selection for subsistence.

Antibiotic therapy and the gut microbiota

The administration of antibiotic therapy in ICU patients has been associated with a reduction in the morbidity, mortality and decrease in the prevalence of ventilator-associated pneumonia. However, the impact of antibiotic therapy on the emergence of antibiotic resistance and infections associated with antibiotic resistant bacteria is still unclear (Plantinga *et al.,* 2015). The studies presented in this thesis (**chapters 4-7**) show that the application of antibiotic therapy has a dramatic impact on the diversity and colonization dynamics of the gut microbiota. From an ecological point of view, the selective pressure of antibiotics induced during the therapy decreases the relative abundance of enterobacteria and increases the relative abundance of *Enterococcus* species.

Moreover, a decrease of several members of the commensal anaerobic bacteria, which can play important roles in metabolic, nutritional and protective process in the host, was observed. Individual-specific variation in colonization dynamics with antibiotic resistant bacteria including Gram-positive and Gram-negative bacteria was detected. This information certainly expands our understanding of the dynamics of the gut microbiota under antibiotic selective pressure and could provide novel targets for therapeutic development.

So far, the level of antibiotic resistance in ICUs in the Netherlands seems to be low, and antibiotic therapy appears to be a useful tool for the control of hospital-acquired infections, which is supported by a microbiological monitor of antibiotic resistance development (Plantinga *et al.,* 2015). However, the results obtained in this thesis indicated that a careful control and monitoring of the development of antibiotic resistance in Gram-positive bacteria, especially *Enterococcus* species that harbour antibiotic resistance and virulence genes, should be monitored since the rate of colonization apears to increase during SSD therapy. The implementation of antimicrobial practices, use of broad spectrum antibiotics only under strict conditions, selection of narrow spectrum antibiotics whereever possible, administration of laxatives or promotors of gut motility and prevention of horizontal transmission through hand-washing, glove use and improving the workflow in the health care units, could reduce the emergence and dissemination of antibiotic resistant bacteria.

Novel cultivation approaches to study the commensal reservoir of antibiotic resistance

Currently, there is an increased interest to isolate, identify and characterize members of the commensal anaerobic gut microbiota that have not yet been cultivated. Such uncultured species could be important as a reservoir of antibiotic resistance genes.

Traditional and novel cultivation approaches such as minibioreactor arrays (MBRAs), culture enriched molecular profiling, culturomics methods, microcapsules and Bio-chips (Auchtung *et al.,* 2015; Lau *et al*., 2016; Dubourg *et al.,* 2014; Rettendal *et al*., 2014; Zengler *et al*., 2005; Ingham *et al.,* 2007) combined with high throughput sequencing techniques are increasingly being used to study a relatively poorly explored ecosystem present in the human gut microbiota: the anaerobic microbiota. The ability and capacity to cultivate and isolate pure cultures of these microorganisms can contribute to understanding their role in human health and disease. This also includes the possibility to use available isolates for the generation and study of synthetic microbial communities that allow addressing ecological questions regarding microbiota composition and functioning, as well as the application of synthetic consortia for microbial theraphies building on the success of fecal microbial transplantation (de Vos, 2013). Also, phenotypic insights into these poorly characterized species, especially regarding their resistance profiles, could provide useful information in response to antibiotic treatment of the gut microbiota and will contribute to improve infection control measures, by making them more targeted to the detrimental species, while leaving the beneficial microbiota intact.

Although the development of novel culture techniques is still required to increase the ability to explore the microbial gut ecosystem, the initial strategies already implemented should incorporate the study of the resistome as a key component to understand the interplay between the gut microbiota and antibiotics.

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APPENDICES

Acknowledgments

About the Author

“Everything we hear is an opinion, not a fact.

