Making yourself at home in the intestinal tract

Dynamics of the microbiome, resistome and host-pathogen interactions

Paul Bastiaan Stege

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Dynamics of the microbiome, resistome and host-pathogen interactions

Inwendig vertrouwd voelen
De dynamiek van de darmmicrobiota, het resistoom
en van gastheer-pathogene interacties
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction and thesis outline

Dynamics in the gut microbiome

The human intestinal tract is densely colonized by a complex ecosystem of bacteria, fungi, viruses and phages, that together form the gut microbiome (1). The dominant bacterial taxa in the human gut microbiome belong to the order of *Clostridiales,* followed by the genera *Faecalibacterium*, *Bacteroides*, *Clostridium* and *Prevotella* (2, 3). These are all strict anaerobic bacteria that together contribute to the intestinal catabolic pathways, allowing the absorption of additional nutrients by the intestinal cells (4, 5). Microbes start colonizing the intestines as soon as we are born and one of the first exposures to microbes takes place during labour, when microbes from the mother are transferred to her child (6). The gut microbiome heavily fluctuates the following month and continues to be susceptible to change for approximately 24 months. During this period of time, the gut microbiome slowly reaches an equilibrium, after which it becomes less prone to changes driven by environmental exposure (7). In this state, the microbiome is less susceptible to colonization and rapid growth by newly introduced bacteria, due to the active competition among bacteria for both space and nutrients. Not only does this involve competition for energy sources, but also strategies to deplete the supply of trace metals, which are critical for bacterial metabolic pathways (8). Moreover, some bacterial species can actively kill or inhibit the growth of competitors by the production of bacteriocins, that act as antimicrobial peptides (9, 10). The apparent stable status, a result of this complex interaction among bacterial species that are adapted to this complex ecological environment, is called intestinal homeostasis (11). Together with the host immune system, a balanced gut microbial consortium provides colonization resistance against new and often harmful pathogens (12, 13).

Internal factors such as genetics, age and gender, as well as external factors such as medication, smoking and consumption of alcohol, are known to influence the microbiome composition during adulthood (14-18). These factors may induce gradual changes (e.g. age) or abrupt shifts (e.g. antibiotics) in the composition of the gut microbiome (19). Diet is another factor known to influence the gut microbiome composition, although studies report controverse findings on the extend that diet affects the gut microbiome composition (20-22). Short-term diet intervention studies have observed correlations between diets rich in protein and animal-fats and the increased relative abundance of *Bacteroides*, as opposed to carbohydrate-rich diets and the increased relative abundance of *Prevotella* (23, 24). Another short-term diet intervention study by Kovatcheva-Datchary et al., attributed the increased abundance of *Prevotella* to fiber- and vegetable-rich diets (25). Additional studies further investigated differences in nutrient intake, such as fibers and related changes in the abundance of *Bifidobacteria* and *Lactobacilli* to the amount of consumed non-digestible carbohydrates that are rich in whole grain and wheat bran (26, 27). Alternatively, non-digestive carbohydrates such as resistant starch and whole

grain barley were found to affect the abundance of *Bifidobacteria*, *Eubacterium rectale*, *Roseburia* and *Ruminococcus* (28). However, it is important to note that the majority of these studies involve short term dietary intervention studies or compare participants from different geographic areas. Especially, when participants from different geographic areas are included, it is unclear if all of these effects can be specifically attributed to diet induced effects. When studying the effect of long-term dietary habits on the gut microbiome of vegans, vegetarians and omnivores within a single community, Losasso et al. observed no major differences in microbiome composition between the different diet groups. Minor differences were observed for OTUs affiliated with *Bacteroides*, *Lachnospiraceae*, and *Ruminococcaceae*, that were, however, only present in low abundance (29).

The gut resistome as reservoir of antibiotic resistance genes

The human gut microbiome is also an important reservoir of antibiotic resistance genes (ARGs), called the resistome. Specifically, gut commensal bacteria belonging to phyla Firmicutes and Bacteroidetes are known to carry an array of ARGs and could therefore potentially be involved in transmission of ARGs to important pathogens (30-32). The gut resistome of healthy humans can typically contain over 100 unique ARGs, with the most abundant ARGs being those that encode for tetracycline resistance, followed by macrolide and beta lactam resistance genes (33, 34). As a result, antibiotic administration directly alters the gut resistome of humans. The oral administration of antibiotics in patients selects for bacteria that are resistant to the administered antibiotics and rapidly increased the abundance of ARGs in the gut resistome (35, 36). Moreover, the accumulation of ARGs is facilitated through genetic linkage of resistance genes. When resistance genes are located on the same mobile genetic element, treatment with a single antibiotic can enrich for multiple resistance genes through co-selection (37). Plasmid-mediated co-selection of quinolone resistance has been observed in gut microbiome of children receiving 12 different combinations of antibiotics, not including quinolones (38). The treatment of patients in primary care with amoxicillin to treat respiratory tract infections, also increased the prevalence of amoxicillin and ciprofloxacin resistant *Escherichia coli* isolates in urine samples, indicating that the relationship between antibiotic use and resistance can be more complex. In this case, the gut resistome is likely to play role in co-selection, since the vast majority of urinary tract infections originate from *E. coli* in the gut microbiome (39). Antibiotic-driven enrichment of pathogens that carry ARGs can take place in the general population but is more frequently observed in clinical settings, where effects on the resistome were observed in a matter of days and can lead to the intestinal outgrowth of (potential) pathogenic bacteria (30, 40, 41).

As the gut resistome is represented by a subset of the gut microbiome, the gut resistome composition can therefore also be influenced by lifestyle factors such as international travel and living conditions (42). Changes in the gut resistome have also been observed when comparing urbanized with agricultural populations, suggesting a potential impact of the use of antibiotics in livestock on the human resistome (43, 44). Antibiotic use in animals will, although unintentionally, enrich for antibiotic resistant bacteria, which in turn increases the risk of zoonotic transmission of antibiotic resistant bacteria (45). When comparing Chinese swine farms that used antibiotics with farms that did not, Zhu et al. observed an increased abundance of aminoglycoside, tetracycline, sulphonamide and florfenicol ARGs (46). Moreover, the country-specific use of antibiotics was correlated to the prevalence of ARGs in the microbiome of broilers and pigs, including linezolid-resistance gene *optrA* and chloramphenicol resistance gene *cat* (47). The gut microbiome of pet animals forms another putative reservoir of pathogens and ARGs. Since pets come in close contact with their owners on a daily basis, they form a potential risk for zoonotic transmission to humans (48-50). *Salmonella* isolates that carry gentamicin and colistin resistance genes were frequently detected in the skin and gut microbiome of pet reptiles from different countries (51). Fluoroquinolone-resistant and methicillin-resistant *Staphylococcus aureus* were isolated from the skin microbiome of dogs and cats (52-54). In addition to the isolation of multi-drug resistant *S. aureus* from dogs, fluoroquinolone-resistant extraintestinal pathogenic *Escherichia coli* (ExPEC) were isolated from dog urine samples in Australia (55). Dogs were furthermore observed to be carrier of ampicillin-resistant *E. faecium* in their gut microbiome and are suspected to play a role in the transmission of vancomycin-resistant enterococci (VRE) (56-59).

The role of the gut microbiome in colonization resistance

Bacteria colonizing the intestinal tract of humans can be roughly divided into bacteria that inflict damage to the cell tissue, called pathogens, and those that live in symbiosis with the host cells (60). The latter can be further subdivided into commensals that act as true symbionts and offer a mutualistic interaction and opportunistic pathogens, or pathobionts, that require a certain degree of immunological or microbiome impairment to induce disease (61). Antibiotic treatment can induce perturbation of the gut microbiome, often referred to as dysbiosis (62, 63). This may result in the depletion of gut commensals allowing the proliferation of intestinal opportunistic pathogens such as *Clostridium difficile* and *Salmonella enterica* serovar Typhimurium (64, 65). Colonization and overgrowth by *C. difficile* are typically followed by the production of toxins that can cause severe intestinal inflammation, diarrhoea, and pseudomembranous colitis (66, 67). *C. difficile* furthermore excretes highly transmissible and resistant spores, thereby acting as a source of environmental transmission and challenging hospital infection

control measures (67). *S. enterica* serovar Typhimurium on the other hand, applies type III secretion system to invade epithelial cells, induce inflammation, and breach the epithelial barrier. The large inflammatory host-response results into diarrhoea, creating the perfect conditions for subsequent faecal-oral transmission (68, 69). Previous studies have tried to determine antagonistic bacteria that compete with these opportunistic pathogens for both space and nutrient in the gut environment. Ducarmon et al., highlighted the potential role of *Dorea*, *Atopobiaceae* and *Lachnospiraceae* ND3007 group in colonisation resistance against opportunistic pathogens *E. coli* and *Enterobacter cloacae*, when studying the gut microbiome of elderly in a nursing home setting (70). Commensal strains of *E. coli* were furthermore found to prevent colonization of toxin-producing *E. coli* in the gut microbiome of mice, via competition for the same nutrients, while Bifidobacterium strains were shows to successfully prevent mice against death as a result of toxin-producing *E. coli* (71, 72). In germ-free mice, gut colonization with murine Lachnospiraceae strains was found to offer colonization resistance against *C. difficile* (73). The colonization resistance that is offered by these antagonists might be caused by means of competition for both nutrients or space or, alternatively, by the active secretion of inhibiting compounds. As such, *Bacteroides* species were determined to induce colonization resistance against *Salmonella enterica* serovar Typhimurium in the gut microbiome of mice, through the production of the short-chain fatty acid propionate (74).

In contrast to *C. difficile* and *S. enterica* serovar Typhimurium, important multi-resistant pathogens such as extended-spectrum beta-lactamase-producing ExPEC (ESBL-ExPEC) and VRE reside in the gut microbiome and do not seem to inflict damage to the host, up until the moment of invasion and infection in other body sites (75-77). ESBL-ExPEC and VRE are important examples of opportunistic multidrug resistant pathogens where gut colonization precedes infections (76, 78). Especially in hospitalized patients, that frequently receive antibiotics, initial colonization by these opportunistic pathogens may lead to intestinal overgrowth increasing the risk of causing infections and outbreaks (75, 76, 78-84). A factor contributing to their importance, is the shared trait to rapidly acquire genes, located on mobile genetic elements, such as plasmids, allowing rapid transmission of resistance to clinical important antibiotics (85-89). Because of the high prevalence of resistant clinical isolates, both *E. coli* and *E. faecium* are listed as priority pathogens by the WHO (85).

Escherichia coli

While *E. coli* is a recognized opportunistic pathogen, it commonly accounts for < 0.1% of the gut microbiome of the general population (90, 91). ExPEC is the most important cause of urinary tract infections and blood stream infections (i.e. bacteraemia) and these infections are predominantly community-acquired and the prevalence of ESBL-

ExPEC has increased over the last decade (92-96). *E. coli* frequently acquire and share genes through horizontal gene transfer and identical plasmids are frequently observed in different clones (97). This allows for the rapid acquisition of ARGs and strengthens their additive response to antibiotic treatment, in addition to resistance that results from high genomic mutation rates (88, 98, 99). Plasmids furthermore facilitate the distribution of resistance genes to clinically relevant antibiotics such as third-generation cephalosporins, carbapenems and fluoroquinolones (85, 100, 101). *E. coli* that are resistant to third-generation cephalosporins, such as ESBL-ExPEC, are of particular interest since they are sponsible for a large part of multidrug resistant bacterial infections in Europe (102). The most prevalent ESBL enzymes are encoded by genes belonging to the classes of CTX-M, TEM and SHV (96). Over the past few years, CTX-M and TEM are the most commonly observed ESBL types and mainly detected in *E. coli* associated with clinical infections (96, 103). In addition to adaptation to antibiotic therapy in patients, *E. coli* have strategies to survive in the hospital environment outside of the body as well (104). They are known colonizers of surfaces and are specialized in the colonization of sinks and drainpipes by means of biofilm formation, contributing to their resilience (105-107). ESBL-ExPEC are not only found in the before mentioned reservoirs but are also frequently detected in companion animals like dogs.

Companion animals may represent an important source of ESBL-producing *E. coli*, thereby introducing a transmission risk of these multidrug-resistant opportunistic pathogens to humans on daily basis (108, 109). Companion cats of Dutch households were shown to carry ESBL-producing *E. coli* in their gut microbiome, although detected in low frequency (1,4%) (48). When studying the gut microbiome of diarrheic cats, an increased prevalence of ESBL-producing *E. coli* was observed (25%)(110). Dogs of Dutch households were shown to be frequent carriers of ESBL-producing *E. coli* in their microbiome as well, mostly containing beta-lactamase genes $bla_{\text{CTX-M-32}}$ and $\text{bla}_{\text{TEM-52}}$ (48, 111). Furthermore, up to half of the dogs that carried ESBL-producing *E. coli* (43%) were shown to be persistently carriers over the course of several months (48, 111, 112). Although some cases of transmission of ESBL-producing *E. coli* between dogs and owners have been reported, the dynamics and direction of transmission remain unclear (48, 112, 113). When studying the nasal and gut microbiome of cats, dogs and their owners for multidrug-resistant bacteria, Dazio et al. did not observe evidence for co-carriage or interspecies transmission (114). This is in concordance with the study of Baede et al., where transmission between dogs and owners was not detected either (111). While it is still possible that transmission between pet dogs and owners takes place, these findings may indicate that transmission and subsequent carriage of the studied bacteria is relatively rare and that larger study groups or longitudinal studies are required in order to determine the dynamics of transmission.

Enterococcus faecium

Like *E. coli*, enterococci are a minority species accounting for < 0.1% of a healthy gut microbiome. However, these numbers can significantly increase in hospitalized patients where *E. faecium* can even represent the majority bacterial species in the gut (75, 76, 91). *E. faecium* is a frequent cause of healthcare-associated infections and responsible for healthcare-acquired outbreaks (115, 116). The intrinsic low susceptibility of *E. faecium* to broad spectrum antibiotics, the genome plasticity and efficient acquisition of mobile genetic elements contribute to the importance as nosocomial pathogen (117, 118). Emergence of ampicillin and vancomycin-resistance among enterococci, specifically in *E. faecium*, furthermore reduce treatment options and increases mortality in hospitalized patients (81, 119). In addition to its role as pathogen, enterococci are recognized as central hub of mobile genetic elements, thereby facilitating transmission of ARGs to both Gram-positive and Gram-negative species (120). Of major concern was transfer of the *vanA* gene cluster conferring vancomycin resistance to clinical methicillin-resistant *Staphylococcus aureus* resulting in high level vancomycin-resistance *S. aureus* (VRSA) isolates (121). Thereafter, fourteen VRSA isolates have been reported in the United States and in total 52 isolates world-wide (122). Fortunately, VRSA are still rare, despite wide use of vancomycin for treatment of severe MRSA infections (123). The enterococcal hardened cell wall allows for increased persistence to factors like salt, heat, acidity, perturbation and the intrinsic resistance to broad spectrum antibiotics. This improves survival to common disinfectant procedures and furthermore increases their chance of circulation in the hospital environment (124-126). Although enterococci are also capable of biofilm formation, this is mainly observed in implants of patients and it is unknown if biofilm formation is a key step in hospital circulation (127-129). An important distinction between low abundant isolates in the health gut and isolates retrieved from hospitalized patients, lies in differences in their genetic makeup. *E. faecium* strains from hospitalized patients are part of a distinct phylogenetic clade called clade A1 while *E. faecium* isolates colonizing health humans primarily cluster outside this clade (57, 130). *E. faecium* genes unique to clade A1 encode putative virulence factors, factors implicated in antibiotic transport, intestinal colonization factors and determinants implicated in carbohydrate metabolism particularly factors involved in the utilization of amino sugars, like those that occur on cell surfaces and in mucin (131). These acquired genes may provide an adaptive advantage to *E. faecium* strains recovered from the hospital environment, for instance offering advantages during the initial gut colonization, however in general evidence for the selective advantage of these genes for clade A1 *E. faecium* isolates is largely lacking. While *in vivo* studies confirmed that a phosphotransferase system (PTS) acquired by clade A1 strains is important for gut colonization in mice, it is unclear if this applies to humans as well (132). Furthermore, it is unknown if the surface proteins and pili encoded in the genome of *E. faecium* in general and particularly of clade A1 strains, contribute to *E. faecium* colonization (133, 134).

Intestinal mucosal architecture and bacterial adaptation

Whether or not bacteria are commensal or pathogens, the intestinal tissue is designed to form a stringent barrier for bacteria, while allowing the passage of nutrients into the bloodstream. For this purpose, cells are closely linked together using tight junctions and indirectly by zonula adhaerens, which are necessary for tight junction formation (135). Cells furthermore prevent bacteria from reaching the epithelial layer by the excretion of mucus. This forms a mucus layer, which acts like a tight net-like structure on the apical site of intestinal epithelial cells, but gradually becomes looser towards the lumen, to the point that bacteria are able to colonize it. The major functional components of mucus are the mucin glycoproteins. Mucus is however for 90% composed of water and contains electrolytes, lipids and various other proteins. The intestinal tract is known to produce at least eight different types of mucins, from which MUC2 is thought to be the mucin of main importance (136). Mice that were deficient for the *muc2* gene were observed to have a diminished colon mucus layer and developed intestinal inflammation. Moreover, bacteria were able to reach the epithelial layer and mice eventually developed colon cancer (137, 138). While the colon has a thick mucus layer that forms a strong barrier for bacteria, the mucus layer of the small intestine is thinner, generally loose in structure and relies on rapid renewal to shed bacteria (139, 140). The barrier function of the intestines is furthermore enhanced by components of the innate and adaptive immune system, such as secretion of IgA, causing bacteria and viruses to aggregate upon binding, resulting in reduced diffusion in the mucus layer. In the small intestines, Paneth cells are located in crypts, near stem cells and secrete antimicrobial peptides (AMPs) to keep bacteria at bay. The most prevalent AMPs are defensins, lysozymes and C-type lectins, which are generally designed to target cell wall components of bacteria (141, 142). The highest concentration of AMPs is found in the crypts of the small intestines and impede bacteria from reaching the stem cells. The stem cells are responsible for the regeneration of the intestinal epithelial layer and are therefore the most critical cells (143). The colon lacks Paneth cells, but instead contain deep crypt secretory cells that function as the equivalent of Paneth cells (144).

The competition for space and nutrients has driven bacteria to adapt to the intestinal environment. In order to colonize the intestines more efficiently bacteria express surface proteins, such as pili and mucus-binding proteins (145). Moreover, a selection of specialists, such as *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *S. enterica serovar* Typhimurium and *C. difficile*, are even able to cleave parts of the mucus chains and/or feed from it (146-148). In turn, the host cells are known to respond on mucus degradation by secreting additional mucus (149). This is an example of microbial-host crosstalk, resulting into the adaptive evolution of both bacteria as the host. A subset of bacteria is known to be motile in mucus using flagella, allowing bacteria from reaching

into the thicker layers of mucus and eventually to the cell layer (150). This is a common trait for pathogenic bacteria such as Enterohemorrhagic *E. coli* (EHEC), *S. enterica serovar* Typhimurium and *C. difficile* and is commonly joined by the expression of toxins, causing disruption of the cell layer and can allow for bacterial translocation into the bloodstream (151, 152).

It is not entirely understood how the intestines can adhere to a strict management that allows for symbiosis with bacteria, but meanwhile prevent colonization by pathogens. Microbial-host crosstalk is however expected to play a central part in distinguishing good bacteria from bad. Human-derived colonic organoids have recently been used as novel *ex-vivo* model to study human specific bacteria-host interactions (153). Colonic organoids are kept as stem cells during passaging, but can be differentiated into different cell types, including colonocytes, mucus-producing goblet cells and important cell structures like tight junctions (154-156). These different cell types offered new insights into pathogen dynamics in the intestines. When using co-cultures of differentiated intestinal organoids and *S. enterica* serovar Typhimurium, additional insights were gained into the mechanism of action of pathogenicity island 1 (157). Colonic organoids were used in a comparable setup to study the effect of pathogenicity island *pks* of *E. coli* and its ability to induce a mutational signature in the organoid genome, similar to that observed in colorectal cancer (158). Colonic organoids were furthermore used to study the MUC2 binding properties of *C. difficile* and how this can reduce MUC2 production (159). This model was additionally used to study the effects of tissue damage introduced by *C. difficile* excreted toxins (160). The before mentioned studies apply colonic organoids that are cultured in matrigel and form spheroids (i.e. 3D cultures). This model has the additional advantage that the apical side of cells is located on the inside of spheroids and allows for the survival of oxygen-sensitive anaerobic bacteria (161). Colonic epithelium forms an alternative to this model and is generated by differentiation of organoid cells into a monolayer. Colonic epithelium has the advantage of increased accessibility, but it is challenging to apply this system for anaerobic bacteria (162). Colonic epithelium has been used to study the effects of mucin degrader *Akkermansia muciniphila* on cell proliferation, the damaging effects of *Campylobacter concisus* on tight junctions and to determine the cell receptor targeted by *C. difficile* toxin B (163-165).

Aim and outline of this thesis

In this thesis, we studied the gut microbiome as reservoir of pathogenic bacteria and ARGs. The gut microbiome is of particular interest since it is one of the most densely populated microbial habitats in humans. Multi-drug resistant opportunistic pathogens, such as VRE, transmit among hospitalized patients and depend on the initial gut colonization for their downstream pathogenicity. A second goal was therefore to study host-pathogen crosstalk during initial stages of gut colonization, using *E. faecium* as a model organism and human-derived colonic epithelium as a model organ.

In part I of this thesis, we investigated the dynamics of gut microbiome and resistome in humans and pet animals. In chapter 2 we characterized the gut microbiome and the resistome dynamics of the general Dutch population with different dietary habits using metagenomic shotgun sequencing (MSS) and a resistome targeted capture-based sequencing (ResCap) approach, respectively. This revealed that vegans had a distinct gut microbiome composition, compared to other diet groups with a lower abundance of *Streptococcus thermophilus* and *Lactococcus lactis* compared to pescatarians and a lower abundance of *S. thermophilus* when compared to omnivores. Analysis of the resistome revealed that among all diet groups, 119 and 145 unique ARGs were detected by MSS or ResCap, respectively. Five or fifteen ARGs were shared between all diet groups, based on MSS and ResCap, respectively. However, the total number of detected ARGs by MSS or ResCap was not significantly different between the groups. In chapter 3, we studied the microbiome and resistome of companion animals (dogs) over the course of six weeks using 16s rRNA gene sequencing and ResCap. We confirmed that a large proportion of dogs are carrier of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC). The prevalence of ESBL-EC furthermore coincides with blooms of *Clostridium sensu stricto 1*, *Enterococcus*, *Lactococcus* and the shared genera of *Escherichia-Shigella*, which potentially include opportunistic pathogens species. Resistome analysis revealed that ESBL-EC prevalence was correlated with the abundance of the ARGs *cmlA*, *dfrA*, *dhfR*, *floR* and *sul3*. In summary, this study showed that ESBL-EC carriage in dogs is associated with a distinct microbiome and resistome composition.

In part II of this thesis, we describe the development of novel techniques to functionally characterize the opportunistic intestinal pathogen *E. faecium*. In chapter 4, we integrated CRISPR-Cas9 to genetically modify *E. faecium* and demonstrate its efficiency by generating a gene deletion mutant without genomic scarring. Subsequently, we set out to study VRE-host crosstalk by using a recently developed human-derived colonic epithelium in chapter 5. This revealed large-scale differential gene expression upon interaction between vancomycin-resistant *Enterococcus faecium* and colonic epithelium. In *E. faecium* we observed upregulation of pili genes, downregulation of vancomycin

resistance genes and the reconfiguration of large metabolic pathways for energy supply. In colonic epithelium, we observed a large number of genes (219 genes) to be differentially expressed upon co-culturing with *E. faecium* strain E8202, including GDF-15 involved in activation of the inflammation pathway, upregulation of genes encoding GTP-binding proteins, allowing the detection of outside cell stimuli and expression of lysozyme and differentially expression of genes involved in cell death. Finally, in chapter 6 we summarize and discuss the findings in this thesis, the limitations and future perspectives of the applied methods and models to study the resistome dynamics of the gut microbiome and the functional consequences thereof.

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Part I

Dynamics of the gut microbiome and resistome

Chapter 2

Impact of long-term dietary habits on the human gut resistome in the Dutch population

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Abstract

The human gut microbiome plays a central role in health and disease. Environmental factors, such as lifestyle and diet, are known to shape the gut microbiome as well as the reservoir of resistance genes that these microbes harbour; the resistome. In this study we assessed whether long-term dietary habits within a single geographical region (the Netherlands) impact the human gut resistome. Faecal samples from Dutch omnivores, pescatarians, vegetarians and vegans were analysed by metagenomic shotgun sequencing (MSS) (n=149) and resistome capture sequencing approach (ResCap) (n=64). Among all diet groups, 119 and 145 unique antibiotic resistance genes (ARGs) were detected by MSS or ResCap, respectively. Five or fifteen ARGs were shared between all diet groups, based on MSS and ResCap, respectively. The total number of detected ARGs by MSS or ResCap was not significantly different between the groups. MSS also revealed that vegans have a distinct microbiome composition, compared to other diet groups. Vegans had a lower abundance of *Streptococcus thermophilus* and *Lactococcus lactis* compared to pescatarians and a lower abundance of *S. thermophilu*s when compared to omnivores. In summary, our study showed that long-term dietary habits are not associated with a specific resistome signature.

Introduction

The human gut microbiome is a complex ecosystem composed of bacteria, fungi, viruses and phages. It not only plays a central role in nutrient acquisition, but it also affects our state of health and disease (1-3). Many factors are known to influence its composition, which can be either host-derived, such as age or immunological and pathological disorders (4, 5), or exposure to environmental factors (the exposome), including diet (6-12). Correlations have been observed between diets rich in protein and animal-fats and the high relative abundance of *Bacteroides*, as opposed to carbohydrate-rich diets and the high abundance of *Prevotella* (13, 14). In addition, increased abundance of *Prevotella* and *Lachnospira* was correlated with fiber- and vegetable-rich diets (15, 16). The majority of studies that have observed these diet-induced effects on the microbiome either compare participants from different geographic areas or involve short term dietary intervention studies (14, 17-19). When studying the effect of long-term dietary habits within a single community on the gut microbiome of vegans, vegetarians and omnivores, Losasso et al. only observed differences in bacteria that are present in low abundance, and part of the families *Bacteroides*, *Lachnospiraceae*, and *Ruminococcaceae* (20). This study used 16S rRNA gene sequencing to determine the microbiome composition, which has insufficient resolution to allow for comparisons at the species level.

The human gut microbiome is also an important reservoir of antibiotic resistance genes (ARGs) (21-24). It is therefore important to understand how long-term dietary habits not only impact the microbiome composition, but also the composition of the total of ARGs, the resistome, in the human gut. Advances in high-throughput sequencing have allowed in depth studies of the human gut resistome. The gut resistome of healthy humans can typically contain over 100 unique ARGs, with the most abundant ARGs being those that encode for tetracycline resistance, followed by macrolide and beta lactam resistance genes (25, 26). Just as with the microbiome, several factors are known to alter the resistome composition. Orally administered antibiotics are known to select for bacteria that are resistant to these antibiotics, therefore increasing the abundance of these ARGs in the gut (27, 28). This antibiotic-driven enrichment can take place in either the general population or more specific, in clinical settings, where effects on the resistome were observed in a matter of days (29-31). Additionally, the human gut resistome can be affected by environmental factors such as international travel and living conditions. This has been shown in studies comparing urbanized with agricultural populations, in which the use of antibiotics plays an important role (32, 33). Finally, meat contaminated with bacteria carrying ARGs as a result of antibiotic usage in livestock, has been highlighted as a possible transmission route for resistant bacteria and could therefore influence the gut resistome as well (34-38). More specifically, zoonotic pathogens such as species of *Salmonella* and *Campylobacter* and certain types of *Escherichia coli* (*e.g.* Shiga-toxin-

producing *E. coli* (STEC)) are known for causing foodborne infections and are frequent carriers of ARGs (39-42).

In this study we assessed whether long-term dietary habits within a single geographical region impact the human gut resistome in the general Dutch population. Using metagenomic shotgun sequencing (MSS), we were able to detect 877 unique bacterial species and an extensive resistome composed of 119 unique ARGs, in the gut microbiome of healthy Dutch residents. resistome capture sequencing approach (ResCap) was applied for a subset of samples and revealed 145 unique ARGs, thereby surpassing the detection limits of MSS. Despite the high resolution of the sequencing data, the total number of ARGs detected by MSS or ResCap per diet group was not significantly different in the general Dutch population.

Results

Diet-associated differences in the gut microbiome

Before determining diet-associated resistome differences, we first assessed the effect of long-term dietary habits on the gut microbiome, as this ecological niche is an important reservoir of antibiotic resistance genes. Faecal samples from 149 Dutch individuals were selected based on their dietary habits and categorized in four matched diet groups: 1) omnivores, 2) pescatarians, 3) vegetarians and 4) vegans (table 1). Faecal samples from these four groups were used for metagenomic shotgun sequencing (MSS) in order to study the effect of dietary habits on the gut microbiome. The mOTUs2-based taxonomic binning method revealed that the top 10 most abundant genera did not differ between the diet groups (figure 1a). In all our study groups, the most abundant taxa belong to the order of *Clostridiales* and genera *Faecalibacterium*, *Bacteroides*, *Clostridium* and *Prevotella*, which matches with the gut microbiome composition observed in previous studies (43, 44). In addition, the gut microbiome diversity, expressed as Shannon index, was not significantly different between diet groups, indicating that the total species diversity is highly similar among diet groups (figure 1b). Statistical analysis of the inter-diet group beta diversity based principal component analysis (PCA) using Aitchison distance, Bray-Curtis distance, or Jaccard distance further revealed that diet was not a main driver of the observed variance in microbiome composition between the samples (figure s1).

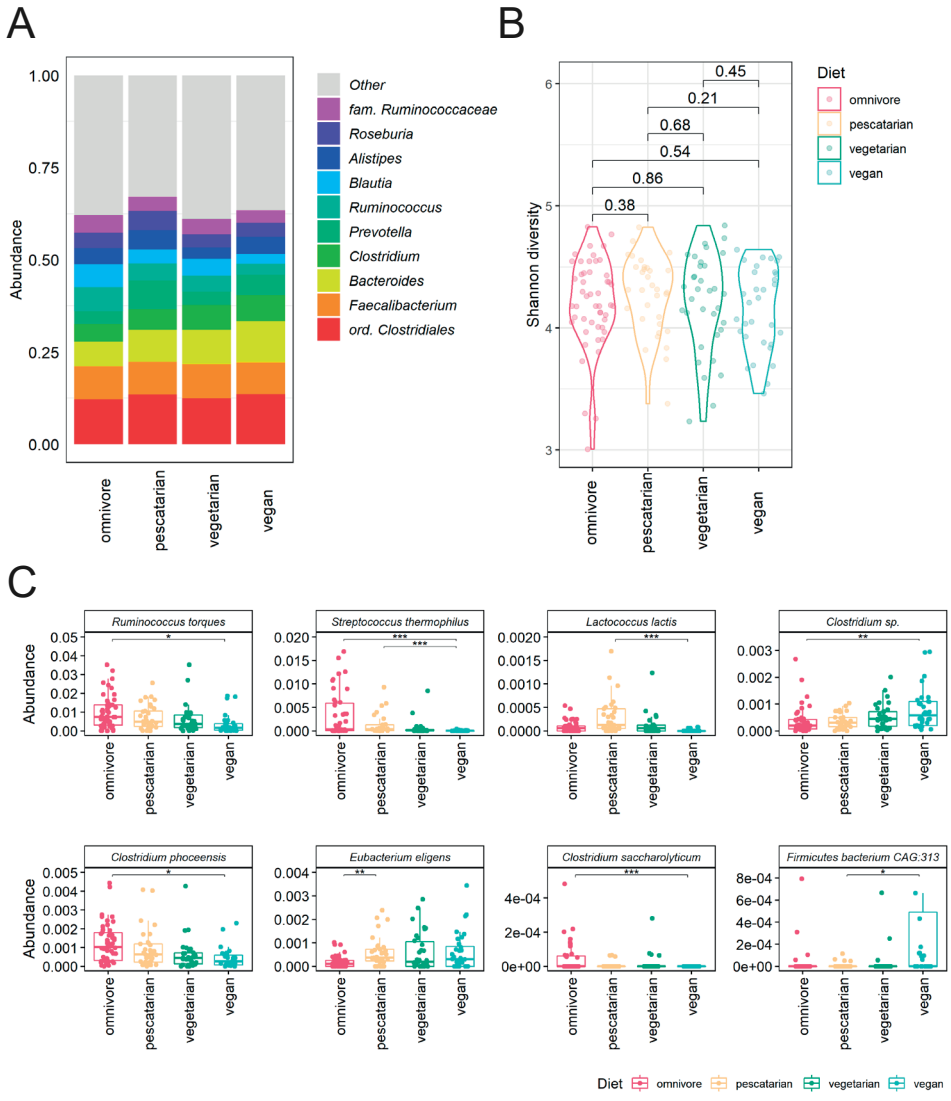

Figure 1 | Association of dietary habits and the gut microbiome composition. (A) Relative abundance of the 10 most abundant bacterial genera per diet group (B) Alpha diversity per diet group expressed by Shannon index. Differences in alpha diversity between diet groups were compared using Wilcoxon rank-sum tests. (C) ANCOM-BC analysis for differential relative abundance of bacterial species between diet groups. Abundance was plotted on the relative abundance scale from 0.00 to 1.00. Adjusted p-values are indicated by *< 0.05, **< 0.01 and ***< 0.001.

Table 1 | Study participants characteristics included in metagenomic shotgun sequencing.

Characteristics	omnivore	pescatarian	vegetarian	vegan
Participants, n= 149	50	33	34	32
Age in years, *median*	47	51	45	37
(10^{th}-90^{th} percentile)	(29-59)	(29-62)	(28-62)	(29-56)
Male participants,	16	12	11	11
(percentage)	(32%)	(36%)	(32%)	(34%)
Participants with pets,	28	16	18	18
(percentage)	(56%)	(48%)	(53%)	(56%)
Participants using medication*,	15	15	7	8
(percentage)	(30%)	(45%)	(21%)	(25%)

* Medication other than antibiotics, proton pump inhibitors, insulin and cancer treatment.

We further explored potential differences in the gut microbiome composition between diet groups by using supervised analysis to compare the abundance of bacterial species using ANCOM-BC (45). Compared to the omnivores, vegans had lower abundance of *Ruminococcus torques* (p_{adj} = 5.0E-02), *Streptococcus thermophilus* (p_{adj} = 4.7E-06), *Clostridium sp.* (p_{adj} = 6.5E-03), *Clostridium phoceensis* (p_{adj} = 3.7E-02) and *Clostridium saccharolyticum* (p_{adj} = 1.6E-03) (figure 1c). Similarly, *Streptococcus thermophilus* (p_{adj} = 1.5E-04), *Lactococcus lactis* (p_{adj} = 7.0E-07) and *Firmicutes bacterium* CAG:313 (p_{adj} = 2.7E-02) were less abundant in vegans compared to pescatarians. Finally, *Eubacterium eligens* (p_{adj} = 2.9E-03) was more abundant in the microbiome of pescatarians when compared to omnivores (figure 1c).

In addition to mOTUs2 we also applied MetaPhlAn3 to profile the microbiome composition. In concordance with the results of the mOTUs2, using MetaPhlAn3 we did not observe differences in the top 10 most abundant genera between the diet groups (figure s2). Using MetaPhlAn3 for differential abundance analysis, we also found a lower relative abundance of *R. torques*, *S. thermophilus* and *C. saccharolyticum* in vegans compared to omnivores and higher abundance of *E. eligens* in pescatarians compared to omnivores (figure s3). Furthermore, the MetaPhlAn3 approach detected a higher relative abundance of *Lactobacillus delbrueckii*, *Coprococcus comes*, *Dorea formicigenerans*, *Dorea longicatena*, *Lawsonibacter asaccharolyticus* and *Phascolarctobacterium* CAG:266 in omnivores compared to vegans (figure s3).

Composition of the gut resistome across diet groups based on metagenomic shotgun sequencing

We next set out to investigate whether long-term dietary habits impacted the gut resistome composition. Using MSS we were able to identify 119 unique antibiotic resistance genes (ARGs) among all diet groups. The total number of detected ARGs was not significantly different

between the groups, with an average of 17±4 genes found in omnivores, 16±5 in pescatarians, 17±4 in vegetarians and 17±5 in vegans (figure 2a). Among these, five were consistently detected in all diet groups (detected in 95% of the samples), namely the aminoglycoside resistance gene *ant(6)-Ia*, the macrolide-lincosamide-streptogramin B resistance gene *erm*(B), and the tetracycline resistance genes *tet*(40), *tet*(Q) and *tet*(W) (figure 2b).

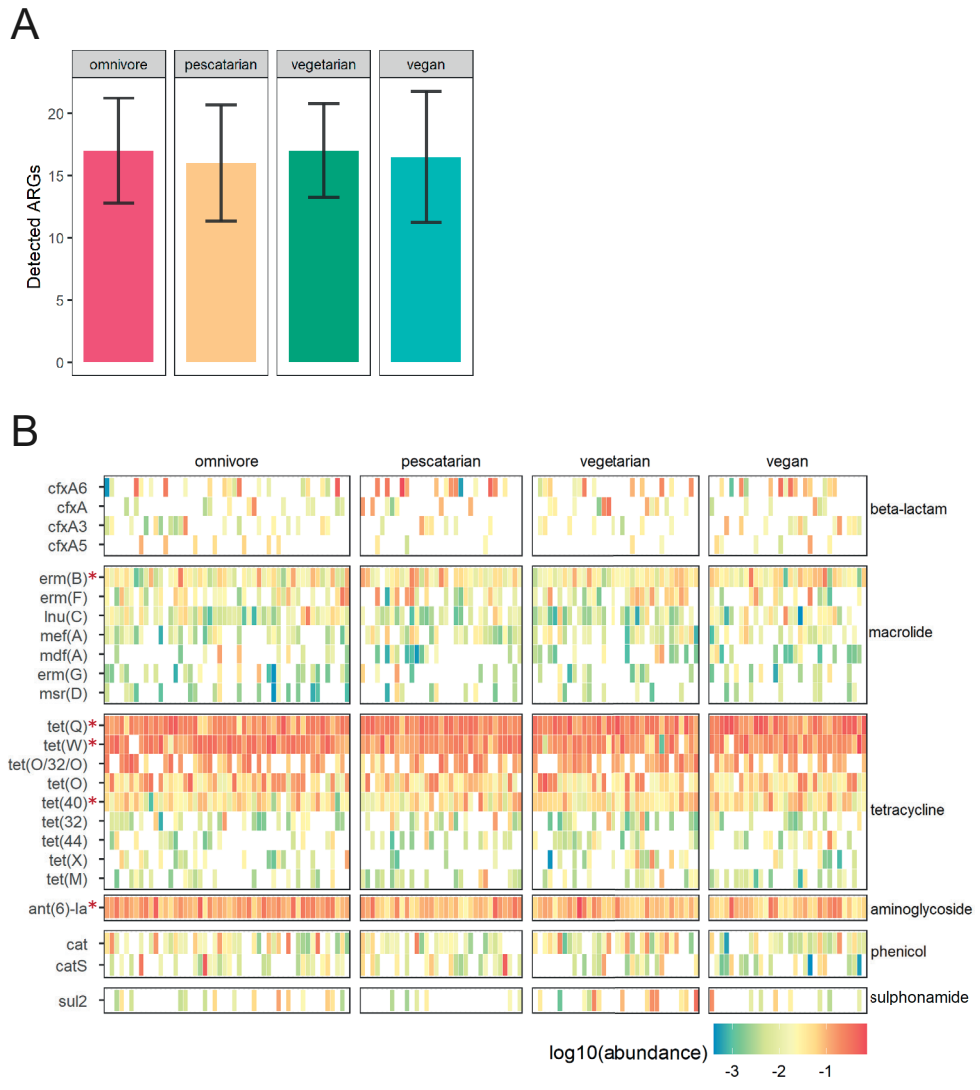

Figure 2 | Antibiotic resistance gene distribution in the gut resistome through metagenomic shotgun sequencing. (A) Average number and standard deviation of antibiotic resistance genes (ARGs) detected in each diet group. (B) Heatmap depicting ARGs abundance per participant. Each column denotes a study participant, clustered per diet group. Rows represent the relative abundance of ARG classes. ARGs present at least in 10% of the participants are shown. ARGs present in the resistome of at least 95% of the participants are indicated by the asterisks.

The most abundant genes detected encode for resistance to the classes of tetracyclines, macrolides, beta-lactams, aminoglycosides and phenicols. There were no differences when comparing the overall top 10 most abundant ARGs between the diet groups (figure 3a). Also, the resistome diversity, expressed by Shannon index, was not significantly different between the diet groups (figure 3b). In addition, diet does not seem to be the main driver of the observed variance in resistome composition between the samples when analysed by beta diversity based principal component analysis (PCA, figure s4).

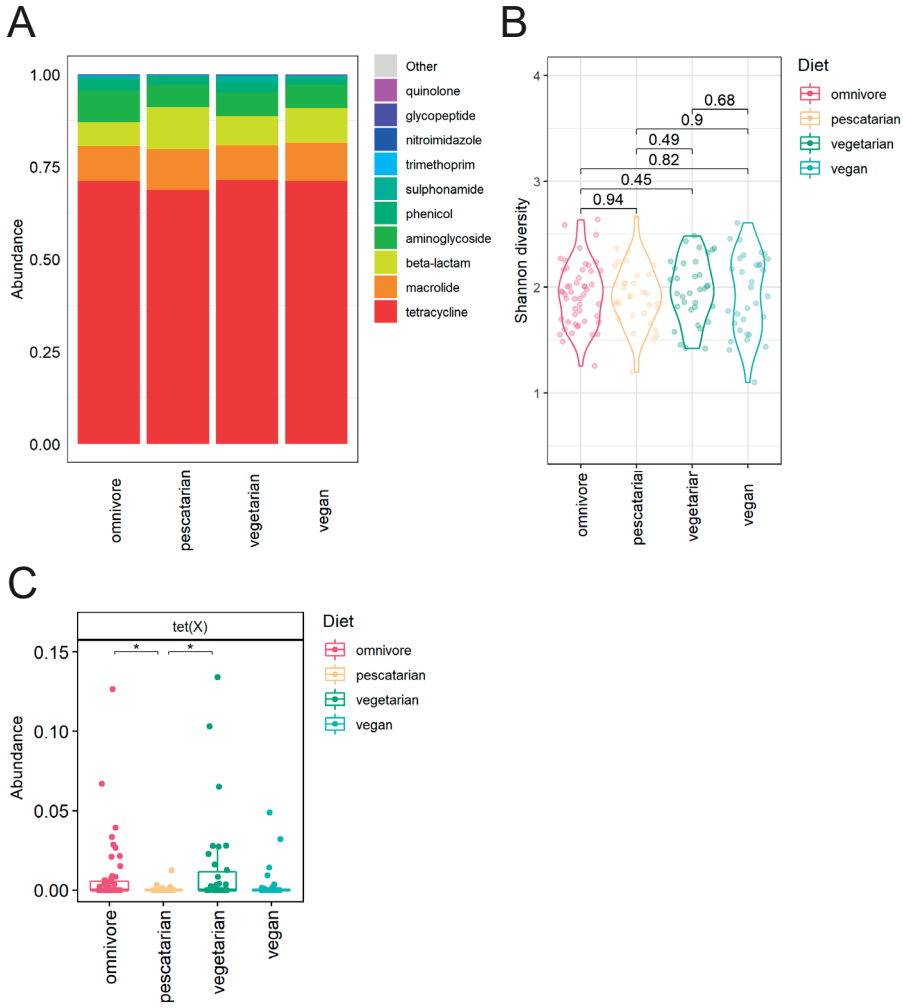

Figure 3 | Association of dietary habits and the gut resistome composition through metagenomic shotgun sequencing. (A) Relative abundance of the 10 most abundant gene classes encoding antibiotic resistance, summarized per diet group. (B) Alpha diversity per diet group expressed by Shannon index. Differences in alpha diversity between diet groups were compared using Wilcoxon rank-sum tests. (C) Differential abundance analysis using ANCOM-BC plotted on relative abundance scale from 0.00 to 1.00. Adjusted P-values below 0.05 are indicated by *.

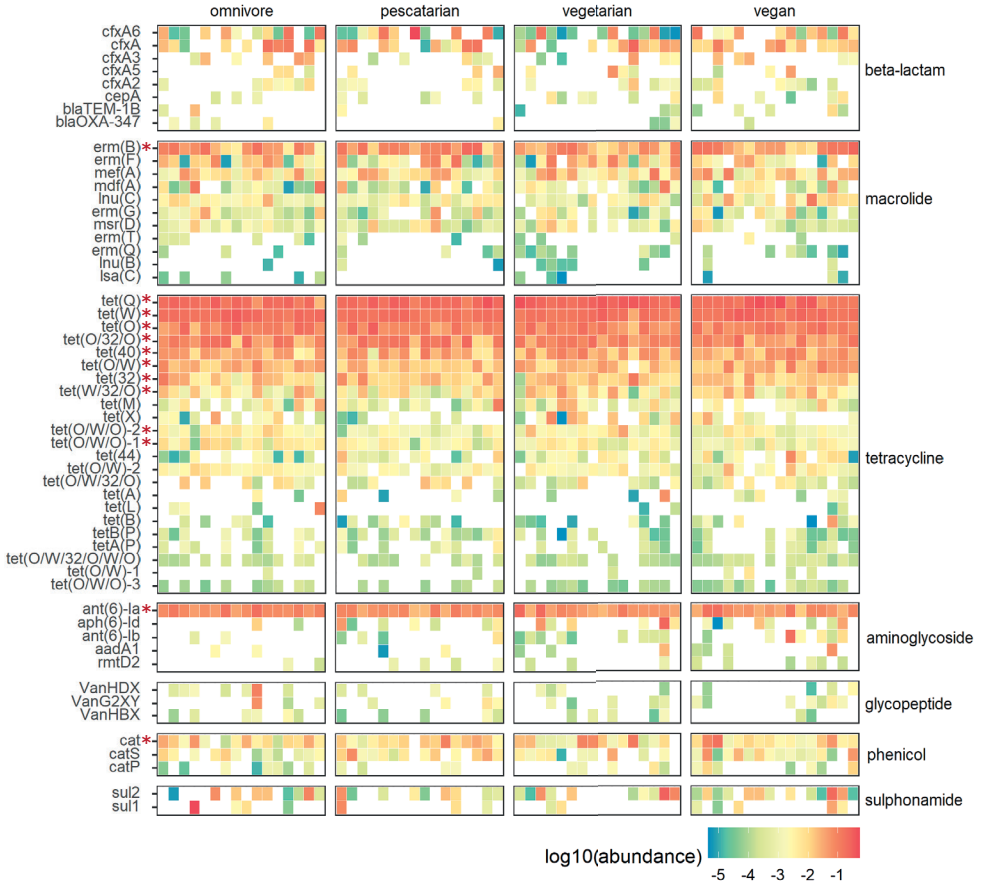

Figure 4 | Antibiotic resistance gene distribution in the gut resistome as detected by ResCap. Each column denotes a participant, clustered in columns according to diet group. Rows are categorized by antibiotic resistance gene (ARG) classes. ARGs present at least in 10% of the participants are shown. ARGs that are present in the resistome of at least 95% of the participants are indicated by the asterisks.

Supervised analysis using ANCOM-BC only revealed a significant difference of the abundance of *tet*(X), which is present in low abundance in pescatarians in comparison to omnivores (p_{adj} = 4.4E-02) and vegetarians (p_{adj} = 1.0E-02) (figure 3c). Overall, based on MSS, the resistome was highly similar among the studied diet groups.