Everything we see is a perspective, not the truth”

Marcus Aurelius

Achieving my PhD led me along a very exciting journey and I am very pleased that I had the privilege to be able to reach this goal. It has been a learning curve full of joy, with some tough moments too. However, there was a key phrase that I kept repeating to myself and that was, “Keep on going”. Such a simple expression, but it gave me the strength that I needed to complete this task. During this time, many people from both inside and outside the Microbiology Department helped me obtain such a successful end to my years of study.

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“A journey of a thousand miles begins with a simple step”

Lao Tzu

Teresita de Jesus Bello Gonzalez, was born on May 16th in Caracas, Venezuela. In 2004, she obtained her bachelor diploma on Bioanalysis at The Central University of Venezuela, Caracas – Venezuela (UCV). Subsequently, she started to work as an analyst and junior researcher on the topic of Sporicidal and Bactericidal activity of Disinfectants and Antibiotic Resistance on mycobacterial species at the Tuberculosis Laboratory, Biomedicine Institute, Caracas – Venezuela (IBM). At that time, she established some important findings whilst collaborating with the Venezuelan Institute of Scientific Research (IVIC), Autonoma University of Mexico (UNAM) focusing on the control of infections, clinical microbiology and antibiotic resistance. Later, she took up the position of professor (instructor) in the Parasitology Department at the Bioanalysis School at The Central University of Venezuela (UCV). After gaining significant experience in the research area, she decided to start her Master degree in Biomedical Science. In 2009, she obtained her diploma as Magister at Andes University, Merida – Venezuela (ULA). The topic of her thesis was entitled "The Prevalence of pneumococcal associated pneumonia in a children's hospital in Caracas – Venezuela". She performed her first MsC internship at the Laboratory of Pediatric Infectious Diseases (Radboud University Nijmegen Medical Centre, Netherlands) investigating the prevalence of antigens and antibodies expressed during pneumococcal associated pneumonia. In her second MsC internship at Centre d'Ingénierie des Protéines (Université de Liege, Belgium) she worked on the detection of antibiotic resistance genes on *St. pneumoniae* isolates. In 2011, she moved to the Netherlands and started her PhD at the Laboratory of Microbiology, Molecular Ecology Group at Wageningen University. During her PhD, she studied the interplay between gut microbiota and antibiotics as part of the Evotar and SEDAR project under the supervision of Prof. Dr. Hauke Smidt and Dr. Mark van Passel. The results of her PhD project are now presented in this thesis.

List of publications

Teresita d.J. Bello Gonzalez, Phu Pham, Janetta Top, Rob J.L. Willems, Willem van Schaik, Mark W.J. van Passel, and Hauke Smidt. Dynamics of *Enterococcus* colonization in intensive care unit hospitalized patients receiving prophylactic antibiotic therapies. *Submitted*

Elena Buelow*, **Teresita de Jesús Bello González***, Susana Fuentes, Wouter A.A. de Steenhuijsen Piters, Leo Lahti, Jumamurat R. Bayjanov, Eline A.M. Majoor, Johanna C. Braat, Maaike S. M. van Mourik, Evelien A.N. Oostdijk, Rob J.L. Willems, Marc. J.M. Bonten, Mark W.J. van Passel, Hauke Smidt, Willem van Schaik. Gut microbiota and resistome dynamics in intensive care patients receiving selective digestive tract decontamination. *Manuscript in preparation*

T.D.J Bello Gonzalez, E.G. Zoetendal, M.W.J. van Passel, H. Smidt. Mapping the diversity and colonization dynamics of gut antibiotic resistant bacteria in ICU patients by culture dependent and independent approaches. *Manuscript in preparation.*

Claudia Cortesia, **Teresita Bello**, Gustavo Lopez, Scott Franzblua, Jacobus de Waard, Howard Takiff. Use of GFP labeled NTM to evaluate the activity QACs disinfectants and antibiotics. Brazilian Journal of Microbiology, 2016; Oct *in press*