Higher antibiotic resistance gene detection sensitivity of ResCap compared to metagenomic shotgun sequencing

Although MSS was able to detect a large variety of ARGs, these only represented 0.06±0.03% of the total number of reads. This indicates that the proportion of ARGs is relatively low when compared to the total gene pool present in the samples. In order to improve the sensitivity to detect ARGs, we applied the probe based resistome capture sequencing approach (ResCap), on a subset (64/149; 43%) of samples (table 2) (46). ResCap was able to greatly enrich the number ARGs specific reads, with 40.5±15.2% of the total number of reads sequenced mapping to ARGs.

Table 2 | Participant characteristics of the subset selected for ResCap.

Characteristics	omnivore	pescatarian	vegetarian	vegan
Participants, n= 64	16	16	16	16
Age in years, *median*	46	42	44	43
(10^{th}-90^{th} percentile)	(27-57)	(31-59)	(30-59)	(33-56)
Male participants,	8	8	8	9
(percentage)	(50%)	(50%)	(50%)	(56%)
Participants with pets,	9	11	8	8
(percentage)	(56%)	(69%)	(50%)	(50%)
Participants using medication*,	4	5	6	2
(percentage)	(25%)	(31%)	(38%)	(13%)

* Medication other than antibiotics, proton pump inhibitors, insulin and cancer treatment.

To evaluate the sensitivity of ResCap compared to MSS, we compared the observed number of ARGs identified per sequencing depth, by using rarefaction curves in the same 16 samples per diet group, subjected to both MSS and ResCap. Overall, ResCap was able to detect a higher number of ARGs than MSS. Even at 70M reads per sample, MSS did not reach the level of sensitivity that ResCap was able to achieve (figure s5a). Where MSS detected 16±5 to 18±4 ARGs per diet group with a sequencing depth of 70M reads, ResCap resulted in the detection of 33±8 to 39±7 ARGs per diet group with a sequencing depth of 20M reads (figure s5b).

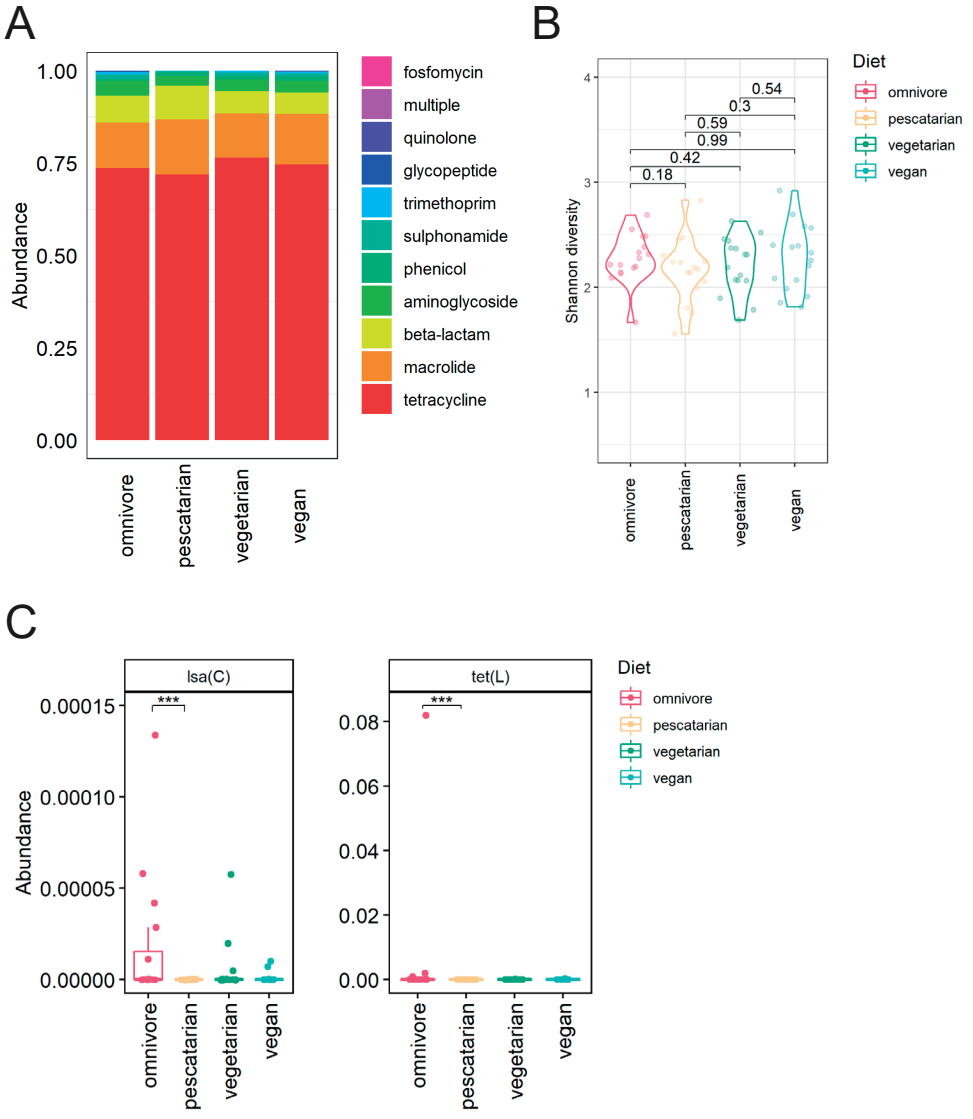

Figure 5 | Association of dietary habits and the gut resistome composition through ResCap. (A) Relative abundance of the ten most abundant antibiotic resistance gene classes, summarized per diet group. (B) Gene diversity per diet group, shown by Shannon index. (C) Differential abundance analysis using ANCOM-BC plotted on the scale 0.00 to 1.00%. Adjusted p-values below 0.001 are indicated by ***.

Dietary habits are not associated with differences in resistome composition assessed by ResCap

A total of 145 ARGs were detected using ResCap, from which 86 ARGs were also detected by MSS (table s1). The majority of genes detected by both methods included tetracycline resistance genes (19 genes, 22%), followed by beta-lactam resistance (19 genes, 22%) and macrolide resistance (15 genes, 17%). The ARGs detected by ResCap and not MSS include mainly beta-lactam (28/59; 47%), tetracycline (9/59; 15%) and aminoglycoside resistance genes (9/59; 15%). In contrast, MSS revealed 32 genes that went undetected by ResCap, including beta-lactam resistance (10 genes, 31%), nitroimidazole resistance (8 genes, 25%), and tetracycline resistance (4 genes, 13%). Of the 145 genes detected by ResCap, 15 ARGs were detected between all diet groups (detected in 95% of the samples) (figure 4). These 15 genes included the five genes (*ant(6)-Ia*, *erm*(B), *tet*(40), *tet*(Q) and *tet*(W)) that were also detected in all diet groups by MSS. In addition to these five, the chloramphenicol resistance gene *cat*, the lincomycin resistance gene *lnu*(C), the macrolide resistance gene *mef*(A), and the tetracycline resistance genes *tet*(32), *tet*(O), *tet*(O/32/O), *tet*(O/W), *tet*(O/W/O)-1, *tet*(O/W/O)-2 and *tet*(W/32/O) were detected in all diet groups using ResCap (figure 4).

The total number of ARGs detected by ResCap per diet group was not significantly different, with an average of 36±5 genes found in omnivores, 32±9 in pescatarians, 40±10 in vegetarians and 40±10 in vegans (figure s5b). The most abundant ARGs identified encode for tetracycline, macrolide, beta-lactam and aminoglycoside resistance (figure 5a). No differences were observed when comparing the overall top 10 most abundant ARG. No significant differences were observed when comparing the resistome diversity as calculated by Shannon index (figure 5b). Beta-diversity based PCA further confirmed that diet is not a main driver of the observed variance in resistome composition between the samples (figure s6). Supervised analysis using ANCOM-BC revealed that the abundance of two ARGs, *lsa*(C) and *tet*(L), were significantly different between the diet groups pescatarians and omnivores (figure 5c). However, these genes are present in very low abundance (median below 0.05%) and in low prevalence (< 15%). Based on these results we can conclude that differences in dietary habits did not result in significant differences in resistome composition.

Discussion

By using the combined power of metagenomic shotgun sequencing (MSS) and ResCap we were able to detect over 850 different bacterial species and more than 100 unique antibiotic resistance genes (ARGs) in the gut microbiome of participants from the

general Dutch population with distinct dietary habits. Our results show that long-term dietary habits are not associated with specific resistome signatures.

To investigate the gut resistome in humans, we used two techniques, MSS and ResCap. Using genomic DNA from human faecal samples, Lanza et al. found that ResCap results in a two-fold increase in gene diversity compared to MSS. In addition, they reported a 279-fold increase in the amount of reads that mapped to the ResCap targeted genes (46). Results of our study are comparable to Lanza et al., as we observed a two-fold increase in gene diversity when comparing the number of ARGs detected by ResCap to MSS. Moreover, ResCap resulted in 40.5±15.2% of the reads mapping to ARGs, compared to 0.06±0.03% for MSS, thus indicating a 675-fold change. In a similar way, Macedo et al. also reported an increase of mapped reads of 200-fold, when comparing ResCap to MSS performed on genomic DNA from soil and manure samples (47). Lastly, Guitor et al. applied a custom ARG probe-database and compared its efficiency to capture the resistome over MSS. They compared both systems using a DNA pool composed of four bacterial species and observed a 100-fold increase of target specific reads (48). ResCap and MSS sequencing results differed in the number of ARGs that they were able to detect. The 59 ARGs that were detected by ResCap and not MSS suggests that the capture-based approach offers increased sensitivity. However, MSS revealed 32 genes that went undetected by ResCap. From these, 14 genes (44%) were not included in the probe library, including nitroimidazole resistance genes *nimA*, *nimB*, *nimC*, *nimD*, *nimE*, *nimF*, *nimH* and *nimJ* (table s1). We currently have no explanation why we were not able to detect the 14 genes for which probes were present in the ResCap library and that were detected by MSS but not by ResCap.

We observed limited differences in the resistome composition between the diet groups, independently of the detection approach. A previous MSS based comparison of the gut resistome in the Chinese, Danish and Spanish general population, revealed the overall high abundance of ARGs that confer resistance to tetracycline, followed by macrolide, β-lactam and aminoglycoside resistance (26). This is also observed in our study which included samples from only Dutch population. When using ResCap as high-resolution method to profile the gut resistome, only two ARGs (*lsa*(C) and *tet*(L)) were differentially abundant between omnivores and pescatarians. Since these genes are observed in low abundance and prevalence, the biological relevance of this finding is unclear. MSS revealed that *tet*(X) was more abundant in the resistome of omnivores compared to pescatarians. It is most likely that the increased sample size (n= 149 vs n=64 samples included for MSS and ResCap respectively) resulted in the detection of differentially abundant *tet*(X) by MSS, but not by ResCap. This suggests that the small sample size for ResCap is a potential limitation of the current study.

Two methods, mOTUs2 and MetaPhlAn3 were used to study the impact of dietary habits on the gut microbiome (49, 50). This showed that long-term dietary habits did not result in significant differences in the top 10 most abundant genera, nor in differences in gut microbiome diversity and that diet was not a main driver of the observed variance in microbiome composition between the samples. Both methods were largely in concordance regarding which genera were differentially abundant using a supervised analysis where only a few compositional differences between the microbiome of omnivores, pescatarians and vegetarians were detected. Variation between methods include the detection of differentially abundant *Clostridium* species by the mOTUs2, compared to differentially abundant species from the family of *Lachnospiraceae*, by the MetaPhlAn3. This variation could be attributed to the distinct ways in which the tools correct for genomic sequences from yet unknown species. While mOTUs2 makes use of marker genes, using its clusters of orthologues groups of proteins approach (COGs), MetaPhlAn3 instead estimates the 'unknown' portion by using the average gene length and genome length

As part of a short-term diet intervention study, David et al. associated the consumption of animal derived products with an increased relative abundance of *Alistipes*, *Bilophila* and *Bacteroides* and a decrease in the relative abundance of *Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii* (14). Although our study included similar diet groups with omnivores consuming animal derived products, and vegans that do not, we did not observe similar findings. When compared to pescatarians, we detected a lower relative abundance of *Eubacterium* in omnivores, namely *Eubacterium eligens*. Chung et al. described *E. eligens* to be a pectin degrading specialist, which is a major component of plant cell walls and increased plant consumption might therefore explain the lower abundance of *E. eligens* in omnivores (51). The non-concordance between the studies by David et al. and our study might be explained by differences in resolution of 16S rRNA gene sequencing used in David et al., as compared to MSS in our study, but maybe more importantly this illustrates the difference between an intervention-based study compared to our study design, where long-term diet habits were studied.

More recently, De Angelis et al. compared the gut microbiome, proteome and metabolome of Italian omnivores, vegetarians and vegans based on 16S rRNA gene profiling. Similar to our study, they showed only slight differences in the abundance of bacterial families between the diet groups. *Ruminococcaceae* were found to be most abundant in omnivores, while *Lachnospira* were associated with vegans and vegetarians (52). Similarly, Losasso et al. observed differences in the relative abundance of *Ruminococcaceae* when comparing the microbiome of vegans, vegetarians, and omnivores (20), using 16S rRNA profiling. Although the observations are limited to the family level, these studies align with our results, where omnivores were found to have a higher relative abundance of *R. torques*

when compared to vegans. In contrast to these dietary studies, we observed differences in the relative abundance of lactic acid bacteria when comparing the microbiome between diet groups. This difference could be explained by the relatively high consumption of dairy products in the Netherlands (53). Omnivores and pescatarians showed higher relative abundance of *Streptococcus thermophilus* when compared to vegans. Similarly, *Lactococcus lactis* was present in higher relative abundance in the microbiome of pescatarians compared to vegans. Both *S. thermophilus* as *L. lactis* have been associated with the consumption of dairy products (54). Zhernakova et al. also detected a specific association between the consumption of buttermilk and the abundance of *Leuconostoc mesenteroides* and *L. lactis*, when assessing factors contributing to variation of the gut microbiome composition in the Dutch and Belgium population (12, 55). Since dairy products are excluded from the vegan diet, it could explain the low abundance of these lactic acid bacteria in this group.

To conclude, using ResCap we were able to increasingly detect resistance genes in complex samples like human faeces in our study, compared to MSS, and found that the gut microbiome of humans included in this study represented a large reservoir of 145 different antibiotic resistance genes in the studied population. However, differences in long-term dietary habits in the Dutch population did not result in significant differences in resistome composition between the four diet groups. When comparing the microbiome composition of diet groups in the Dutch population, mainly the vegan diet was associated with a distinct taxonomic composition. Since geographic location likely has an impact on the microbiome and resistome composition in the general population, future studies involving human populations from other geographic regions, are needed to determine the generalizability of our findings in the Dutch population.

Methods

Participant inclusion

In this study, faecal samples from 149 Dutch individuals from a previous study in the general population (the "NLD-VEGA-study") were selected based on their dietary habits (table 1) and categorized in four different diet groups: 1) omnivores, 2) pescatarians, 3) vegetarians and 4) vegans (56). Groups were matched for sex, age, education level, medication usage and keeping animals (table 1). Differences between groups were compared by the Kruskal-Wallis or X^2 test where appropriate. All participants had a Dutch nationality, were born in the Netherlands and lived in urban areas. None of them had used antibiotics, insulin, proton pump inhibitors or drugs related to cancer treatment and chemotherapy in the past three months.

Ethical approval

The original "NLD-VEGA-study" protocol was approved by the medical ethics committee of the University Medical Center Utrecht, the Netherlands (no. 15-561/C). All experiments were performed in accordance with relevant guidelines and regulations.

Classification of diet groups

Diet groups were defined based on the following criteria: Omnivores consumed meat at least three times per week, for the past six months or more. Pescatarians consumed fish and animal derived products but did not consume meat in the past six months or more. Vegetarians consumed animal derived products but did not consume meat or fish in the past six months or more. Vegans did not consume meat, fish, or animal derived products in the past six months or more. Furthermore, pescatarians, vegetarians and vegans did not prepare the restricted products described for house members or pets or have house members that consumed those.

Sample collection, storage and DNA extraction

Faecal samples were sent by regular mail and transported for a maximum of 24 hours before storage at -80°C. Samples were divided into aliquots of 0.2 g, thereby introducing one freeze-thaw cycle. Samples were thawed a second time and used for DNA extraction, using a modified protocol of the QIAamp fast DNA stool mini kit (Qiagen, Venlo, the Netherlands) as described by Knudsen et al. (57). In brief, 0.2 g faeces were added to 'lysing matrix A, 2 ml tubes' (MP biomedicals, Landsmeer, the Netherlands), containing 1 ml InhibitEx buffer (Qiagen). Beat beating was applied at 3.5 m/s for 30 s, followed by 30 s incubation on ice and one final beat beating step, using the FastPrep-24 (MP biomedicals). After 7 minutes of incubation at 95°C, the fast DNA stool mini kit protocol (Qiagen) was resumed at the proteinase K treatment step. Total DNA was quantified by Picogreen assay (Thermo Fisher Scientific, Waltham, MA, USA).

Metagenomic shotgun sequencing and microbiome data processing

Samples were sent to Baseclear B.V. (Leiden, the Netherlands) for metagenomic shotgun sequencing (MSS), together with a DNA extraction (negative) control and mock control (ZymoBIOMICS Microbial Community Standard). The NovaSeq 6000 (Illumina, San Diego, USA) was used, with the S1, 2 x 150 bp paired-end kit (Illumina) and the company protocol/standard settings. Raw reads were trimmed by Trimmomatic v0.39 (options: slidingwindow:4:15 minlen:70) and used for taxonomic profiling with either mOTUs2 version 2.5.0 or MetaPhlAn3 using default settings (49, 50, 58). Samples contained on average 70.7M ± 11.3M reads, while the negative control contained less than 300k reads. The MSS results of the mock control contained a total of 89M reads, and matched with the expected mock composition (table s2).

ResCap sequencing

Sixteen samples per diet group were selected for in-depth resistome analysis using the ResCap targeted sequence capture panel consisting of probes targeting genes that confer resistance to antibiotics, metals, biocides and included probes that target relaxase genes (46). The capture panel contains probes against 7963 resistance genes and was expanded, by the addition of probes against the following *mcr* genes (*mcr1.1*, *mcr1.2*, *mcr1.3*, *mcr1.4*, *mcr1.5*, *mcr1.6*, *mcr1.7*, *mcr1.8*, *mcr1.9*, *mcr1.10*, *mcr2.1*, *mcr2.2*, *mcr2.3*, *mcr3.1*, *mcr3.2.1*, *mcr3.2.2*, *mcr3.3.1*, *mcr3.3.2*, *mcr3.4.1*, *mcr3.4.2*, *mcr3.5.1*, *mcr3.5.2*, *mcr3.6, mcr4*, *mcr5*, *mcr6*, *mcr7* and *mcr8*) (Roche ID: OID41815). The subset of samples selected per diet group were matched for sex, age, education level, medication usage and having pets (table 2). Differences between groups were tested by the Kruskal-Wallis or X^2 test where appropriate. ResCap was performed according to the supplied protocol. In brief, 0.8-1.0 μg DNA was used for fragmentation using the KAPA HyperPlus Kit v4.17 (Roche, Woerden, The Netherlands) to generate 400 bp fragments. End repair, A-tailing and adapter ligation were performed as described by the SeqCap EZ HyperCap User's Guide v2.3. Pools of 12 samples were used for hybridization and capture using an extended version of the ResCap probe collection as described in the original publication (46). Sample pools were sequenced on a NextSeq500 (Illumina), using high output and paired-ends of 2 x 150 bp.

Resistome data processing

ResCap and MSS data were trimmed using Trim Galore version 0.6.4 with standard settings (59). KMA version 1.3.4 was used to align sequences to the Resfinder database version of 2020-06-02 (60). For KMA, paired-end reads were used as input by using *-ipe*, together with the options: *-tmp, -1t1*, *-cge*, *-apm p*, *-ef*. The resulting list of detected genes and their abundance was trimmed by applying a cut-off of 90% identity (called query Identity) and of 80% coverage (called template coverage). Finally, the output value *depth* was used for subsequent analysis, which represents the amount of aligned base pairs, while correcting for gene length. Analysis of the MSS negative control for antibiotic resistance genes, containing less than 300k reads, revealed the low abundance of antibiotic resistance genes *tet(L)* and *aadD*.

Data analysis

Analysis of sequencing data was performed in R version 4.0 and the functions of the packages phyloseq and ggplot2 (61-63). The top 10 abundances of the microbiome and resistome were plotted using aggregate top taxa and plotting functions of the microbiome package (64). Shannon index was calculated using the alpha diversity functions of the microbiome package and plotting functions of the microbiomeutilities package (65). Aitchison, Bray-Curtis and Jaccard distance PCA were generated using the transform function of the microbiome package and ordinate (RDA) and plot ordination functions

of the phyloseq package. Correlations of sample dissimilarity and diets were tested using PERMANOVA with the adonis function at 999 permutations of the vegan package (66). Basic accessor functions of phyloseq were used to generate heatmaps. Differential abundance analysis was performed using ANCOM-BC version 1.0.2, with Bonferroni correction for false discovery rate and an alpha of 0.05 as a threshold for significance (45).

Data availability

The 149 MSS and 64 ResCap sequencing files have been deposited in the European Nucleotide Archive repository under the study accession no. PRJEB45944 and PRJEB46230, respectively. R scripts to reproduce the analysis reported in this study can be found at; https://gitlab.com/PB_Stege/diet_microbiome_resistome/

List of abbreviations

ARGs	Antibiotic resistance genes
MSS	metagenomic shotgun sequencing
PCA	Principal component analysis
ResCap	resistome capture sequencing approach

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Author contributions

PS, FP, RW, EF, EG, CD, EvD and SF all contributed to the study design and implementation. Lab work was performed by PS, RvdP, MV and MCV. PS, JH, SS, MR, RW and FP performed data analysis, statistical analysis and interpretation. All authors read and approved the final manuscript.

Competing interests

All authors declare no conflict of interests.

Consent to publish

In this study, faecal samples from 149 Dutch individuals from a previous study in the population at large (the "vegastudy") were selected. All participants provided written informed consent for the initial vegastudy and had given permission to use their faecal samples for further research.

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Supplementary data

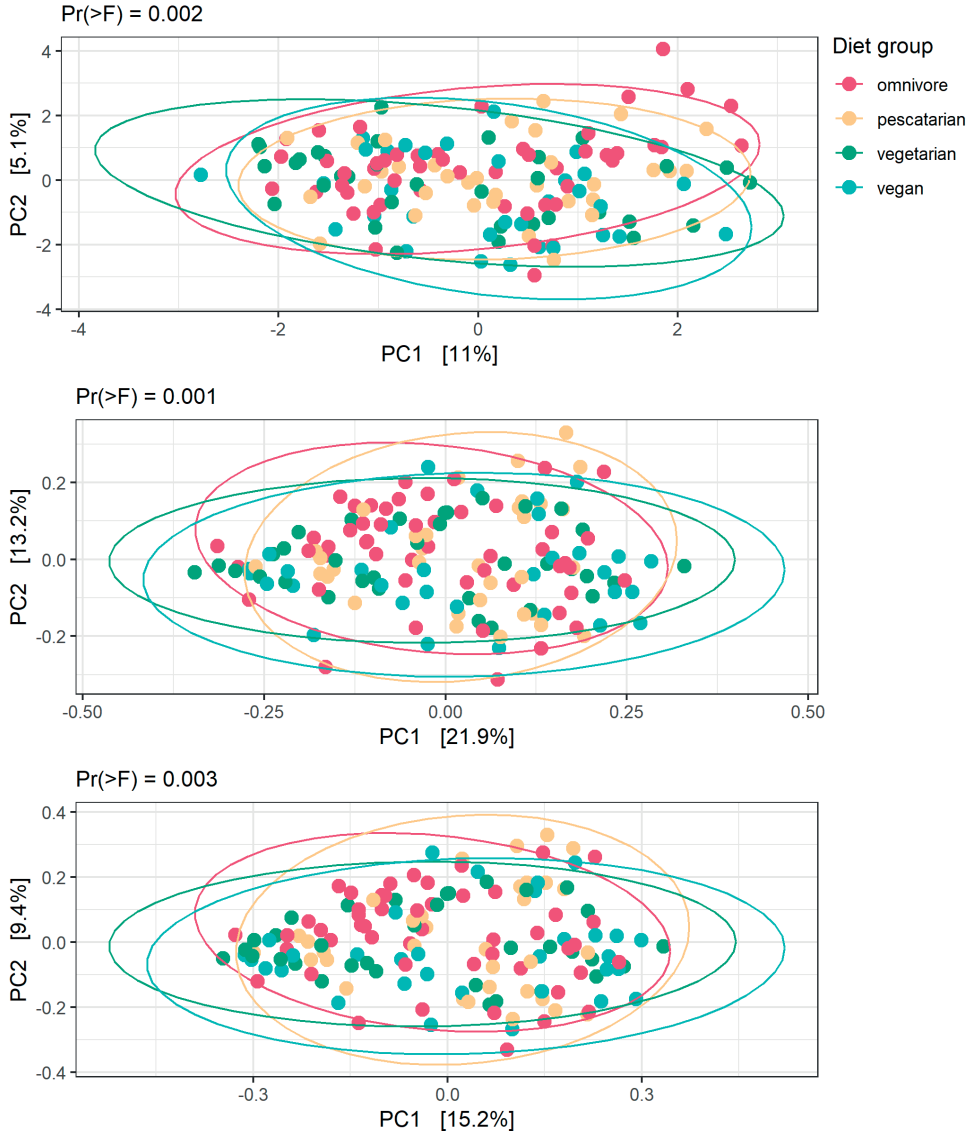

Figure s1 | Overall gut microbiome dissimilarity beta-diversity of all study samples. PCA on bacterial species with data points and ellipses coloured according by diet group, based on (A) Aitchison distance. (B) Bray-curtis distance. (C) Jaccard distance.

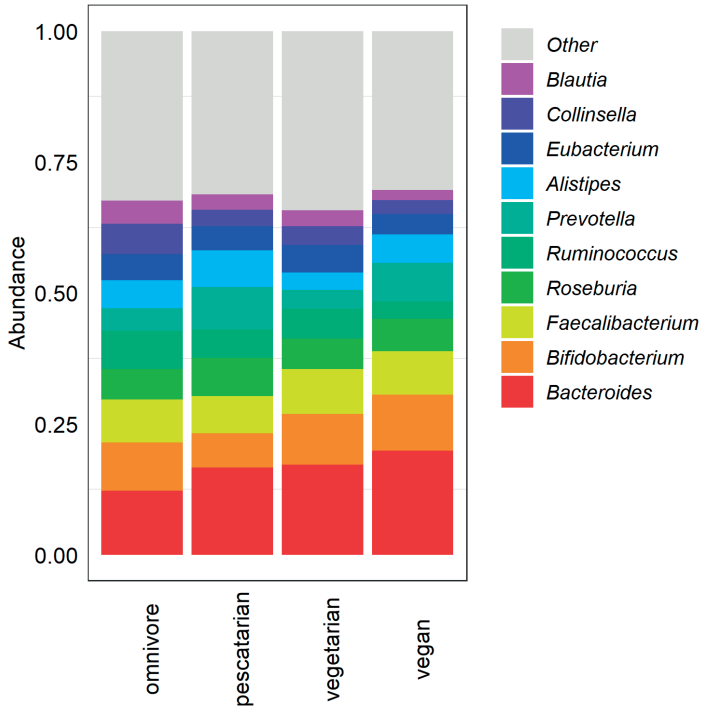

Figure s2 | Association of dietary habits and the gut microbiome composition using MetaPhlAn3. Relative abundance of the 10 most abundant bacterial genera per diet group.

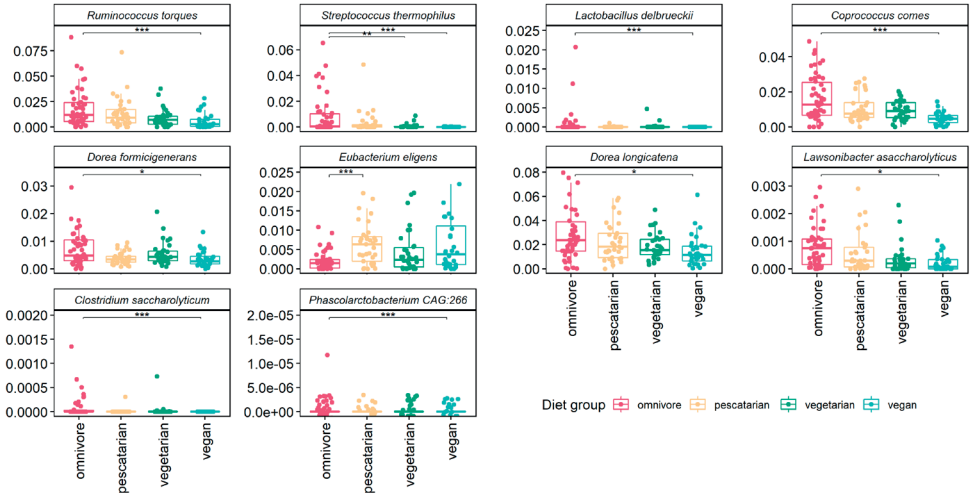

Figure s3 | Differential abundance analysis of gut microbiome composition using MetaPhlAn3 and ANCOM-BC. Plotted on relative abundance scale from 0.00 to 1.00. Adjusted P-values below 0.05 and 0.001 are indicated by * and ***, respectively.

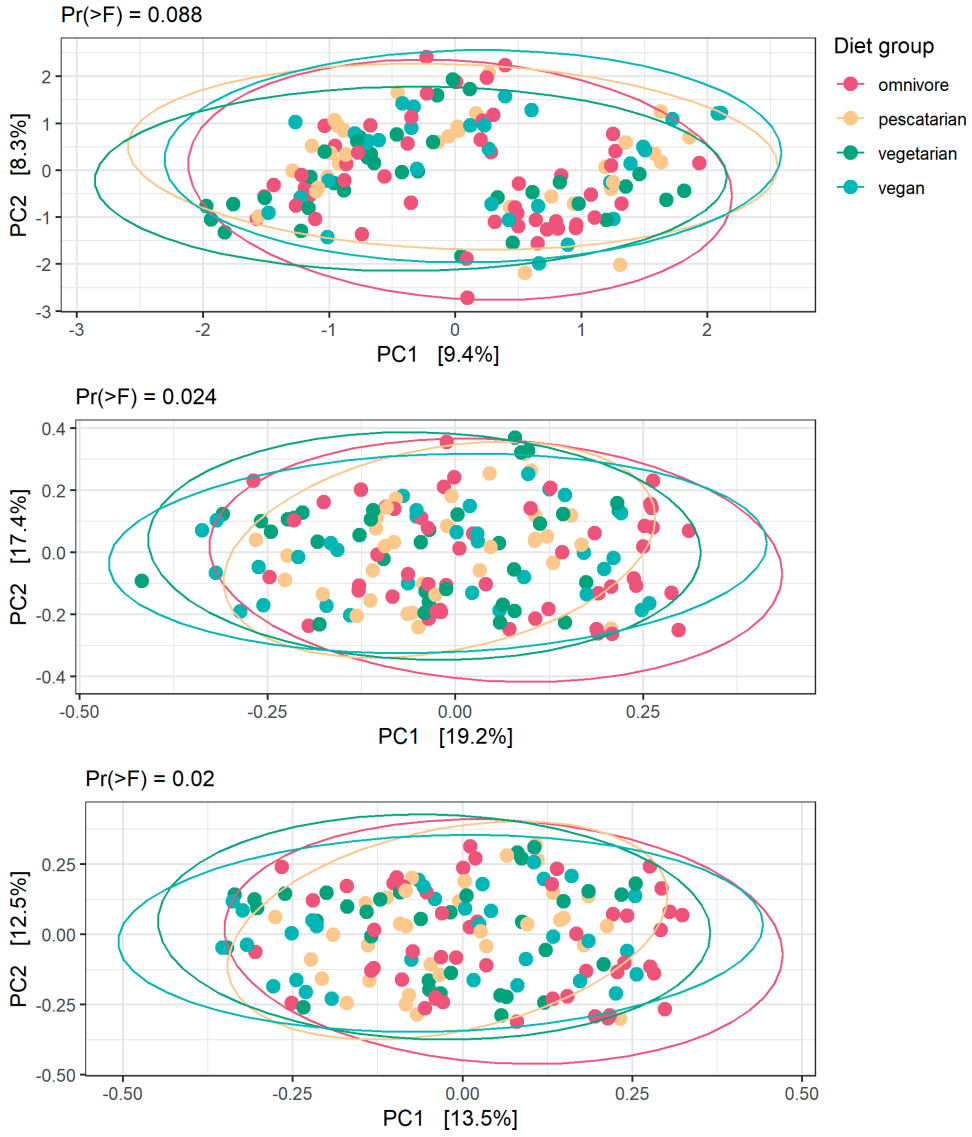

Figure s4 | Overall gut resistome dissimilarity between samples using Metagenomic shotgun sequencing. PCA on antibiotic resistance genes with data points and ellipses coloured according by diet group, based on (A) Aitchison distance. (B) Bray-curtis distance. (C) Jaccard distance.

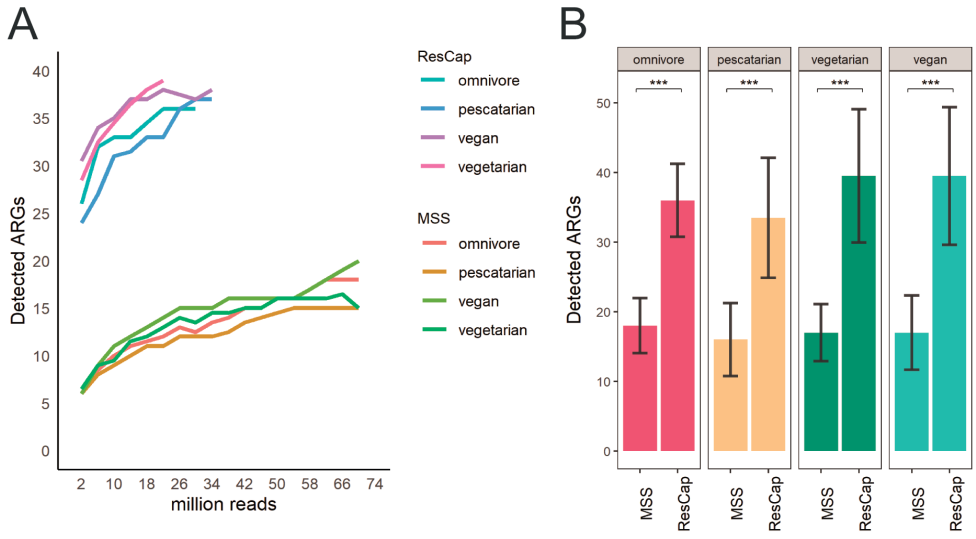

Figure s5 | Comparison of antibiotic resistance genes detected by ResCap and Metagenomic shotgun sequencing. (A) Using 64 identical samples, the efficiency to detect antibiotic resistance genes (ARGs) was compared between methods in a rarefaction curve. The number of detected ARGs represents the median of 16 sixteen samples per diet group. Sequencing data was subsampled by steps of four million reads in samples containing up to 70 million reads. (B) The average number of ARGs detected in the 16 samples per diet group, per sequencing methods, with matching standard deviation.

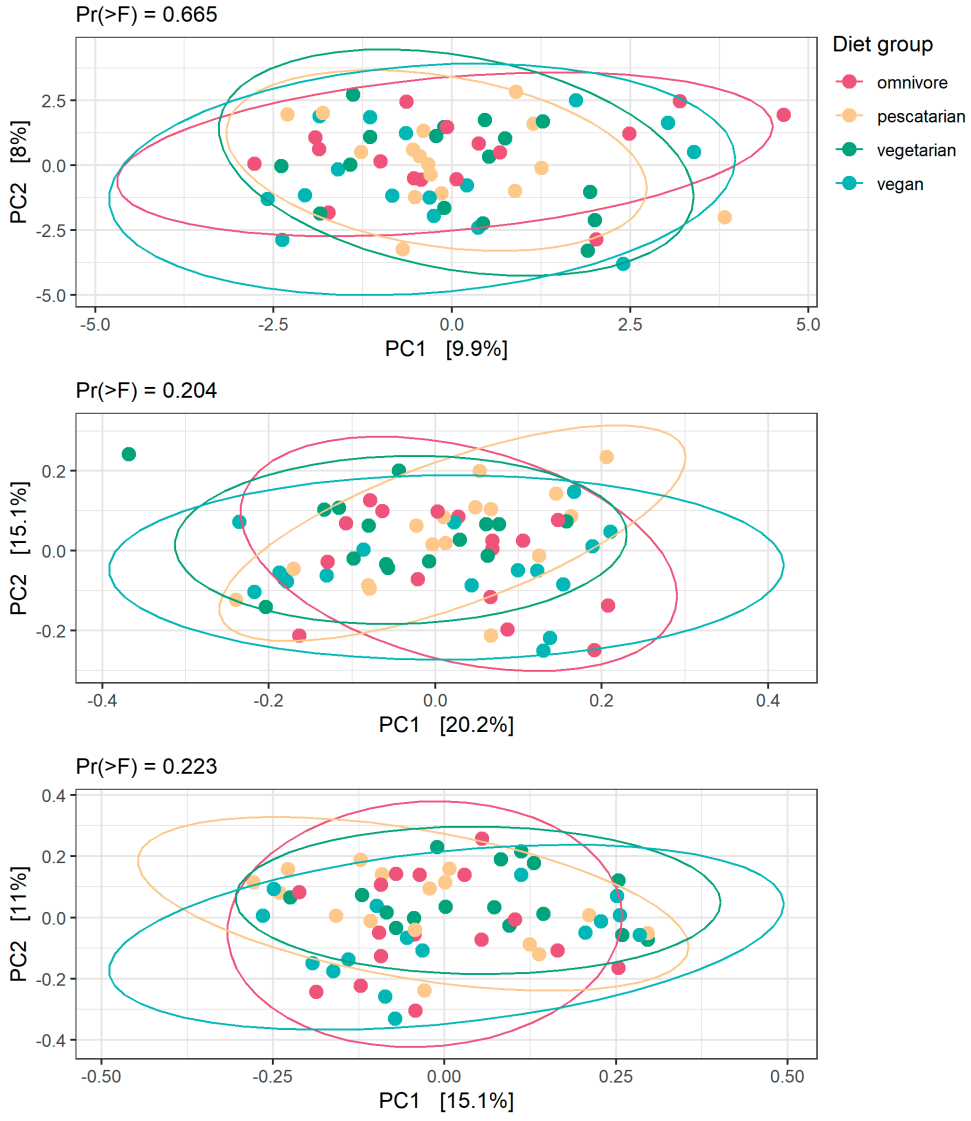

Figure s6 | Overall gut resistome dissimilarity between samples using ResCap. PCA on antibiotic resistance genes with data points and ellipses coloured according by diet group, based on (A) Aitchison distance. (B) Bray-curtis distance. (C) Jaccard distance.

Table s1 | Antibiotic resistance genes detected by ResCap and Metagenomic shotgun sequencing.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter2-table_s1_-_concordance_mss_rescap.xlsx

Table s2 | Detected bacteria in mock community.

Mock community composition (mOTUs2)	
detected species	**relative abundance (%)**
s__Salmonella enterica/bongori	18
s__Bacteria s.	18
s__Proteobacteria sp.	17
s__Pseudomonas	11
s__Staphylococcus aureus	11
s__Lactobacillus fermentum	9
s__Bacteria sp.	8
s__Listeria monocytogenes	7
other	1

Mock community composition (Metaphlan3)	
detected species	**relative abundance (%)**
s__Salmonella_enterica	20
s__Enterococcus_faecalis	18
s__Escherichia_coli	21
s__Pseudomonas_aeruginosa_group	4
s__Staphylococcus_aureus	12
s__Lactobacillus_fermentum	10
s__Bacillus_intestinalis	7
s__Listeria_monocytogenes	6
other	1

Expected mock commnunity composition	
species	**relative abundance (%)**
Salmonella enterica	12
Enterococcus faecalis	12
Escherichia coli	12
Pseudomonas aeruginosa	12
Staphylococcus aureus	12
Lactobacillus fermentum	12
Bacillus subtilis	12
Listeria monocytogenes	12
Saccharomyces cerevisiae	2
Cryptococcus neoformans	2

ZymoBIOMICS Microbial Community Standard

Chapter 3

Gut colonization by ESBL-producing *Escherichia coli* in dogs is associated with a distinct microbiome and resistome composition

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Abstract

The gut microbiome of humans and animals acts as a reservoir of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC). It has been shown that dogs have a high prevalence of ESBL-EC, although their ESBL-EC carrier status often shifts over time. We hypothesized that the gut microbiome composition of dogs is implicated in ESBL-EC colonization status. Therefore, we assessed whether ESBL-EC carriage in dogs is associated with changes in the gut microbiome and resistome. Faecal samples were collected longitudinally from 57 companion dogs in the Netherlands every two weeks for a total of six weeks. Carriage of ESBL-EC was determined through selective culturing and PCR and in line with previous studies, we observed a high prevalence of ESBL-EC carriage in dogs. Using 16s rRNA gene profiling we found significant associations between detected ESBL-EC carriage and an increased abundance of *Clostridium sensu stricto 1*, *Enterococcus*, *Lactococcus* and the shared genera of *Escherichia-Shigella* in the dog microbiome. A resistome capture sequencing approach (ResCap) furthermore revealed associations between detected ESBL-EC carriage and the increased abundance of the antibiotic resistance genes: *cmlA*, *dfrA*, *dhfR*, *floR* and *sul3*. In summary, our study showed that ESBL-EC carriage is associated with a distinct microbiome and resistome composition.

Introduction

In the last decade, the global emerge of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC) is compromising the efficacy of antibiotic therapy and increasing the chance of therapy failure (1). The main reservoir of ESBL-EC is the gut microbiome of healthy humans and animals. Companion animals, often considered family members in households, may be an important source of multidrug-resistant organisms, including ESBL-EC (2-4). The most frequent observed beta-lactamase genes in ESBL-EC in the human gut microbiome include: $bla_{\text{CTX-M-15}}$, followed by $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$ and $bla_{\text{CTX-M-3}}$ *(5, 6)*. This partially overlaps with the occurrence of beta-lactamase genes in the dog gut microbiome, which include $bla_{\text{CTX-M-1}}$, followed by $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$ (3, 4). Additionally, van den Bunt et al. detected $bla_{\text{CTX-M-32}}$ and $\text{bla}_{\text{TEM-52}}$ resistance genes in dogs (7). Longitudinal analysis of ESBL-EC carriage in dogs revealed persistent carriage in 57% of the dogs for one month and in 43% of the dogs for six months (7). In another study, Baede et al., observed persistent carriage of ESBL-EC in 24% of the dogs studied (3). However, both studies also reported temporal shifts in ESBL-EC carrier status, with the vast majority of dogs shifting over time between positive and negative ESBL-EC carrier statuses, some even showing three or more shifts in a 6-week period (3, 7). These shifts probably reflect significant differences in colonization levels of ESBL-EC in dogs, potentially leading to differences in sensitivity levels of detecting ESBL-EC colonization, or uptake or loss of ESBL-EC strains caused by factors not well understood (3).

The composition of the gut microbiome of dogs is potentially one of the factors involved in ESBL-EC colonization. The intestinal tract of humans and animals is densely colonized by hundreds of different species of bacteria that together with fungi, viruses, and phages, represent the gut microbiome (8). This complex ecosystem does not only play a role in host nutrient acquisition but is also involved in health and disease status of the host (9, 10). As a result of the continuous competition for both nutrients and space, commensal gut microbes provide protection against pathogenic bacteria by preventing colonization and subsequent infections, termed colonization resistance (11, 12). This competition furthermore drives the gut microbiome towards an equilibrium, despite the daily exposure to new microbes from numerous environmental sources. Some of the most abundant bacterial genera in the dog gut microbiome include *Peptostreptococcus*, *Bacteriodes*, *Prevotella*, *Faecalibacterium* and *Blautia* (13-15). The gut microbiome furthermore represents an important reservoir of antibiotic resistance genes, called the resistome. Resistance genes that are frequently found as part of the dog intestinal resistome are genes encoding for tetracycline resistance (*tet*(W), *tet*(O), *tet*(Q), *tet*(M)), macrolide resistance (*mefA*, *mel*) aminoglycoside resistance (*aph(3″)-Ib*, *aph(6)-Id*), lincomycin resistance (*lnuC*) and beta-lactamase resistance (namely $bla_{\text{OXA-85}}$) (16).

To unravel whether ESBL-EC carriage in dogs is associated with microbiome and resistome changes, we studied the dog gut microbiome and resistome composition longitudinally in relation to the ESBL-EC status (positive or negative) among 57 dogs, over the course of six weeks. We confirmed by culturing, matrix-assisted laser desorption/ionization-time of flight (MALDITOF) and PCR screening that in this study in a large proportion of these dogs (68%) ESBL-EC carriage could be detected. 16S rRNA gene sequencing revealed that detected ESBL-EC carriage is associated with an increased abundance of *Clostridium sensu stricto 1*, *Enterococcus*, *Lactococcus* and the shared genera of *Escherichia-Shigella*. Target resistome analysis revealed that dogs in which colonisation with ESBL-EC was detected also have a higher abundance of *cmlA*, *dfrA*, *dhfR*, *floR* and *sul3* resistance genes. These findings highlight that detected ESBL-EC gut carriage is associated with a distinct gut microbiome and resistome composition, which may include bacteria with pathogenic potential.

Results

Detected ESBL-EC carriage is associated with specific changes in the gut microbiome

To investigate the association between carriership of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC) and gut microbiome composition, faecal samples were collected from 57 companion dogs in the Netherlands every two weeks for a total of six weeks. From these 57 dogs, 37 were part of households with two or more dogs. In a relatively high number of dogs (39, 68%) ESBL-EC were detected at least once over the course of six weeks. In nine dogs ESBL-EC (16%) were detected at all time points, while in 30 dogs (52%) ESBL-EC were detected intermittently. In the remaining dogs (18 dogs, 32%) no ESBL-EC were detected over the course of six weeks (figure 1).

16S rRNA sequencing was performed to determine gut microbiome composition. The top 20 most abundant bacterial genera in dogs included *Peptoclostridium*, *Blautia*, *Prevotella*, *Faecalibacterium* and *Bacteroides* (figure 2). While individual dogs showed shifts in microbial composition between different time points, the largest difference in gut microbiome composition was observed between dogs in which ESBL-EC carriage was detected at all time points and dogs in which ESBL-EC were not detected (figure 2). More specifically, the relative abundance of *Escherichia-Shigella*, *Enterococcus* and *Clostridium sensu stricto 1* was higher in dogs in which ESBL-EC were detected at all time points compared to dogs where ESBL-EC carriage was not detected during all time points (figure 2b, 2c). These differences in relative abundance between time points were not as apparent when ESBL-EC carriage was intermittently detected (figure 2a).