Bello Gonzalez TdJ, Zuidema T, Bor G, Smidt H and van Passel MWJ. Study of the aminoglycoside subsistence phenotype of bacteria residing in the gut of humans and zoo animals. Frontiers in microbiology, 2016; 6: 1-7

Teresita Bello Gonzalez, van Passel MW, Tims S, Fuentes S, De Vos WM, Smidt H, Belzer C. Application of the Human Intestinal Tract Chip to the nonhuman primate gut microbiota. Beneficial Microbes 2014; 17 (3): 1-6

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Mendoza R, De Donato M, de Waard JH, Takiff H, **Bello T**, Chirinos. Susceptibility of *Mycobacterium tuberculosis* to antituberculosis drugs as determined by two methods, in the Sucre state, Venezuela. G. Invest Clin. 2010 Dec; 51 (4): 44555

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Ismar A. Rivera-Olivero, Debby Bogaert, **Teresita Bello**, Berenice del Nogal, Marcel Sluijter, Peter W.M. Hermans and Jacobus H. de Waard. Pneumococcal Carriage among Warao Amerindian Children in Venezuela: Serotypes, Susceptibility Patterns and Molecular Epidemiology". Clinical Infectious Diseases. 2007:45; 1427-1434

T. Bello, I. Rivera, J de Waard. Inactivation of mycobacteria by disinfectants with a tuberculocidal label. Enfermedades Infecciosas y Microbiologia Clinica. 2006; 24(5):319-21

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I. Rivera, **T Bello**, B del Nogal, M Sluijter, D Bogaert, P Hermans, J de Waard Epidemiology of pneumococcal carriage among Warao children in the Delta Amacuro in Venezuela. Clinical Microbiology and Infection, 15th European Congress of Clinical Microbiology and Infectious Diseases Volumen 11, Supplement 2, 2005

Overview of completed training activities

Discipline specific activities

Meetings

- 13th Gut Day Symposium (2011, Wageningen, Netherlands)
- 14th Gut Day Symposium (2012, Leuven, Belgium)
- Scientific Spring Meeting KNVM (2012, Arnhem, Netherlands)
- ASM conference (2012, Aix de Provence, France)
- Scientific Spring Meeting KNVM (2013, Arnhem, Netherlands)
- Annual conference of the association for general and applied microbiology (VAAM, KNVM) (2013, Bremen, Germany)
- 36th International Congress of the Society for Microbial Ecology and Disease (SOMED) (2013, Kosice, Slovakia)
- Symposiun on Microbial Ecology (ISME) (2014, Seoul, South Korea)
- Scientific Spring Meeting KNVM (2014, Arnhem, Netherlands)
- EvoTAR annual meeting (2014, Copenhagen, Denmark)
- ENGIHR "The gut microbiota throught life" (2014, Karlsruhe, Germany)
- International Conference ICETAR (2015, Amsterdam, Netherlands)
- ASM conference (2015, Washington, United States of America)
- Scientific Spring Meeting KNVM (2015, Arnhem, Netherlands)

Courses

- Functional metagenomic of the intestinal tract and food-related microbes (2011, Helsinki, Finland)
- Carbapenems producing organisms (2012, Rotterdam, Netherlands)
- Metagenomics approaches and data analysis (NCBI) (2013, Leiden, Netherlands)
- Symposium novel anaerobes (2014, Wageningen, Netherlands)

General courses

- VLAG PhD week (2011, Venlo, Netherlands)
- Techniques for writing and presenting a scientific paper (2012, Wageningen, Netherlands)
- Course "R" (2012, Wageningen, Netherlands)
- Training in metagenomic libraries UMC (2012, Utrecht, Netherlands)
- ARB/SILVA basic training (2014, Wageningen, Netherlands)

Optionals

- Preparation of PhD research proposal
- Molecular Ecology group meetings (weekly)
- PhD/Post doc meetings (biweekly)
- Microbiology PhD trip (2013, Canada and United States of America)

COLOPHON

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