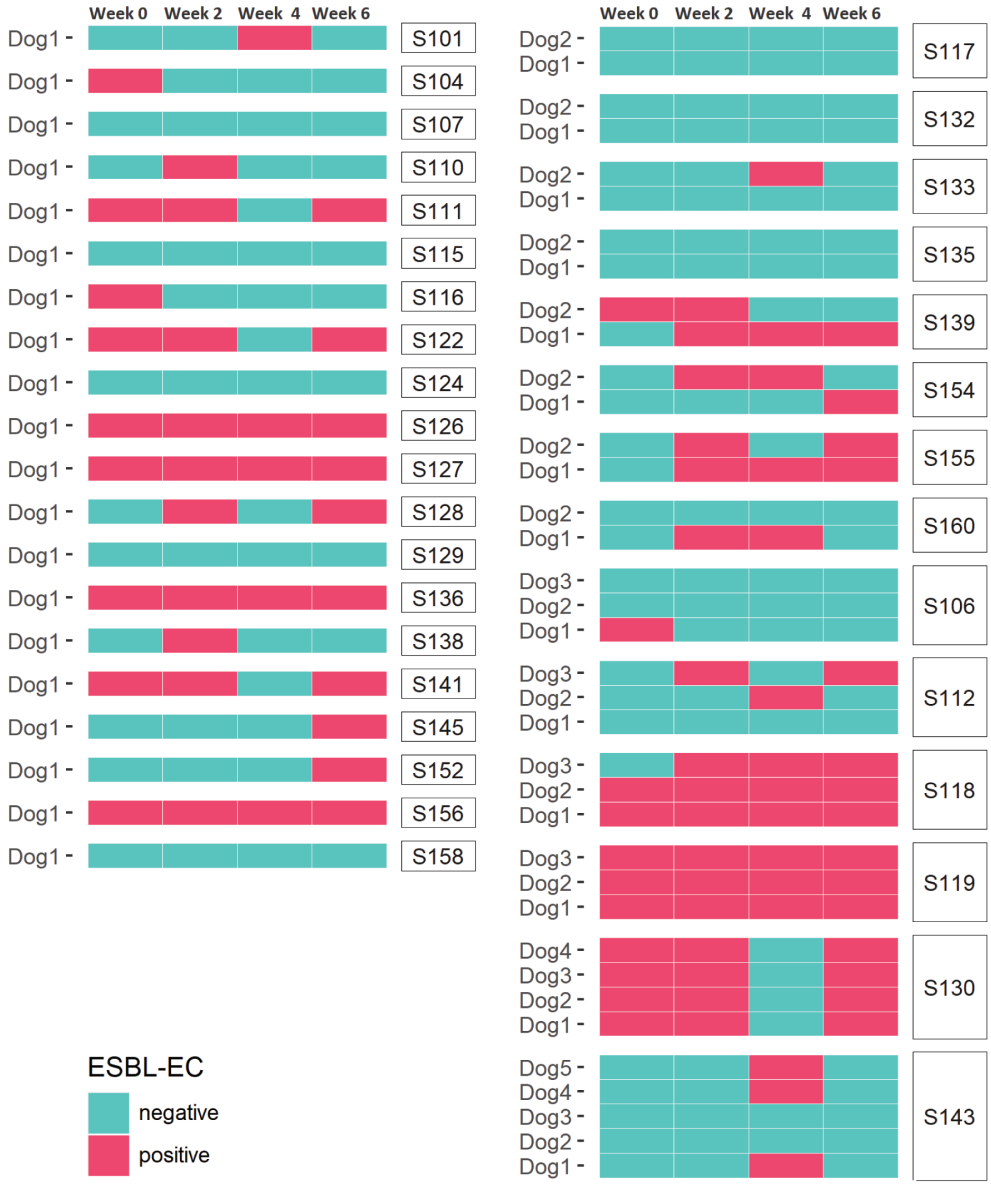

Figure 1 | Detection of ESBL-producing *Escherichia coli* in dogs. Rows represent individual dogs and samples from these dogs are grouped by household, while columns indicate time points with two-week intervals. S-numbers indicate the households. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

Figure 2 | Detected ESBL-EC carriage and the gut microbiome composition. Relative abundance of the 20 most abundant bacterial genera per dog. Each row represents a time point and ESBL-EC detection is

marked by '+' when ESBL-EC were detected or '-' when this was not the case. Rows are grouped per dog. A) Dogs in which ESBL-EC were detected intermittently, B) Dogs in which ESBL-EC were detected during all time points. C) Dogs in which ESBL-EC were not detected during at any time point. S-numbers indicate the households and D-numbers the dogs. Red boxes indicate bacterial genera that show large differences in relative abundance between dogs in which ESBL-EC were detected and dogs in which ESBL-EC were not detected during all timepoints.

The gut microbiome diversity, expressed as Shannon index, was not significantly different between faeces samples of dogs in which ESBL-EC were detected or where this was not the case, indicating that the total species diversity was similar regardless ESBL-EC detection status (figure 3a). Principal component analysis (PCA) based on Aitchison distance was applied to disclose differences in the structure of gut microbiome across all individual dog samples (figure 3b). The first two principal components explain a large proportion of the observed variance in gut microbiome composition (31%), suggesting grouping of dogs based on ESBL-EC detection status, although differences were not statistically significant (p=0.053).

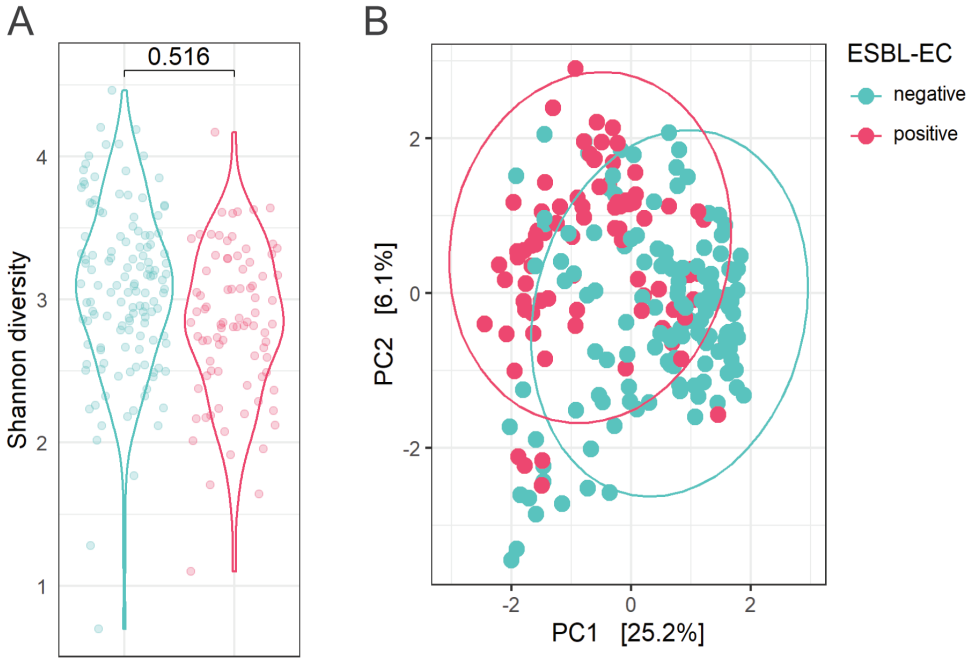

Figure 3 | ESBL-EC carriage and the gut microbiome diversity. A) Alpha diversity expressed by Shannon index and grouped based on dog ESBL-EC carriage. Differences in alpha diversity between groups, expressed by Shannon index, were tested using linear mixed-effects models. B) Aitchison distance PCA based on bacterial genera with data points and ellipses coloured according to dog ESBL-EC carriage, tested with PERMANOVA. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

Univariate generalized linear mixed-effects models (GLME) were used to test for associations between the bacterial genera abundance and detected ESBL-EC carriage in dogs table s1. Univariately, detected ESBL-EC carriage was associated with increased abundance of *Clostridium sensu stricto 1*, *Enterococcus, Lactococcus* and the shared genera of *Escherichia-Shigella* (figure 4a, 4c). In contrast, detected ESBL-EC carriage was associated with decreased abundance of *Colidextribacter*, *Faecalibacterium*, *Fournierella*, *Holdemanella*, *Muribaculaceae*, *Negativibacillus*, *Peptococcus* and *Prevotella* (figure 4a, 4c). Multivariate analysis confirmed that the increased abundance of *Clostridium sensu stricto 1*, *Enterococcus* and *Lactococcus*, are together associated with detected ESBL-EC carriage (figure 4b, 4c, table s1).

Detected ESBL-EC carriage is associated with the increased abundance of specific antibiotic resistance genes.

Since the gut microbiome is an important reservoir of resistance genes, differences in the observed microbiome composition that were found to be associated with detected ESBL-EC carriage, may also result in changes in the resistome composition. To investigate this, 10 households for which samples on four time points were available and contained no more than a single dog were selected for resistome profiling using resistome capture sequencing approach (ResCap) (figure s1). Single dog households were selected to avoid possible clustering of resistome features among dogs living in the same household. We first set-out to compare the results of ESBL gene detection using ResCap with the detection of ESBL genes using culture-based method followed by conventional PCR screening. PCR screening of the 40 samples used for ResCap detected ESBL genes in 19 samples (48%) (table s2). From the 40 samples sequenced by ResCap, 7 (18%) contained $bla_{\text{CTX-M}}$ genes. In addition, 1 sample contained bla_{SHV}, a potential ESBL gene (table s2). Of note, the applied MEGARes database used in ResCap analysis does not distinguish between different CTX-M groups or SHV variants. In the 14 samples where ResCap did not detect an ESBL gene, but the selective culturing and PCR confirmation did, $bla_{\text{CTX-M-1}}$ was detected in 7 samples, $bla_{\text{CTX-M-14/18}}$ in 4 samples, $bla_{\text{CTX-M-15}}$ in 7 samples and $bla_{\text{CTX-M-32}}$ in 2 samples (table s2). In contrast, ResCap detected $bla_{\text{CTX-M}}$ resistance genes in two samples that were not detected using the culture-based method followed by PCR screening (table s2).

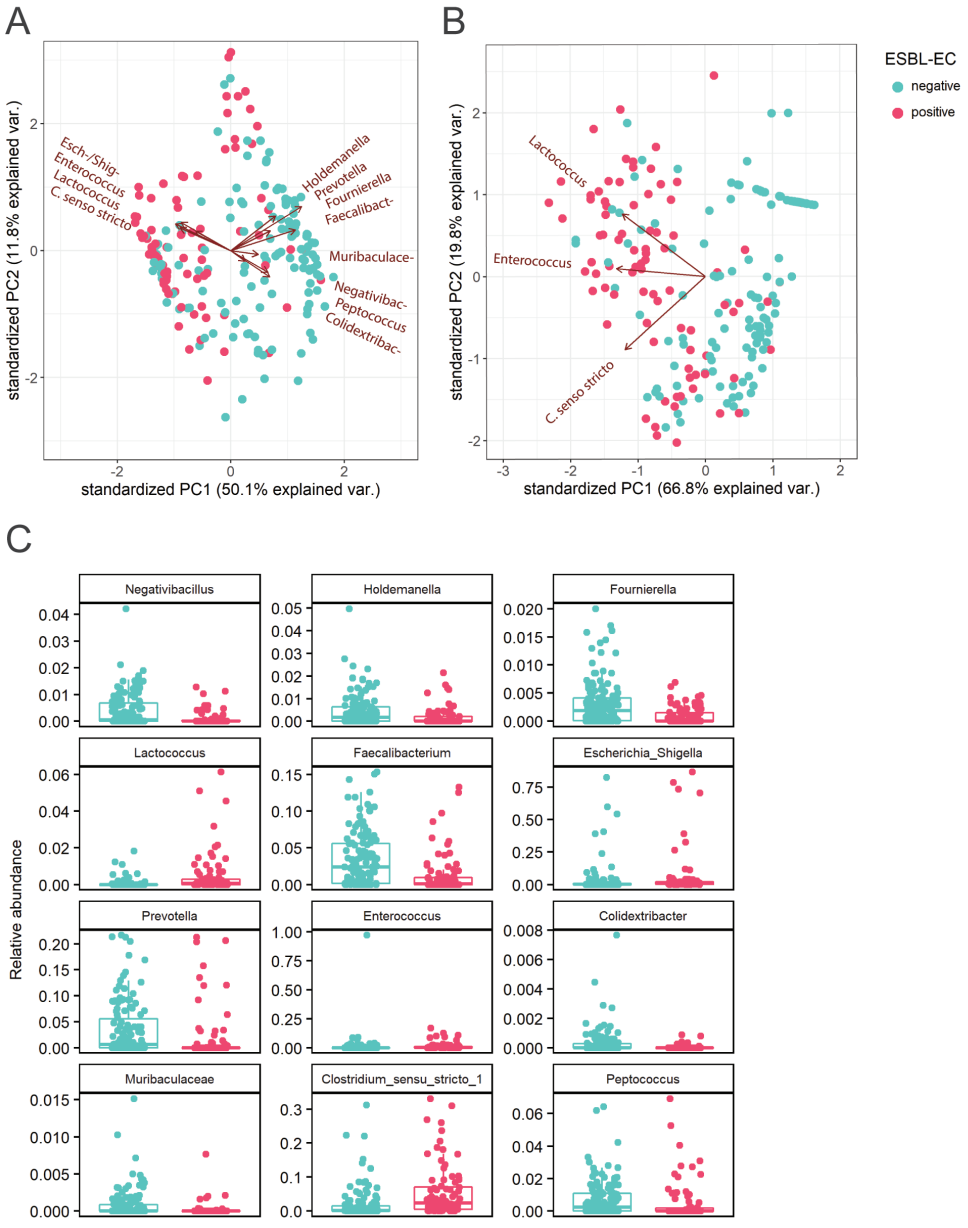

Figure 4 | Association of detected ESBL-EC carriage and individual bacterial genera. PCA composed of only the bacterial genera that are significantly associated with detected ESBL-EC carriage or the group where ESBL-EC carriage was not detected, as determined by (A) univariate longitudinal analysis and (B) multivariate longitudinal analysis. (C) Individual abundance of bacterial genera that are significantly associated with detected ESBL-EC carriage. Abundance was plotted on the relative abundance scale from 0.00 to 1.00. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

In total, 133 unique acquired antibiotic resistance genes (ARGs) were identified by ResCap in 40 samples (table s3). The 20 most abundant ARGs included 10 different tetracycline resistance genes, the macrolides/lincosamides/streptrogramin (MLS) resistance genes *mefA* and *ermB*, lincomycin resistance gene *lnuC*, beta-lactamase resistance genes *ampH*, *cfx* and bla_{EC}, aminoglycoside resistance genes *ant(6)*, *aph(2")* and *aac(6')* and sulphonamide resistance gene *sul2* (figure 5). No clear association was observed between the prevalence of these top 20 most abundant ARGs in dog samples and detected ESBL-EC carriage.

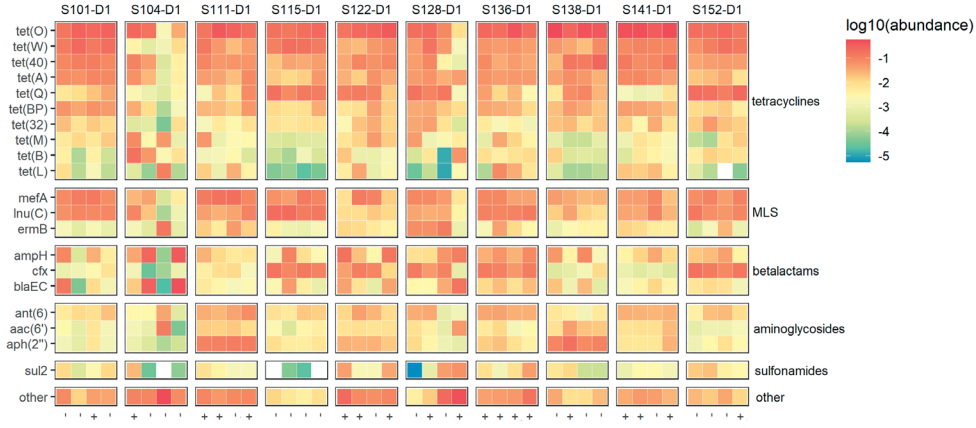

Figure 5 | Relative abundance of the gut resistome. Relative abundance of the 20 most abundant ARGs per dog. Each column represents a time point and detected ESBL-EC carriage is marked by '+' or '-' when ESBL-EC carriage was not detected. Columns are grouped per dog and S-numbers indicate the households and D-numbers the dogs.

The gut resistome alpha-diversity, expressed as Shannon index, was not significantly different between dog samples in which ESBL-EC carriage was detected and in samples where this was not the case (figure 6a, p=0.332). Differences in the beta-diversity of the gut resistome across all individual dog samples were also not statistically significant (figure 6b, p=0.282).

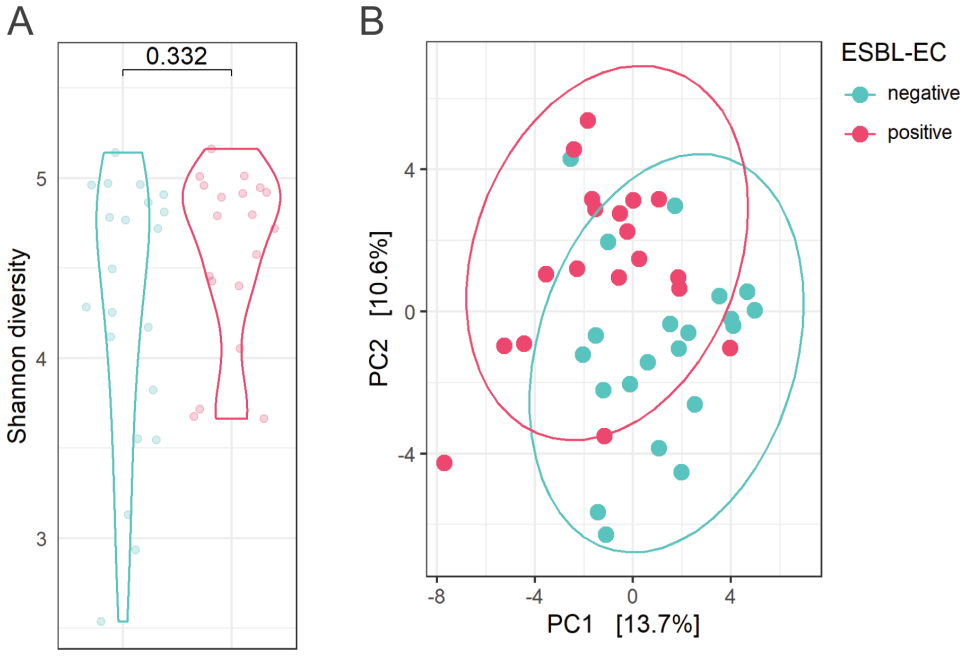

Figure 6 | Association of dog gut resistome composition and detected ESBL-EC carriage. Alpha diversity expressed by Shannon index and grouped based on detected ESBL-EC carriage. Differences in alpha diversity between groups, expressed by Shannon index, were tested using LME models. B) Aitchison distance PCA based on bacterial species with data points and ellipses coloured according to the detected ESBL-EC carriage status in the dogs, tested with PERMANOVA. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

Univariate GLME analysis showed significant associations between detected ESBL-EC carriage and increased abundance of the *cmlA*, *dfrA*, *dhfR*, *floR* and *sul3* genes (figure 7a, table s4). Multivariate analysis confirmed that the increased abundance of *dhfR* was associated with detected ESBL-EC carriage (table s4). Although *dhfR* was present in low abundance in the resistome, the difference in gene abundance of *cmlA, dfrA, dhfR, floR* and *sul3* was mainly due the presence of these genes in dogs in which ESBL-EC carriage was detected, compared to an absence of these genes in dogs were ESBL-EC carriage was not detected (figure 7b).

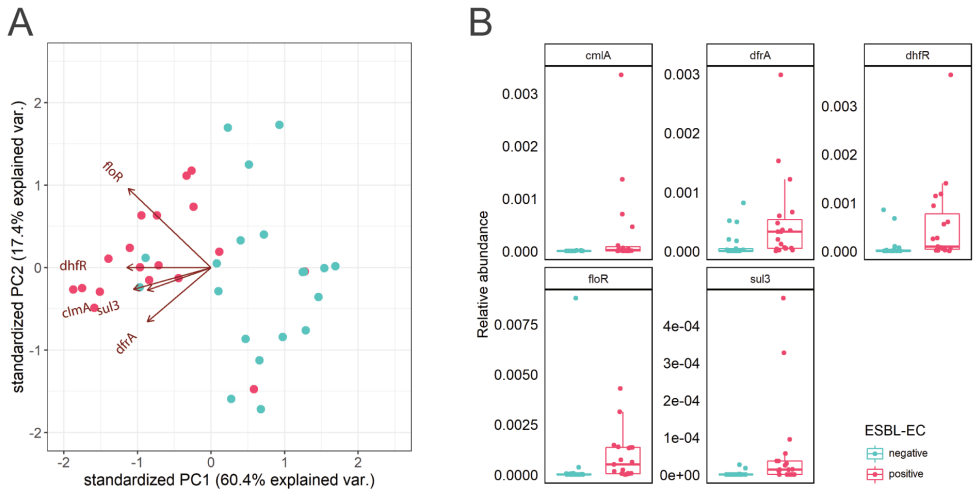

Figure 7 | Association of detected ESBL-EC carriage and the resistome. PCA composed of only the ARGs that are significantly associated with detected ESBL-EC carriage, as determined by (A) univariate longitudinal analysis. (B) Individual abundance of antibiotic resistance genes that are significantly associated with detected ESBL-EC carriage. Abundance was plotted on the relative abundance scale from 0.00 to 1.00. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

Long-read metagenomics of two dog samples.

To obtain more detailed species information and genetic context of resistance genes, faecal samples of two dogs belonging to household S111 and S128 were subjected to long-read metagenomic sequencing. Samples of two dogs that carried the bacterial genera *Clostridium*, *Enterococcus* and *Lactococcus* and for which samples on four time points were available, were selected for this analysis. Long-read metagenomics of faecal DNA yielded 13.5 million reads (sample median of 1.7 million reads) with an N50 of 2839 bp (total throughput: 17.8 Gbp). Species within the combined Escherichia-Shigella genus could not be accurately assigned because of the close genetic relatedness between the two species (17, 18).

Metagenomic analysis revealed that in both dogs the genus Clostridium (9.2% - 10.2% total relative abundance) was primarily represented by *Clostridium hiranonis* (66.3% - 70.0%), and *Clostridium perfringens* (9.7% - 12.3%) (figure s2), while for *Lactococcus* (0.04% - 0.2% total relative abundance), we mainly detected reads representing *Lactococcus garvieae* (24.4% - 43.9%) and *Lactococcus lactis* (25.8% - 32.6%). Finally, for *Enterococcus* (0.3% - 2.3% total relative abundance), the species distribution in the two analysed dogs was highly dissimilar, with *Enterococcus hirae* dominating in sample S128 (68%), while not being detected in sample S111.

With respect to resistance gene context, the analysis revealed in the dog of household S128 co-localization of the florfenicol resistance gene *floR* with the trimethoprim resistance gene *dfrA36*, the sulphonamide resistance gene *sul2* and the class 1 integron specific recombinase *intI1* on a single 13902 bp read (figure s3). In the dog of household S111, the trimethoprim resistance gene *dfrA1* appeared to be co-localized with the streptothricin resistance gene *sat2_gen*, the streptomycin resistance gene *aadA1*, the beta-lactam resistance gene $bla_{\text{TEM-10}}$, and the sulphonamide resistance gene *sul2* on a single 11439 bp read (figure s3).

Discussion

In this longitudinal study, we assessed carriage of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC) in dogs and determined whether detected ESBL-EC carriage is associated with distinct changes in microbiome and resistome composition. Detection of ESBL-EC carriage was highly prevalent in dogs, with 68% of the dogs found positive for ESBL-EC at least once over the course of six weeks. This high prevalence of ESBL-EC in dogs is in line with previous findings where 84% of the dogs carried ESBL-producing *Enterobacterales* over the course of six months (3). Furthermore, we observed persistent detection of carriage of ESBL-EC during six weeks of time in 16% of the dogs, which is slightly lower than described in previous longitudinal studies where 24% and 43% of dogs were found positive for ESBL-producing *Enterobacterales* (3, 7). In the current study, in a large percentage of dogs (44%) ESBL-EC carriage was detected intermittently during the study. This is in concordance with previous findings where 61% of the dogs switched between ESBL-EC carriage and, to a lesser extent, with others where 18% of the dogs switched between ESBL-EC carriage (3, 7).

Factors that explain intermittent detection of ESBL-EC carriage by dogs, *i.e.* fluctuation in the relative abundance of ESBL-EC over time, are not well understood. We hypothesized that the composition of the gut microbiome might be one of the factors explaining this. Therefore, we set out to study the gut microbiome and resistome composition using faeces samples from dogs in which ESBL-EC were detected or not using both 16S rRNA sequencing and ResCap, respectively. Within the selection of Dutch companion dogs, we were able to detect 244 different bacterial genera and 133 unique acquired antibiotic resistance genes (ARGs) in the dog gut microbiome. The most abundant bacterial genera in dogs included *Peptoclostridium*, *Blautia*, *Prevotella*, *Faecalibacterium* and *Bacteroides*, which was in line with gut microbiome diversity observed in dogs in previous studies (13-15). Comparing the gut microbiome of dogs in which ESBL-EC were detected to dogs where this was not the case at the time of sampling, revealed associations between detected ESBL-EC carriage and an increased

abundance of the bacterial genera *Clostridium sensu stricto 1*, *Enterococcus*, *Lactococcus* and the shared genera of *Escherichia-Shigella.* Multivariate analysis confirmed the observed associations of *Clostridium sensu stricto 1*, *Enterococcus* and *Lactococcus* with detected ESBL-EC carriage.

Long-read metagenomic sequencing of faecal samples of two dogs, using Oxford Nanopore Technology, suggest that these genera may include the opportunistic pathogens *Clostridium perfringens* and *Lactococcus garvieae*. *C. perfringens* is a potential toxin producer that is associated with dog gastrointestinal diseases (19). Previous studies have associated the increased abundance of *E. coli* and *Enterococcus* with dogs that had chronic signs of gastrointestinal disease and confirmed inflammatory changes, while *C. perfringens* is specifically associated with dogs diagnosed with haemorrhagic diarrhoea (20-23). The association of *Enterococcus* and ESBL-EC carriage is of particular relevance, since dogs, sharing living space with their owners with commonly very close physical contact, are suspected of playing a role in the zoonotic transmission of these potential pathogenic multidrug resistant bacteria (24, 25). *L. garvieae* is the only known pathogenic *Lactococcus* and a recognized human pathogen, although it is mainly found in dairy products and as a pathogen of fish (26, 27).

Detected ESBL-EC carriage was associated with decreased abundance of *Colidextribacter*, *Faecalibacterium*, *Fournierella*, *Holdemanella*, *Muribaculaceae*, *Negativibacillus*, *Peptococcus* and *Prevotella*. Species of *Faecalibacterium*, *Holdemanella* and *Prevotella* are considered commensals, or even symbionts (28-31). Similarly, *Muribaculaceae*, *Negativibacillus* and *Peptococcus* are associated with short-chain fatty acid (SCFA)-production and are also considered beneficial bacteria (32-34). Currently little is known about *Colidextribacter* and *Fournierella* (35). The only cultured species of *Fournierella* is *F. massiliensis*, which was shown to produce butyrate which has a beneficial role to the host (36).

These results show that detection of ESBL-EC carriage in dogs is associated with a specific microbiome signature with increased abundance of potential harmful bacteria and a decreased abundance of bacterial taxa associated with health.

To investigate whether a distinct microbiome signature also affects the composition of resistance genes we applied ResCap to investigate the gut resistome in dogs. We first compared the sensitivity of ResCap to detect ESBL genes to that of culture-based method, followed by conventional PCR screening. While culturing and PCR was able to detect ESBL genes in 19 out of 40 samples (48%), ResCap was able to detect ESBL genes in only 7 samples (18%). Most probably, the low abundance of these genes explains this discrepancy and is a limiting factor for detecting these genes by ResCap. On the other

hand, using ResCap we were able to detect bla_{CTX-M} resistance genes in two samples in which ESBL genes were not detected using the culture-based method followed by PCR screening. Explanations for this discrepancy might be that in these samples the bla_{CTX-M} gene is carried by bacteria that were not selected during the culture step, because they are for instance strict anaerobes or because bla_{CTX-M} genes were not expressed.

Resistome analyses revealed associations between the abundance of specific ARGs and ESBL-EC carriage detection. These ARGs encode resistance to trimethoprim (*dfrA*, *dhfR*), phenicol (*cmlA*, *floR*) and sulphonamides (*sul3*). Multivariate analysis confirmed the observed associations of *dhfR* with detected ESBL-EC carriage. The combination of trimethoprim and sulfamethoxazole is a widely used clinical and veterinary medicine to treat bacterial infections and co-resistance is commonly observed in ESBL-producing *Enterobacterales* (37). Specifically, the genes *sul1*, *sul2*, *sul3*, *dfrA* and *dhfr* are commonly located on plasmids and are found in close association with class 1 integrons or ISCR mobile genetic elements (38-40). While *floR* is also commonly observed in *Enterobacterales*, it specifically encodes resistance to florfenicol, which is a derivative of chloramphenicol and used almost exclusively for treatment of infections in aquaculture and livestock (41-44). In a similar way, *cmlA* encodes resistance to chloramphenicol which is associated with class 1 integrons and carried on plasmids in *Enterobacterales* (45, 46).

Metagenomic Nanopore sequencing reveals that the gut microbiome of dogS111 contains genes *dfrA1*, *sat2_gen*, *aadA1*, bla_{TEM-10}, *sul2*, that are located on a single genetic element. For dogS128 ARGs *floR*, *dfrA36*, *sul2* and the class 1 integron specific recombinase *intI1* are located on a single genetic element, which is in concordance with previous findings, and may result in the increased circulation of ARGs in the environment through co-selection (37, 47).

To conclude, our findings suggest that the gut microbiome composition of dogs is implicated in ESBL-EC carriage detection in dogs. It is yet unknown if these changes in the gut microbiome and resistome facilitate ESBL-EC colonization levels or are driven by ESBL-EC colonization levels. Previous findings indicate that diet has a greater impact on the gut microbiome composition than dog breed, age or weight and that consumption of raw meat increases the risk of ESBL-EC carriage, compared to a diet of dry food (3, 48). Raw meat diets were also found to increase abundances of *Lactobacillus* and *Clostridium* in the dog gut microbiome (13, 49). Data on dietary habits were unfortunately not available for the dogs included in this study.

Co-enrichment of antibiotic-resistant bacteria with pathogenic potential, such as ESBL-EC, *Clostridium, Enterococcus* and perhaps even *Lactococcus* in companion animals, as

we found in this study, highlights the importance to study the microbial and resistome composition in companion animals, as gut colonization of potential pathogens might be an indication of more profound changes in gut microbiome composition in which multiple antibiotic resistant potential pathogens are enriched. This may pose a risk for zoonotic transmission of antibiotic resistance bacteria and genes from companion animals to humans.

Materials and methods

Ethics statement

Animal sampling was performed in accordance with the guidelines of the Dutch Animals Act (stb-2011-345) and the Animal Welfare Body Utrecht. No additional license was required.

Household inclusion

In this study, faecal samples were collected from 57 dogs that were part of 34 households in the general Dutch population (figure 1). Four samples were collected longitudinally per participant, using two-week intervals for a total of six weeks. Samples were collected over a five-month period. For recruiting participants, a cohort described in a prior study was approached by e-mail (50). In the prior study these participants did not object to be approached for future research. No underlying health conditions were reported for the participating dogs at the same of sample collection.

Sample collection, storage and DNA extraction

Faecal samples were sent by the dog owner by regular mail, which is typically delivered within 24 to 48 hours. Upon arrival at the lab samples were used for selecting culture ESBL (described in the next section in details), while the remaining faecal material was stored at -80°C. One freeze-thaw cycle was introduced when dividing samples into aliquots of 0.2 ml. Aliquoted samples were thawed a second time for DNA extraction, using a modified protocol of the QIAamp fast DNA stool mini kit (Qiagen, Venlo, the Netherlands). In brief, 0.2 g faeces were added to 500ul 0.1mm zirconium beads (Lab Services) and 1 ml InhibitEx buffer (Qiagen) in a 2ml Sarstedt tube. Beat beating was performed two times at 3800 rpm for 2 minutes, using a Mini-beadbeater-24 (Biospec, Rijswijk, the Netherlands) and applying 2-minute ice cooling steps in between. Samples were subsequently incubated at 95°C for 7 minutes, followed by 1 minute centrifugation at 16.000 x g. The supernatant was removed and stored, while 1 ml InhibitEx buffer was added to the bead-beating tube with left over material. The bead-beating, incubation and centrifugation steps were repeated and the supernatant removed. Both supernatant fractions were treated with proteinase K and pooled by passing both fraction through

a single spin column, according to the fast DNA stool mini kit protocol (Qiagen). DNA elution was performed using 100ul Buffer EB (Qiagen) and DNA LoBind microcentrifuge Eppendorf tubes (VWR International, Amsterdam, the Netherlands). Total DNA was quantified by Picogreen assay (Thermo Fisher Scientific, Eindhoven, the Netherlands).

ESBL-EC characterization

Faecal samples were used to inoculate MacConkey agar plate supplemented with 1 mg/L cefotaxime (MacC+) and incubated overnight aerobically at 37°C, in order to select for third generation cephalosporin resistant bacteria. In addition, 0.5 g faecal material was suspended in 4.5 ml LB broth with 1mg/L cefotaxime (LB+), which was subsequently inoculated on another MacC+ plate after overnight incubation at 37°C. When growth was observed after direct inoculation on the MacC+ plate, five colonies per plate were selected for PCR screening for the presence of ESBL genes. If growth was only observed after selective enrichment in LB+ and subsequent inoculation on MacC+, one colony per plate was selected for PCR screening for the presence of ESBL genes (table s5). When ESBL genes were detected by PCR, the PCR products were sent for Sanger sequencing to determine which ESBL genes were present (BaseClear, Leiden, the Netherlands, table s6). Bacterial species were furthermore confirmed using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass-Spectrometry (MALDI-TOF MS). When both the presence of ESBL genes and the bacterial species were confirmed, a sample was considered to contain ESBL-producing *E. coli*, here referred as ESBL-EC.

16S rRNA gene sequencing

All samples were subjected to 16s rRNA gene sequencing to determine gut microbiome composition. The 16S rRNA gene hypervariable regions V3 and V4 were amplified (~430 bp) and sequenced with an Illumina MiSeq reagent Kit v3 (600-cycles) on a MiSeq system (Illumina, Eindhoven, the Netherlands), as was described previously (51, 52). Sequences were taxonomically assigned using QIIME™ 2, DADA2 and the SILVA database, applying a cut-off of 8000 reads (53-55).

ResCap sequencing and data processing

Forty samples were selected for in-depth resistome analysis using the ResCap targeted sequence capture panel consisting of probes targeting 7963 resistance genes, with the addition of probes against the following *mcr* genes (*mcr1.1*, *mcr1.2*, *mcr1.3*, *mcr1.4*, *mcr1.5*, *mcr1.6*, *mcr1.7*, *mcr1.8*, *mcr1.9*, *mcr1.10*, *mcr2.1*, *mcr2.2*, *mcr2.3*, *mcr3.1*, *mcr3.2.1*, *mcr3.2.2*, *mcr3.3.1*, *mcr3.3.2*, *mcr3.4.1*, *mcr3.4.2*, *mcr3.5.1*, *mcr3.5.2*, *mcr3.6*, *mcr4*, *mcr5*, *mcr6*, *mcr7* and *mcr8*) (Roche ID: OID41815) (56, 57). ResCap was performed according to the supplied protocol. In brief, 600-700 ng DNA was used for fragmentation using the KAPA HyperPlus Kit v4.17 (Roche, Woerden, The

Netherlands) to generate 400 bp fragments. End repair, A-tailing and adapter ligation were performed as described by the SeqCap EZ HyperCap User's Guide v2.3. Pools of 12 samples were used for hybridization and capture using an extended version of the ResCap probe collection as described in the original publication (56). Sample pools were sequenced on a NovaSeq 6000 using a S1 reagent Kit (300 cycles) (Illumina). ResCap-based targeted enriched Illumina reads were trimmed using Trim Galore version 0.6.4 with standard settings (58). KMA version 1.3.4 was used to align sequences to the MEGARes 2.0 database (59, 60). The MEGARes database combined previous described databases of antibiotic resistance (ARGs), metal and biocide resistance genes. For KMA, paired-end reads were used as input by using *-ipe*, together with the options: *-tmp*, *-1t1*, *-cge*, *-apm p*, *-ef*. The resulting list of detected genes and their abundance was trimmed by applying a cut-off of 90% identity (named query Identity) and of 80% coverage (named template coverage). The output value depth was used for subsequent analysis, which represents the amount of aligned base pairs, while correcting for gene length.

Nanopore sequencing and data processing

Samples of the dogs from household S111 and S128 were used for metagenomic Nanopore sequencing. R9.4.1 flow cells were used in combination with the ligation sequencing kit (SQK-LSK109) and native barcode expansion kit (EXP-NBD104, all Oxford Nanopore Technologies, Oxford, UK). Data acquisition and base-calling were performed by MinKNOW version 20.10.6 and demultiplexing by Guppy version 4.0.11.

Data analysis

Analysis of sequencing data was performed in R version 4.0 and the functions of the packages phyloseq and ggplot2 (61-63). The abundance of the top 10 gut microbiome taxa and resistance genes were plotted using aggregate top taxa and plotting functions of the microbiome package (64). Shannon index of diversity was calculated using the alpha diversity functions of the microbiome package and plotting functions of the microbiomeutilities package (65). Generalized linear mixed-effects models (GLME) with logit link function and binomial error distribution were used to test differences in alpha diversity between ESBL-EC groups, accounting for the cluster-longitudinal nature of the data, with the same dogs being sampled over time and some of them belonging to the same households. Aitchison distance PCA was applied using the transform function of the microbiome package and ordinate (RDA) and plot ordination functions of the phyloseq package. Correlations of sample dissimilarity and ESBL-EC groups were tested using PERMANOVA with the adonis function at 999 permutations of the vegan package (66). GLME analysis was also used to test for differentially abundant taxa (genus level) and resistance genes associated with ESBL-EC. Normalized 16S rRNA or ResCap data was converted to relative abundance and used for centered log-ratio (CLR) transformation. For the 16S rRNA data, a filtering cut-off of 10% prevalence and 0.01% abundance was

applied and the remaining targets were tested for collinearity using variance inflation factor (VIF) and assessed with GLME. For GLME analysis of the ResCap data, we applied these same filtering steps, but subsequently selected for acquired ARGs only. To control for multiple-hypothesis testing, the Benjamini–Hochberg method was used. Genera that were associated with ESBL-EC at univariate analysis were then entered in a multivariable GLME for ESBL-EC: a backward variable selection procedure was then applied to remove non-significant (p>0.1) targets. Nanopore metagenomic data analysis was performed using Kraken version 2.0.9 with the GTDB_r89_54k_kraken2 database and standard settings, for analysis of the gut microbiome (18). The relative abundance of the species withing a genus, was calculated as the read count per total number of reads. Similarly, ABRicate version 0.9.7 was applied with standard settings for analysis of the resistome, using the NCBI database extended with the class 1 integron specific recombinase *intI1* (47, 67).

Data availability

The 228 16S rRNA gene sequencing, 40 ResCap sequencing and 8 Nanopore sequencing files have been deposited in the European Nucleotide Archive repository under the study accession PRJEB50027. R scripts to reproduce the analysis reported in this study can be found at; https://gitlab.com/PB_Stege/microbiome_resistome

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Conflict of Interest

All authors declare no conflict of interests.

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Supplementary data

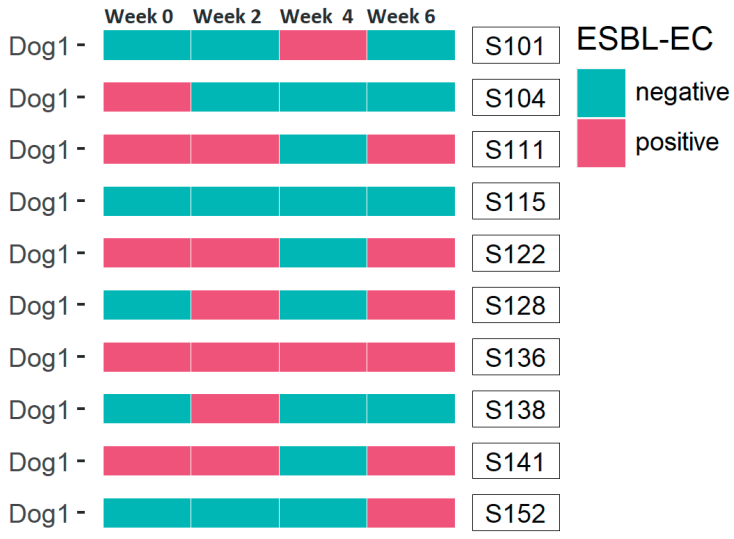

Figure s1 | Detected carriage of ESBL-producing *Escherichia coli* in dogs selected for ResCap analysis. Rows represent individual dogs and S-numbers indicate the households, while columns indicate time points with two-week intervals. ESBL-EC detection is indicated in blue when ESBL-EC were detected at a time point, or red when ESBL-EC were not detected.

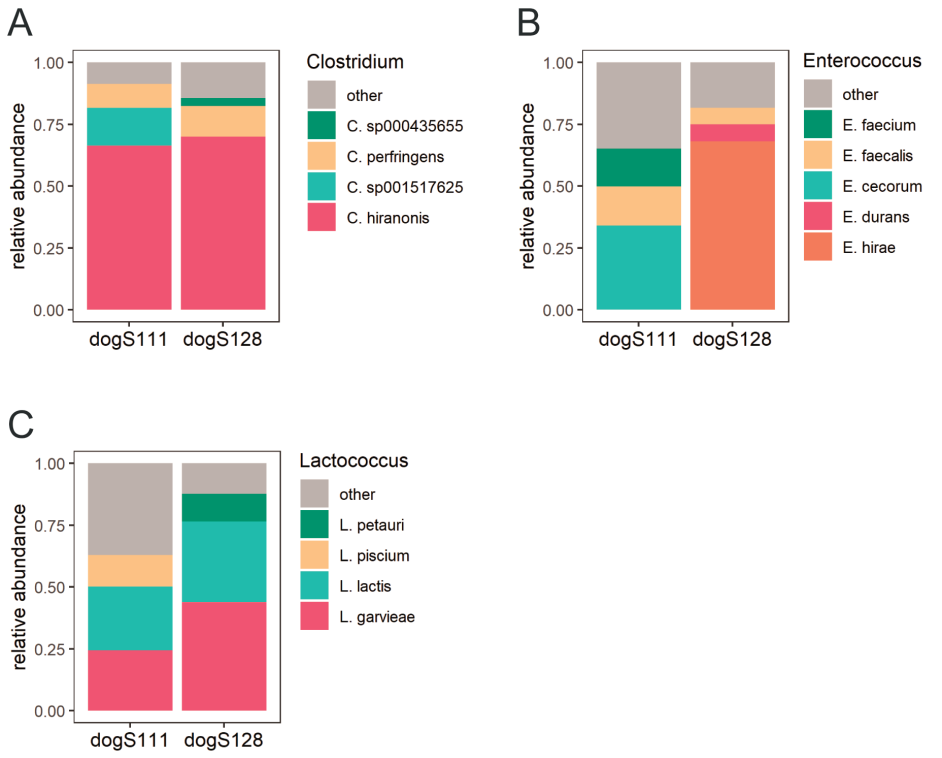

Figure s2 | Relative abundance within ESBL-EC associated genera. Nanopore metagenomic sequencing revealed the relative abundance of the three most abundant species per dog per sample, within the genera of (A) *Clostridium*, (B) *Enterococcus* and (C) *Lactococcus*.

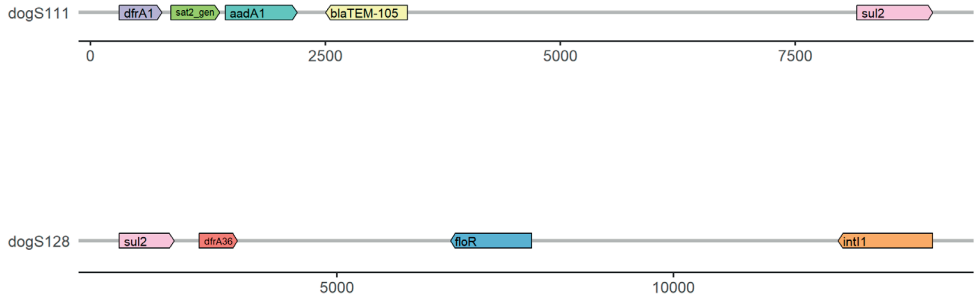

Figure s3 | Antibiotic resistance gene context. Nanopore metagenomic sequencing revealed that dogS111 contained ARGs *dfrA1*, *sat2_gen*, *aadA1* and $bla_{\text{TEM-10}}$ in close physical distance on a single sequencing read. DogS128 contained resistance genes *floR*, *dfrA36*, *sul2*, and the class 1 integron specific recombinase *intI1*, located on a single sequencing read.

Table s1 | Univariate and multivariate longitudinal gut microbiome analysis.

	median abundance (%) ESBL-EC		univariate		multivariate	
Genus	**positive**	**negative**	**estimate**	**p-adj**	**estimate**	**p-adj**
Clostridium_sensu_stricto_1	0.16	2.38	0.301	0.013	0.205	0.011
Colidextribacter	0.00	0.00	-0.513	0.054		
Enterococcus	0.00	0.49	0.312	0.002	0.178	0.022
Escherichia_Shigella	0.07	0.81	0.246	0.038		
Faecalibacterium	2.40	0.09	-0.246	0.044		
Fournierella	0.19	0.00	-0.291	0.052		
Holdemanella	0.18	0.00	-0.262	0.054		
Lactococcus	0.00	0.07	0.372	0.002	0.265	0.006
Muribaculaceae	0.00	0.00	-0.378	0.054		
Negativibacillus	0.05	0.00	-0.261	0.052		
Peptococcus	0.23	0.00	-0.216	0.054		
Prevotella	0.65	0.00	-0.218	0.044		

Table s2 | ResCap ESBL gene detection.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter3-table_s2_-_ResCap_ESBL_gene_detection.xlsx

Table s3 | Acquired resistance genes detected by ResCap.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter3-table_s3_-_Acquired_resistance_genes_detected_by_ResCap.xlsx

Table s4 | Univariate and multivariate longitudinal resistome analysis.

	median abundance (%)		univariate		multivariate	
	ESBL-EC					
Antibiotic resistance gene	**positive**	**negative**	**estimate**	**p-adj**	**estimate**	**p-adj**
cmlA	0.00	0.00	0.480	0.082		
dfrA	0.00	0.03	0.359	0.082		
dhfR	0.00	0.01	0.490	0.065	0.490	0.002
floR	0.00	0.05	0.287	0.082		
sul3	0.00	0.00	0.434	0.082		

Table s5 | Primer list for PCR mediated ESBL gene validation.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter3-table_s5_-primer_list_for_PCR_mediated_ESBL_gene_validation.xlsx

Table s6 | PCR mediated ESBL gene validation.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter3-table_s6_-_PCR_mediated_ESBL_gene_validation.xlsx

Part II

Implementation of novel techniques to study colonization dynamics

Chapter 4

CRISPR-Cas9-mediated genome editing in vancomycin-resistant *Enterococcus faecium*

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Abstract

The Gram-positive bacterium *Enterococcus faecium* is becoming increasingly prevalent as a cause of hospital-acquired, antibiotic-resistant infections. A fundamental part of research into *E. faecium* biology relies on the ability to generate targeted mutants but this process is currently labour-intensive and time-consuming, taking 4 to 5 weeks per mutant. In this report, we describe a method relying on the high recombination rates of *E. faecium* and the application of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas9 genome editing tool to more efficiently generate targeted mutants in the *E. faecium* chromosome. Using this tool and the multi-drug resistant clinical *E. faecium* strain E745, we generated a deletion mutant in the *lacL* gene, which encodes the large subunit of the *E. faecium* β-galactosidase. Blue/white screening using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) could be used to distinguish between the wild-type and *lacL* deletion mutant. We also inserted two copies of *gfp* into the intrinsic *E. faecium* macrolide resistance gene *msrC* to generate stable green fluorescent cells. We conclude that CRISPR-Cas9 can be used to generate targeted genome modifications in *E. faecium* in 3 weeks, with limited hands-on time. This method can potentially be implemented in other Gram-positive bacteria with high intrinsic recombination rates.

Introduction

Antibiotic resistance is currently recognised as a global threat to human health (Ferri et al. 2017). Enterococci are among the most problematic multi-drug resistant bacteria causing infections among hospitalised patients, contributing to 10000 - 25000 deaths per year in the USA alone (McKinnell et al. 2012). Clinically, the two most important enterococcal species are *Enterococcus faecalis* and *Enterococcus faecium*. While historically *E. faecalis* has been the most prominent enterococcal pathogen, since the 1990s *E. faecium* has rapidly emerged as a nosocomial pathogen of major importance. Infections caused by *E. faecium* are generally more difficult to treat as vancomycin resistance is more widespread in *E. faecium* than in *E. faecalis* (Gilmore, Lebreton and Schaik 2013; García Solache and Rice 2019). Until we understand the molecular underpinnings that contribute to the transfer of antibiotic-resistant genes and pathogenicity, we will be hampered in our ability to develop treatment strategies. To drive functional studies, efficient genome editing tools are essential, which are currently lacking. Current methods to generate targeted mutations in *E. faecium* mostly rely on allelic exchange between the chromosome and a temperature-sensitive vector which contains an antibiotic resistance cassette and sequences that flank the target site on the *E. faecium* genome (Maguin et al. 1996; Nallapareddy, Singh and Murray 2006; Zhang et al. 2012). The antibiotic cassette can be removed using the Cre- lox system, but a single lox site remains as a scar (Zhang et al. 2012). These protocols are time-consuming, taking upwards of 4 to 5 weeks. The process involves several days of sub-culturing and selection of colonies on media with different antibiotics, to screen for a double cross-over event and then removal of the resistance marker by Cre-lox. In addition, extensive screening by colony PCR is needed to eliminate false positives and retrieve the desired double cross-over mutant. The process to generate targeted mutants in *E. faecium* was improved by the use of counter-selection system against single cross-over mutants by the use of *pheS**, a mutated allele of the *E. faecalis* phenylalanyl tRNA synthetase α-subunit that confers susceptibility to p-chloro-phenylalanine in enterococci (Kristich, Chandler and Dunny 2007; Thurlow, Thomas and Hancock 2009; Somarajan et al. 2014; Bhardwaj, Ziegler and Palmer 2016).

To further expand the genetic toolbox for multi-drug resistant *E. faecium*, we explored the use of clustered regularly interspaced palindromic repeats (CRISPR) and its associated Cas9 protein to generate mutants in *E. faecium*. The Cas9 nuclease introduces double-strand breaks in DNA that is targeted by a CRISPR and, together with other CRISPR-associated proteins, serves as a defence against invading bacteriophages and plasmids in prokaryotes (Brouns et al. 2008; Marraffini and Sontheimer 2008). The combination of CRISPR and Cas9 has been successfully used for genome editing in eukaryotes where CRISPR-Cas9 drives the generation of mutants by inducing double-

strand DNA breaks, which are then repaired by non-homologous end-joining (NHEJ) (Cong et al. 2013). While some bacteria have NHEJ systems, there is no evidence for their presence in *Enterococcus* and other Lactobacillales and thus *E. faecium* can only escape the lethal effect of CRISPR-Cas9 targeting a chromosomal site by utilising homologous recombination (HR) (Bowater and Doherty 2006). One approach to use CRISPR-Cas to identify recombinant genotypes is to introduce a vector that contains DNA identical to the flanking sequence of the target region while the cell produces Cas9 and a CRISPR-array homologous to the target sequence. Most surviving cells will have undergone a HR event thereby escaping CRISPR-Cas-mediated killing (Jiang et al. 2013; Wang et al. 2015, 2018). Genome editing approaches using HR and CRISPR-Cas9 have been used for numerous bacterial species, including Gram-positive lactic acid bacteria (Mougiakos et al. 2016; Leenay et al. 2019). *Enterococcus faecium* has a high intrinsic recombination rate and readily integrates novel exogenous DNA in its genome (de Been et al. 2013), making it particularly suited for the implementation of CRISPR-Cas9 as a counterselection strategy during the generation of targeted mutants.

In this study, we aimed to develop a CRISPR-Cas9 based genome editing approach for *E. faecium*. We adapted a CRISPR-Cas9-based genome editing approach previously developed for the lactic acid bacterium Lactobacillus reuteri (Oh and Van Pijkeren 2014), relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange combined with CRISPR-Cas9 to counterselect against wild-type cells.

Results and discussion

Implementation of CRISPR-Cas9-mediated genome editing in *E. faecium*

We initially attempted to combine single-stranded DNA recombineering and CRISPR-Cas genome editing in *E. faecium*, as was previously demonstrated in the lactic acid bacterium *Lactobacillus reuteri* (Oh and Van Pijkeren 2014). We were, however, unsuccessful in generating mutants in *E. faecium* using this methodology. Either not enough oligonucleotides were transformed into the cells due to the inherent low transformation efficiency in *E. faecium*, or the activity of the single-stranded DNA binding protein RecT was too low to support incorporation of the oligonucleotide into the chromosome. We then decided to adapt the *L. reuteri* system by relying on the high intrinsic recombination rate of *E. faecium* for allelic exchange and by using CRISPR-Cas9 to counter select against wild-type cells. For this we used the vectors pVPL3004, which encodes Cas9 and pVPL3115, encoding the CRISPR array to which the protospacer target sequence can be added. To facilitate further adaptations needed for genomic modifications we transferred the CRISPR guide RNA section from pVPL3115 to the vector pWS3 to create pVDM1001. This plasmid has the benefit of

having a temperature-sensitive replicon for Gram-positive bacteria and can replicate in *E. coli* EC1000, facilitating further cloning procedures.

The *E. faecium* CRISPR-mediated genome engineering plasmid thus relies on pVPL3004 and the novel vector pVDM1001 being present in the strain of interest (Fig. 1A). The general workflow is depicted in Fig. 1B. The plasmid pVPL3004 was first transformed into *E. faecium* E745 to allow for CRISPR-based genome modifications. We then exchanged the control protospacer in pVDM1001 for one that targets the region on the *E. faecium* chromosome that we intended to manipulate. Third, we added a HR template that contained the desired mutation. Lastly, the resulting pVDM1001-derived plasmid was transformed into E745 containing pVPL3004. Transformants were selected on BHI agar plates containing both erythromycin and spectinomycin, and were subjected to PCR to determine the recombinant genotype. As a proof-of-principle in this study, we generated a deletion mutant in *lacL* (locus tag: EfmE745 01561), the gene encoding the large sub-unit of the *E. faecium* β-galactosidase and we integrated *gfp* in the chromosomal *msrC* gene (Singh, Malathum and Murray 2001)(locus tag: EfmE745 02638) to generate a fluorescently tagged *E. faecium* strain.

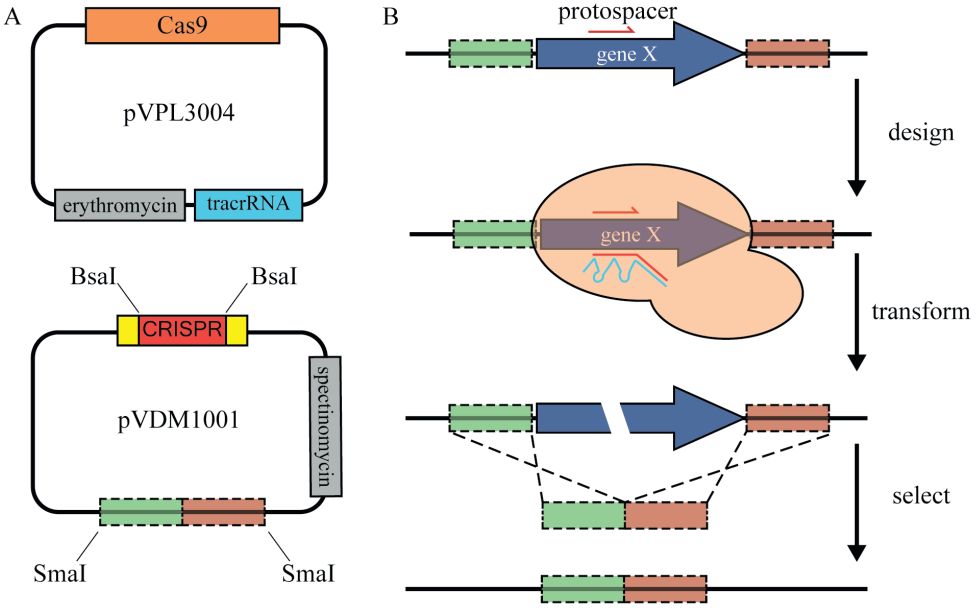

Figure 1 | Schematic overview of the CRISRP-Cas9-mediated genome editing. This system consists of two plasmids (panel A), pVPL3004; which contains *cas9* from *S. pyogenes*, tracrRNA and an erythromycin selection marker, and pVDM1001; which contains a CRISPR targeting the desired region, the template DNA which carries the desired mutation and a spectinomycin selection marker. The general workflow for generating mutants is shown in panel B, and includes the design of the CRISPR- protospacer and repair template which are incorporated in pVDM1001. The second step is the transformation of the plasmids pVPL3004 and the relevant pVDM10001 derivative into *E. faecium*, followed by direct selection of the mutant.

Generation of a deletion mutant and a chromosomal integration mutant

To delete *lacL* we adapted pVDM1001 to contain a CRISPR targeting the wild type locus of *lacL* (pVDM-x*lacL*). The vector pVDM-d*lacL* contained, in addition to the CRISPR targeting *lacL*, a HR template consisting of two regions flanking *lacL*, which allowed the generation of a targeted deletion mutant. To insert *gfp* in the chromosome, we created a HR template containing flanking regions of *msrC* and two copies of *gfp* in tandem as a transcriptional fusion under control of the constitutively expressed Pbac promotor. We cloned the *gfp* HR template and a specific CRISPR targeting *msrC* into pVDM1001 to create pVDM-*msrC*::*gfp*.

In a representative experiment to generate the *lacL* deletion mutant, we transformed E745 + pVPL3004 with various constructs to quantify the emergence of spontaneously resistant or CRISPR escape mutants and the selective efficiency of the CRISPR. We transformed *E. faecium* E745 with dH_2O (background), pVDM1001 (empty vector), pVDM-*xlacL* (carrying a CRISPR that targets *lacL*) and pVDM-*dlacL* (carrying both the *lacL*-targeting CRISPR and the HR template for the generation of the *lacL* deletion mutant). This resulted in 70, 250, 68 and 80 colonies, respectively, after selection on BHI agar plates containing erythromycin and spectinomycin to select for both pVPL3004 and pVDM1001 and its derivatives. The relatively high background in the water control revealed the appearance of spontaneously spectinomycin-resistant colonies. Our data also indicated that we could successfully transform pVDM1001, which lacks an *E. faecium* CRISPR-array or HR template, into *E. faecium*. The addition of a CRISPR that targets the *lacL* gene in pVDM-*xlacL* reduced colony numbers down to background levels (68 colonies versus 70 in the water control), suggesting that CRISPR-Cas9 generated lethal double-strand DNA breaks in the *E. faecium* chromosome. Transformation of pVDM-*dlacL* resulted in a slight increase in colony numbers (80 colonies), potentially indicating successful integration of the HR template. This was confirmed by PCR (Fig. 2A) and subsequent Sanger sequencing as we found that approximately 15% of screened colonies were *lacL* deletion mutants. We obtained comparable results in our attempt to integrate *gfp* in the *msrC* gene, with a background of spontaneously spectinomycin-resistant mutants in the control experiments but a higher number of transformants upon electroporation with pVDM-*msrC*::*gfp* (data not shown). Our overall success rate in generating mutants was considerable higher in comparison to the homologous recombination-based technique we previously developed (Zhang et al. 2012), in which we routinely have to screen 100 or 200 colonies, after several days or even weeks of sub-culturing, before we can isolate the desired mutant that had undergone a double cross-over event. Once we confirmed that we had successfully generated the *lacL* deletion mutant and the *msrC*::*gfp* insertion mutant, the CRISPR-related plasmids were cured by sub-culturing in BHI broth without antibiotics for three days, or between 20 and 25 generations. Between 50 and 100 colonies isolated from this culture were then

transferred to three different BHI agar plates, i.e. BHI agar without antibiotics, BHI agar with spectinomycin and BHI agar with erythromycin to isolate colonies that had cleared both pVDL3004 and the pVDM1001-derivative. Two representative examples of experiments in which we cured the pVPL3004 and the pVDM1001-derivative are shown in Fig. 3. Curing ratios for pVPL3004 were typically around 60%–90% while pVDM1001-derived vectors was more difficult to cure as 1%–5% of colonies had lost the vector. Typically, we obtained 3 to 5 colonies in which both plasmids had cleared per 100 colonies.

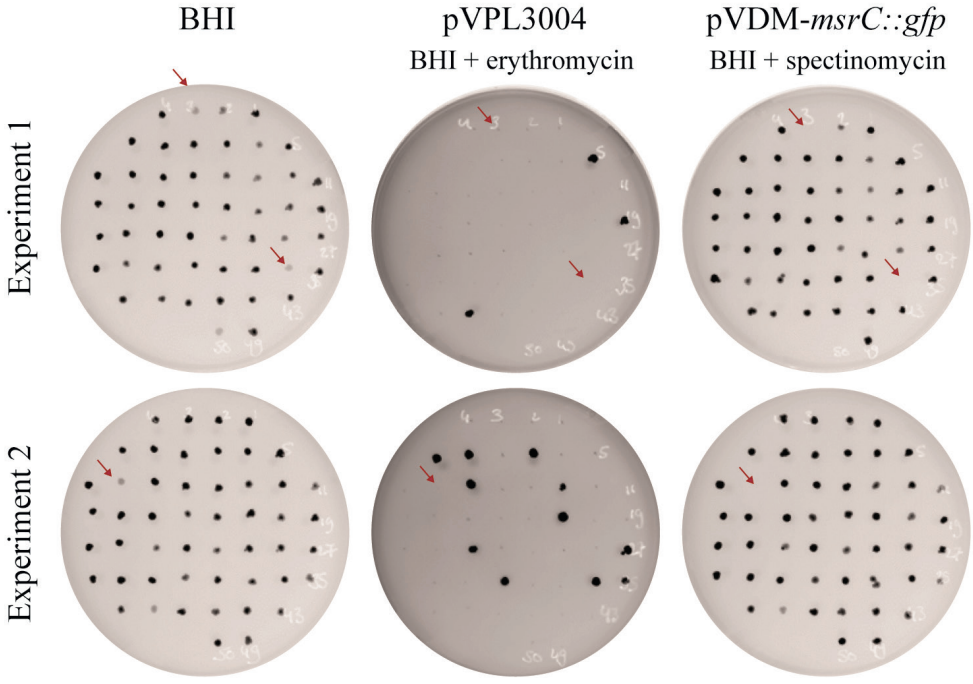

Figure 2 | Clearing efficiency of pVPL3004 and pVDM-*msrC*::*gfp*. After three days of sub-culturing to clear the plasmids, 50 colonies per mutant were transferred to BHI, BHI + 50 µg/ml erythromycin and BHI 200 µg/ml spectinomycin to screen for clones that have lost both plasmids (indicated by the red arrows). The overall clearance of pVPL3004 is 80%–90% and of pVDM-*msrC*::*gfp* is 2%–5%, resulting in at least one colony that has lost both plasmids. The results show results of two independent experiments to clear pVPL3004 and pVDM-*msrC*::*gfp* from the insertion mutant. Colonies were visualized by the ImageQuant LAS4000 imager through their production of GFP. Note that the fluorescent signal is lower in the *gfp* integration mutants than in the colonies where *gfp* is still present on a multi-copy plasmid.

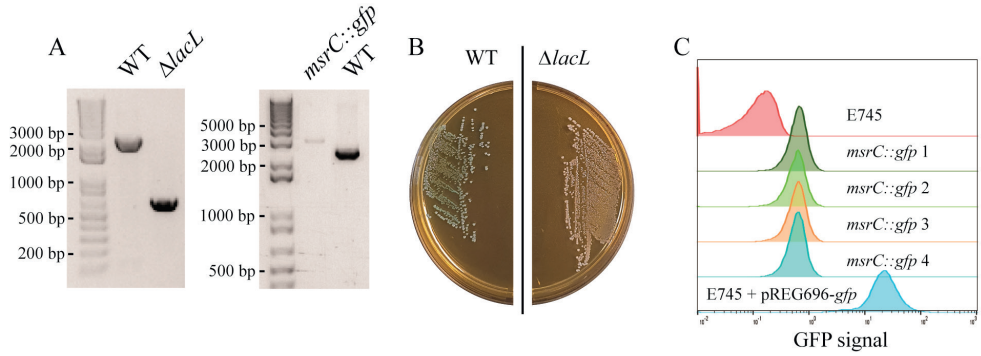

Figure 3 | Generation and phenotypes of the *dlacL* and *msrC::gfp* mutants. (A), Confirmation of *lacL* deletion and *gfp* insertion into *msrC* via PCR. Deletion of *lacL* results in an 1800 bp reduction in size of the PCR product from 2.5 kbp to 0.7 kbp, while insertion of the *gfp* construct into the *msrC* site results in a shift from 2.8 kbp to 3.2 kbp. (B), Growth of wild-type E745 and *dlacL* on BHI with 20 μg/ml X-gal. (C), Flow cytometric analysis of GFP fluorescence levels, from top to bottom, wild-type E745, four different *msrC*::*gfp* clones and, as a positive control, E745 containing pREG696-*gfp*.

Phenotypic characterization of E745 *dlacL* and E745 *msrC::gfp*

Wild-type (WT) E745 and E745 *dlacL*, which were cleared of pVDL3004 and pVDM-*dlacL* as outlined above, were grown on BHI supplemented with the chromogenic substrate X-gal to confirm that the genomic alteration affected β-galactosidase activity. While WT colonies were light blue upon growth on medium containing X-gal, the E745::*dlacL* colonies were creamy white (Fig. 2B), indicating that they could no longer convert X-gal due to the lack of an active β-galactosidase. We determined production of *GFP* by flow cytometry (Fig. 2C) and we found that the GFP signal is higher in E745 *msrC*::*gfp* compared to WT, but considerably lower than the strain in which *gfp* is carried on a plasmid. This most likely reflects differences in copy number of the chromosomally integrated *gfp* construct versus *gfp* carried on the multi-copy pREG696 plasmid.

Conclusions

In this proof-of-principle study we applied CRISPR-Cas9 as a counter-selection strategy to aid in the generation of targeted modifications in the chromosome of a clinical strain of *E. faecium*. Our approach for genome editing in *E. faecium* does not require specialized media and does not leave a scar in the chromosome. Mutants could be efficiently identified by PCR and the plasmids used to generate the mutants were readily cured. In comparison with our previous protocol (Zhang et al. 2012), processing time was reduced by up to 2 weeks and the total number of colonies that need to be screened is reduced by approximately 4-fold. It is important to note that the use of CRISPR-Cas9 allowed us to generate deletion mutants but also to insert genes into

the genome, which can be useful for a number of applications. The stable insertion of fluorescent or bioluminescent tags into the genome can be of particular use during in vivo experiments, e.g. to track colonization and infection by *E. faecium*. We note that the CRISPR/Cas9 system described here can be improved further, e.g. by changing the selection markers to reduce the number of spontaneously resistant colonies or by the addition of phenotypic markers (e.g. genes encoding for bioluminescent or fluorescent proteins) that can facilitate screening for the loss of the plasmid. For unknown reasons, the vector is also not always lost during growth at 37 °C, and this could be another target for further improvement. For any in-depth phenotypic characterization of any mutants generated with this method, or any other method involving genome manipulation, we recommend the use of whole-genome sequencing to rule out the introduction of non-target mutations. We also stress the importance of complementation of mutations upon the generation of mutants. Native CRISPR systems are relatively rare in multi-drug resistant clinical *E. faecium* strains (Palmer and Gilmore 2010; Lebreton et al. 2013) and there is therefore little risk of interference with the system we implemented here. Even though *E. faecium* is broadly recognized as an important multi-drug resistant nosocomial pathogen, there is still a limited mechanistic understanding of its basic biology and the traits that contribute to its transition from gut commensal to opportunistic pathogen. Efficient genome editing tools for *E. faecium* are essential to mechanistically characterise its of resistance to antimicrobials and disinfectants and other adaptations that have contributed to *E. faecium* becoming a globally important nosocomial pathogen. The CRISPR-Cas9-based approach described here improves the current genetic toolbox for *E. faecium* and we anticipate that it will accelerate research into this species. We note that the approach we developed here for *E. faecium* might also be successfully implemented in other enterococci and low-GC Gram-positive bacteria with high recombination rates, including several species in the genera *Lactobacillus*, *Streptococcus* and *Staphylococcus* (González-Torres et al. 2019).

Methods

Bacterial strains, plasmids, growth conditions and oligonucleotides

The vancomycin-resistant *E. faecium* strain E745 (Zhang et al. 2017) was used throughout this study. This strain was isolated from a rectal swab of a hospitalized patient, during routine surveillance of a VRE outbreak in a Dutch hospital. Unless otherwise mentioned, *E. faecium* was grown in brain heart infusion broth (BHI; Oxoid) at 37 °C. The *E. coli* strain EC1000 (Leen-houts et al. 1996) was grown in Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm. *Lactobacillus lactis* MG1363 was grown in M17 broth supplemented with 0.5% w/v lactose. When required, antibiotics were used at the following concentrations: erythromycin 50 μg ml^{-1} for *E. faecium* and 5 μg

ml^{-1} for *L. lactis* and spectinomycin 200 μg ml^{-1} for *E. faecium*, 100 μg ml^{-1} for *E. coli*, and tetracycline 10 μg ml^{-1} for L. lactis. Where indicated, plates were supplemented with 20 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The vectors pREG696 (Grady and Hayes 2003), pWS3 (Zhang et al. 2011) and pET-3α (Novagen) were obtained from our laboratory's culture collection. pREG696-*gfp* was derived from pREG696 by inserting the *gfp* gene under the control of the promoter of the *bacA* gene (Pbac) of *E. faecalis* (Heikens, Bonten and Willems 2007) in the NotI and XhoI restriction sites of pREG696 (J. Top, personal communication). Plasmids pVPL3004 and pVPL3115 were described in previous work (Oh and van Pijkeren 2014). The sequences of the oligonucleotides used in this study are listed in Table 1.

Isolation and transformation of plasmids

Plasmid isolation from *E. coli* was performed using the GeneJET plasmid miniprep kit (Thermo Fischer Scientific, Bleiswijk, the Netherlands) according to the manufacturer's instructions. Isolation of plasmids from *L. lactis* was as described previously (O'Sullivan and Klaenhammer 1993) with slight modifications. In short, 5 ml overnight cultures were centrifuged for 10 min at 3000 g. The cell pellet was resuspended in 250 μl THMS-buffer (30 mM Tris-HCL pH 8, 25% sucrose, 3 mM MgCl2) supplemented with 2 mg ml^{-1} lysozyme. The cell suspension was incubated for 10 min at 37 °C after which 500 μl 1% SDS in 0.2 M NaOH was added. The tubes were mixed gently and incubated on ice for 5 min. About 375 μl ice-cold 3 M potassium acetate pH 5.5 was added and mixed by inversion, followed by incubation on ice for 5 min. Cell debris was pelleted via centrifugation at 20000 g for 5 min, after which the supernatant was transferred to a new tube and an equal amount of isopropanol was added. After a 10-min incubation at room temperature the tubes were centrifuged at 20000 g for 10 min to precipitate the DNA. The pellet was washed with 70% ethanol, air dried and dissolved in sterile dH_2O. Transformation of plasmids into *E. faecium* E745 was performed as previously described (Zhang et al. 2012), typically resulting in 500 - 1000 transformants/μg DNA.

Construction of the pVDM1001 CRISPR delivery vector and generation of *lacL*-deletion and *gfp*-insertion mutants

We first aimed to construct a vector that could be used for genome editing in *E. faecium* E745. This vector, termed pVDM1001, was constructed by cloning a 0.7-kbp fragment, which contains the CRISPR sequences from pVPL3115 in the XhoI and EcoRI sites of pWS3. The fragment was amplified from pVPL3115 using the primers oVDM1001–oVDM1002. The pVDM1001 vector was then implemented for the generation of a *lacL* deletion and *gfp* insertion mutant by modifying the CRISPR sequence via digestion with BsaI and annealing two oligos, oVDM1022-oVDM1023 and oVDM1024-oVDM1025, which contain a protospacer targeting *lacL* or *msrC*, respectively. This created pVDM-*xlacL* and pVDM-*xmsrC*. CRISPRs were designed by identifying 30 bp

Table 1 | List of oligonucleotides used in this study.

Name	Sequence 5 - 3 (restriction sites are underlined)
oVDM1001	AAAACTCGAGCCACTCACCATGGGTACTGCAG
oVDM1002	AAAAGAATTCAACGTTGGCGATTCGTTGGCGATTGA
oVDM1003	/5Phos/GGCGAGTCCTTTTGAAGAAAATATTGCC
oVDM1004	/5Phos/AGCCATTCTTTTCCGTTTTTATTGAGCG
oVDM1005	TCATTGTCGCAACAGATAGC
oVDM1006	GGAACATCTGTGGTATGGCG
oVDM1007	GGCCGAATTGATGACAGTTG
oVDM1008	CTCTCCAGCGATTTGGTAG
oVDM1009	GTAGGCAATCTGTACCACTC
oVDM1011	TGCGTCCTTTGATCCGTTTC
oVDM1012	CACGATGGTACCTGCGTCCTTTGATCCGTTTC
oVDM1013	CATGATGGGCCCCATGTAAAACAACAATTATCG
oVDM1014	CATGATACTAGTATCCGCAAACAAGGAGAAGG
oVDM1015	CTAGATGCGGCCGCGTAGGCAATCTGTACCACTC
oVDM1016	CATGATGAATTCAGGAGGATTAACATATGAGCAAAGGAGAAG
oVDM1018	CATGATGCATGCATGAGCAAAGGAGAAG
oVDM1020	CATGATGGGCCCGCTTGCATCAAAATAAAC
oVDM1021	CACGATGAATTCGTAGAAAATATTTTTGAAATGCATTTC
oVDM1022	AAACGATCTTCAGAGATGTCTTCTTAGTTGCTCGG
oVDM1023	AAAACCGAGCAACTAAGAAGACATCTCTGAAGATC
oVDM1024	AAACTTCCGCTCTGAAGTTTCTTCCAGTCTTAACG
oVDM1025	AAAACGTTAAGACTGGAAGAAACTTCAGAGCGGAA
oVDM1026	CACTATGCATGCTTAGTGGTGGTGGTGGTGGTGGGATC
oVDM1027	CATGATCCCGGGTTAGTGGTGGTGGTGGTGGTGGGATC
oVDM1028	CTAGATCCCGGGGCTGAGCAATAACTAGCATAAC
oVDM1029	CACGATACTAGTCAAAAAACCCCTCAAGACC
oVDM1052	/5Phos/TGCGTCCTTTGATCCGTTTC
oVDM1053	/5Phos/GTAGGCAATCTGTACCACTC
oVDM1054	GGGCGGTGATCACTGATGAATATA
oVDM1055	ACCAATAATTCCTCAGTACCATCCAT
oVDM1056	ATGACCAATTTGATTAACGG
oVDM1057	CTAATTGAGAGAAGTTTCTATA

sequences, that were directly followed by the protospacer adjacent motif (PAM) NGG (Jiang and Doudna 2017). The CRISPR was only used if the last 6 bp, corresponding to the seed sequence, which is crucially important for target site recognition (Jiang and Doudna 2017), did not align to another site on the *E. faecium* E745 genome with an immediately adjacent PAM site. Finally, additional nucleotides were added to create the necessary overhang for ligation into the BsaI site in pVDM1001. The nucleotide sequence of pVDM1001 has been made available on NCBI Genbank with accession number MN580666. To create a *lacL* deletion mutant, a DNA template consisting of a 365 bp upstream region of *lacL* fused together with a 225 bp downstream region of *lacL* (Table S1) was ordered from Integrated DNA Technologies (Leuven, Belgium) and amplified using oVDM1003–oVDM1004. The amplified template was cloned into pVDM-*xlacL* after digestion with SmaI and a blunt end ligation creating pVDM-*dlacL*. To create a *gfp* knock-in construct we amplified 773 bp upstream region of *msrC* and a 507 bp fragment overlapping with the 3' region of *msrC* using primers oVDM1012–oVDM1013 and oVDM1014–oVDM1015, respectively. Each fragment was separately cloned into pWS3 using KpnI-ApaI for the upstream fragment and SmaI-NotI for the downstream fragment, creating pWS3-*msrCup* and pWS3-*msrCdwn*, respectively. Downstream of the *msrCup* fragment a Pbac promotor was inserted. The promotor site was amplified from pREG696-*gfp* using primers oVDM1020–oVDM1021 and inserted after ApaI-EcoRI digestion creating pWS3-*msrCup*-Pbac. To pWS3-*msrCdwn* a T7 terminator was added which was amplified from pET3α using primers oVDM1028-oVDM1029 and digested with SmaI-SpeI to create pWS3-T7-*msrCdwn*. pWS3-*msrCup*-Pbac was then digested with KpnI-EcoRI and the *msrCup*-Pbac fragment was transferred to pWS3-*msrCdwn*-T7 to create pWS3-*msrC*-Pbac-T7. To compensate for the low copy number of the *gfp* integration in the chromosome, we amplified two copies of *gfp* from pREG696-*gfp* (laboratory collection) using primers with different restriction sites, oVDM1016–oVDM1026 (EcoRI-SphI) and oVDM1018–oVDM1027 (SphI-SmaI), and consequently ligated together after digestion with SphI. This construct with two *gfp* genes in tandem was inserted into pWS3-*msrC*-Pbac-T7 via EcoRI-SmaI digestion creating the complete *msrC*::*gfp* template. This template was amplified using oVDM1052-oVDM1053 and transferred to pVDM1001 by digestion with SmaI creating pVDM-*msrC*::*gfp*. To perform the chromosomal modifications, we first transformed E745 with pVPL3004, with selection for transformants by plating on BHI with 50 μg ml^{-1} erythromycin and 24 h incubation at 37 °C. Presence of pVPL3004 in E745 was confirmed via PCR using primers oVDM1005-oVDM1006. A colony positive for pVPL3004 was grown in the presence of 50 μg ml^{-1} erythromycin and made competent to receive pVDM1001 or one its derivates described above. After transformation with these vectors the transformants were selected on BHI agar with 200 μg ml^{-1} spectinomycin and 70 μg ml^{-1} erythromycin and incubated 48–72 h at 30 °C. Successful deletion of *lacL* was confirmed by PCR with primers oVDM1007-

oVDM1008. Insertion of *gfp* was confirmed by PCR with primers oVDM1009-oVDM1011.

Curing of CRISPR and Cas9 plasmids

A colony that was positive for the desired mutation was transferred to 200 ml BHI without antibiotics and incubated overnight at 37 °C at 250 rpm after which 200 µl was transferred to 200 ml pre-warmed BHI and incubated overnight at 37 °C. This process was repeated a third time after which a 100 µl sample was taken and diluted 1000 times of which 25 µl was transferred and spread on a BHI agar plate. After 24 h incubation at 37 °C, 50 colonies were transferred to BHI agar, BHI agar with 200 µg ml^{-1} spectinomycin or BHI agar with 50 µg ml^{-1} erythromycin. After incubation overnight at 37 °C the plates were examined for colonies that were susceptible to both spectinomycin and erythromycin. Curing of the Cas9 delivery vector pVPL3004 and the CRISPR containing vectors derived from pVDM1001 was confirmed via colony PCR using the primer sets oVDM1054-oVDM1055 and oVDM1056-oVDM1057, respectively.

Flow cytometric analysis of GFP fluorescence in E745

To confirm the phenotype of the *gfp* integration mutant, cultures of E745, E745::*msrC*::*gfp*, and E745 + pREG696-*gfp* in 3 ml BHI, supplemented with 250 µg ml^{-1} spectinomycin if required, were started and incubated overnight at 37 °C. The fluorescence of the cultures was then determined by flowcytometric analysis after adjusting the cultures to an OD600 of 0.2. These were then diluted 25-fold in a 2-ml volume of PBS of which 200 µl was transferred to a round bottom 96-well plate, which was placed into a MACSQuant (Miltenyi Biotech) machine. Flow cytometric analysis was performed by measuring fluorescence at 488 nm excitation and 525 nm emission at 35.000 events in total. Bacteria were gated on single cells based on forward and side scatter. Data was further processed in FlowJo (FlowJo LLC).

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Conflicts of interest.

None declared.

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Supplementary data

Table s1 | Sequence of the repair template used for the deletion of *lacL*.

lacL repair template
GAGTCCTTTTGAAGAAAATATTGCCGCATGGATGTTTGTGTCTCC- TAAACAAGATGAAGCTATTGTGTTTTTAGGGAGGATACTAGCTTCAGCT- CAACCAGCGTTCCATGAAGTATATCTGATGGGGTTAGATGATGAGG- CACTTTATCAGGAACAGACCTCGAAGCGGATATTTTCGGGGGCCGAATT- GATGACAGTTGGACTTTACTTCCCCGATTTTCAAGGTGATTTCCAAACA- GAACTGCTTCATTTCAAAAAGTTATGAGAGAGAAGGAAAAAAGTATGAAAG- CAAATATAATGATCCAATCACAGGCAGAGAAGTGATGCGCTATGGCGGTG- ACTTTGACGATAAACCAAGTGACTATGAATTCTCAGGGAATGGGATCGTT- TTTGCAGATGGACAAGAAAAACCCGCCATGCAGGAGGTAAGATATTATTAT- GAAAAATACAGTAAATAAAAGTCATATGGATACGGAAAAAGTTGCAATCGTCT- TCGGCGACTGTACATTAGGTGTCAAATCGGGGAATACGCATTATATTTTT- TCTTATACAAGAGGCGGACTGGAATCGCTCAATAAAAACGGAAAAGAATGGC- TA

Chapter 5

Large-scale differential gene expression in vancomycin-resistant *Enterococcus faecium* upon interaction with human-derived colonic epithelium

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Abstract

Vancomycin resistant *Enterococcus faecium* is an opportunistic pathogen that is able to colonize the intestines of hospitalized patients. This initial colonization is an important step in the downstream pathogenesis, which includes outgrowth of the intestinal microbiota and potential subsequent infection of the host. The impact of intestinal overgrowth on host-enterococcal interactions is not well understood. We therefore applied a dual RNA sequencing approach in order to unravel the transcriptional dynamics of both *E. faecium* and human derived colonic epithelium upon co-culturing. Cultures of colonic epithelium and co-cultures of colonic epithelium and hospital-associated vancomycin resistant (vanA-type) *E. faecium* were first visualized by confocal microscopy, which revealed that *E. faecium* resided on top of the colonic epithelium rather than being positioned between colonic cells. RNA sequencing revealed that exposure to the colonic epithelium resulted in the upregulation of *E. faecium* genes implicated in pili expression, and a downregulation of vancomycin resistance genes. Furthermore, pathway analysis revealed an overall switch in metabolism to amino acid scavenging and reduction. The host response to *E. faecium* colonization included changes in expression of genes involved in inflammation and amino acid degradation, but large changes in pathway expression could not be detected. In summary, our study demonstrates that co-culturing of *E. faecium* with human colonic epithelium initiates an elaborate gene response in both *E. faecium* and host cells which enhances our insight in host-*E. faecium* interactions.

Introduction

Vancomycin resistant *Enterococcus faecium* (VRE) emerged as an important opportunistic pathogens and is recognized by the WHO as a global threat (1). In hospital settings, VRE are able to colonize the intestines of patients, followed by rapid outgrowth, which puts patients at risk of self-contamination during common hospital procedures, and subsequent bacteraemia (2-6). While enterococci are minority species in a healthy microbiota, accounting for < 0.1% of intestinal microbiota, these numbers can significantly increase in hospitalized patients where *E. faecium* can even represent the majority bacterial species in the intestines (7). Extensive molecular epidemiological and comparative genomic analyses revealed that *E. faecium* strains from hospitalized patients are part of a distinct phylogenetic clade that was initially designated clonal complex-17 and later renamed clade A1 (8-10). *E. faecium* genes unique to clade A1 include putative virulence genes, genes encoding antibiotic transport, intestinal colonization factors and genes implicated in carbohydrate metabolism particularly indicating a shift to the utilization of amino sugars, like those that occur on cell surfaces and in mucin (8). Plasmidome analyses indicated that the predicted plasmidome size of isolates from hospitalized patients was considerably larger than that from other isolation sources, such as non-hospitalized persons, dogs, pigs and poultry (9). Plasmid encoded genes specific for isolates from hospitalized patients include a locus of three genes putatively encoding an ABC transport system with similarity to lipoprotein/bacteriocin/macrolide export systems, a bacteriocin gene with homology to *bacA*, and a complete phosphotransferase system putatively involved in carbohydrate uptake (11). The acquisition and enrichment of specific carbohydrate uptake systems in clade A1 strains may provide these isolates a broader and or novel metabolic repertoire allowing clade A1 *E. faecium* to colonize the dysbiotic intestinal microbiota of hospitalized patients. In fact, disruption of the intestinal microbial community in hospitalized patients is thought to be one of the key factors contributing to the outgrowth of *E. faecium* in these patients (2, 12). Antibiotic-driven dysbiosis in the mammalian host does not only lead to *Enterococcus* outgrowth it also results in changes in the architecture of the intestinal epithelial cell lining. In an intestinal colonization model in mice, antibiotic treatment allowing enterococcal outgrowth resulted in a reduction of the mucus-associated intestinal microbiota layer, colon wall, and Muc-2 mucus layer, as well as deformation of E-cadherin adherens junctions between colonic cells and entrapment of *E. faecium* in an extracellular matrix consisting of secretory IgA (sIgA), polymeric immunoglobulin receptor (pIgR), and epithelial cadherin (E-cadherin) proteins (13).

Together, these findings suggest that particular genes acquired by hospital-associated *E. faecium* strains facilitate intestinal colonization in hospitalized patients, which coincides with significant changes in the intestinal architecture. However, detailed mechanistic

insights on *E. faecium* host interaction in the human intestines is still largely lacking. Recently, human-derived colonic organoids have been used as an *ex-vivo* model to study human specific bacteria-host interactions. Differentiated colonic organoids, grown as monolayers of colonic epithelium, express several cell types, including colonocytes, Lgr5+ stem cells, mucus-producing goblet cells and important cell structures like occludin and E-cadherin (14-16). In this study we applied a dual RNA sequencing approach to unravel *E. faecium*-host transcriptional responses during experimental *in vitro* colonization of human-derived colonic epithelium. RNA sequencing data revealed that *E. faecium* responds to colonic epithelium by upregulation of genes involved in pili expression and a downregulation of vancomycin resistance genes. Pathway analysis furthermore revealed an overall switch in metabolism to amino acid scavenging and reduction. The host response to *E. faecium* colonization include changes in expression of genes involved in inflammation and amino acid degradation, but large changes in pathway expression could not be detected. These findings highlight important transcriptional changes of *E. faecium* that may play an important role during intestinal colonization which enhances our insight in host-*E. faecium* interactions.

Results

Establishing *E. faecium*-human colonic epithelium co-cultures

To study *E. faecium*-host interaction we first cultured human derived colonic differentiated epithelium. Using confocal live imaging we visualized both the colonic epithelium and a mucus layer (figure 1a). We then continued to co-culture the vanA-type vancomycin-resistant E. *faecium* strain E8202 for 24 hours with colonic epithelium at a multiplicity of infection (MOI) of 6. *E. faecium* strain E8202 was sequenced and fully assembled previously recovered from a hospitalized patient in 2015 that belongs to clade A1 (ST117) and contains 6 plasmids (table s1)(9). We first established that this *E. faecium* strain was able to grow in both the cell culture medium (i.e., control condition) as well as in the co-culture with colonic epithelium. We observed a 3.4 and 2.6 log fold increase in enterococcal CFU counts, relative to the inoculum in bacterial and cell culture medium, respectively (figure 1b). We then continued to visualize the spatial organization of the co-cultures using confocal live imaging. For this purpose, we used the modified vancomycin-resistant *E. faecium* strain E745 that continuously expressed GPF and co-cultured with colonic epithelium. *E. faecium* strain E8202 did not accept the GFP expression plasmid upon transformation which is why we were forced to use this second *E. faecium* strain. *E. faecium* strain E745 is also a vanA-type vancomycin-resistant strain, recovered from a hospitalized patient in 2000, and also belongs to clade A1 (ST16)(17). We observed that *E. faecium* E745 resided on top of the colonic epithelium, rather than being positioned between colonic cells (figure 1c).

It was unclear if *E. faecium* and the colonic cells were in direct contact or separated by the mucus layer.

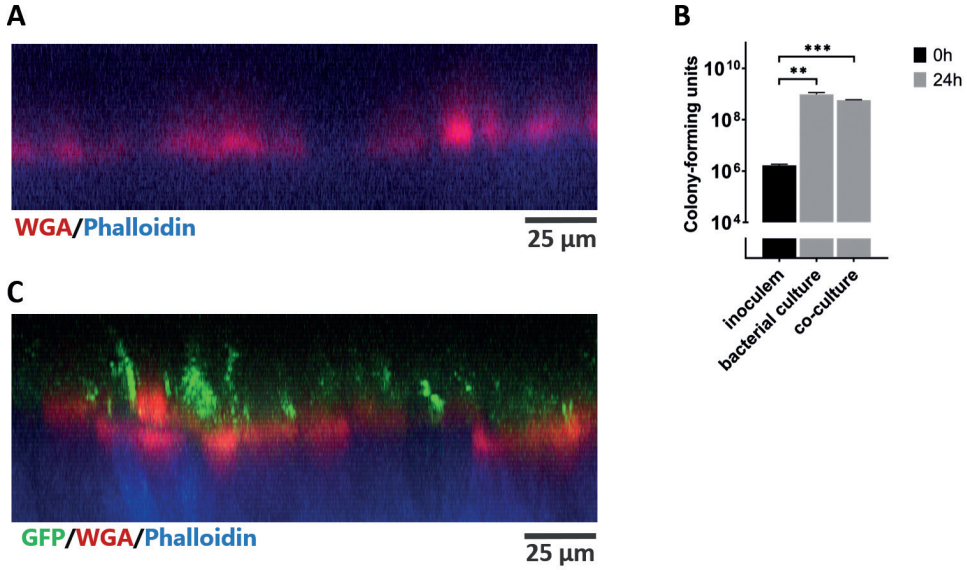

Figure 1 | Co-cultures of colonic epithelium and *E. faecium* reveals spatial organization. (A) Colonic epithelium used for confocal live imaging Images are stack to generate a side view of colonic cells stained by phalloidin (in blue) and mucus stained by WGA (in red). (B) CFU counts of vancomycin-resistant *E. faecium* strain E8202 at time of inoculation, after 24h of incubation in colonic media and after 24h of co-culturing with colonic epithelium, pval = 0.0087, pval = 0.0005, respectively. (C) Confocal live imaging of co-culture consisting of colonic epithelium and a GFP expressing vancomycin-resistant *E. faecium* strain E745 (in green), visualized by a side view of stacked images.

Co-culture of *E. faecium* E8202 with colonic epithelium results in altered patterns of *E. faecium* gene expression

We next investigate the transcriptional response of *E. faecium* E8202 upon co-culturing with colonic epithelium by performing dual RNA sequencing (figure 2). This generated 5.4-29.1 million reads (10^{th}-90^{th} percentile) per sample, from which 3.1-31.2 million reads (10^{th}-90^{th} percentile) mapped to *E. faecium* specific genes (figure s1). This is in accordance with the coverage used in previous studies that applied dual RNA sequencing to measure genome-wide transcriptional changes of both bacteria and host cells on bacteria (18). Furthermore, the read counts for the housekeeping genes *gdh* and *gyrA* indicated a sufficient high number of reads per gene with on average 5300 and 14800 normalized reads, respectively (figure s2a). Principal component analysis (PCA) revealed that culture condition (i.e., either *E. faecium* monoculture or co-culture with human colonic epithelium) is a main driver of the observed variance in gene expression between the samples in *E. faecium* E8202 (figure s3a).

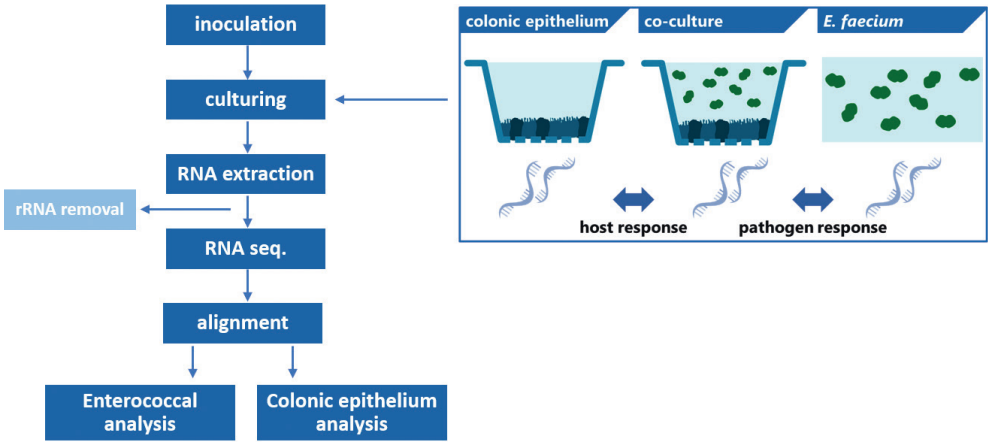

Figure 2 | Schematic representation of culture conditions for RNA sequencing. To determine the host response and pathogen response, respectively, vancomycin resistant *Enterococcus faecium* strain E8202 and colonic epithelium were co-cultured and cultured separately in otherwise identical culture conditions for 24h. Culturing was followed by RNA extraction, rRNA depletion, subsequent library preparation and sequencing. Analysis was split into two parts; one to determine the enterococcal response and a separate one to determine the host response.

Compared to the control condition (here cell culture medium) and while applying batch correction, we observed a total of 520 differentially expressed *E. faecium* genes ($p_{adj} < 0.05$, fold change > 1.5) in response to exposure to the colonic epithelium. From these, 252 genes were upregulated (48.1%) and 268 genes were downregulated (51.9%) (table s2). The top 50 significantly upregulated genes are part of the following five operons : (1) genes *EF_1900 – EF_1917* are involved in pili synthesis (*fms21*, *EF_1901*, *fms20*, *EF_1905*, *EF_1907* to *EF_1915*, *EF_1917*); (2) genes *EF_620 – EF_628* are involved purine metabolism (*purC*, *purS*, *purQ*, *purL*, *purF*, *purM*, *purN*, *purH*, *purD*) (figure 3); (3) genes *EF_1448 – EF_1458* are involved in pyrimidine metabolism (*pyrE*, *EF-1449*; *EF_1451*, *carB; carA, pyrC*, *pyrB*, *EF_1456, pyrR*); (4) genes *EF_939 – EF_942* encode a Two-component system (*agrB*, *EF_0940*, *EF_0942*); (5) genes *EF_1755 – EF_1758* are part of an operon with a yet unknown function (figure 3 and table s2). In addition to these specific operons, the top 50 significantly upregulated genes included genes that are not part of a gene operon: *EF_615* and *EF_617* (*purB* and *purE*), are predicted to be part of purine pathways as well, *EF_2954* (*sspP*), a putative C47 peptidase and nine genes with the following putative functions: aldehyde alcohol dehydrogenase (*EF_0991*), amino acid permease family protein (*EF_1408*), hypothetical proteins (*EF_1923*, *EF_2434*), DNA topoisomerase III (*EF_2435*), hypothetical protein (*EF_2450*), xanthine/uracil/vitamin C permease (*EF_2554*), hypothetical protein (*EF_3023*), class II bacteriocin (*EF_3034*).

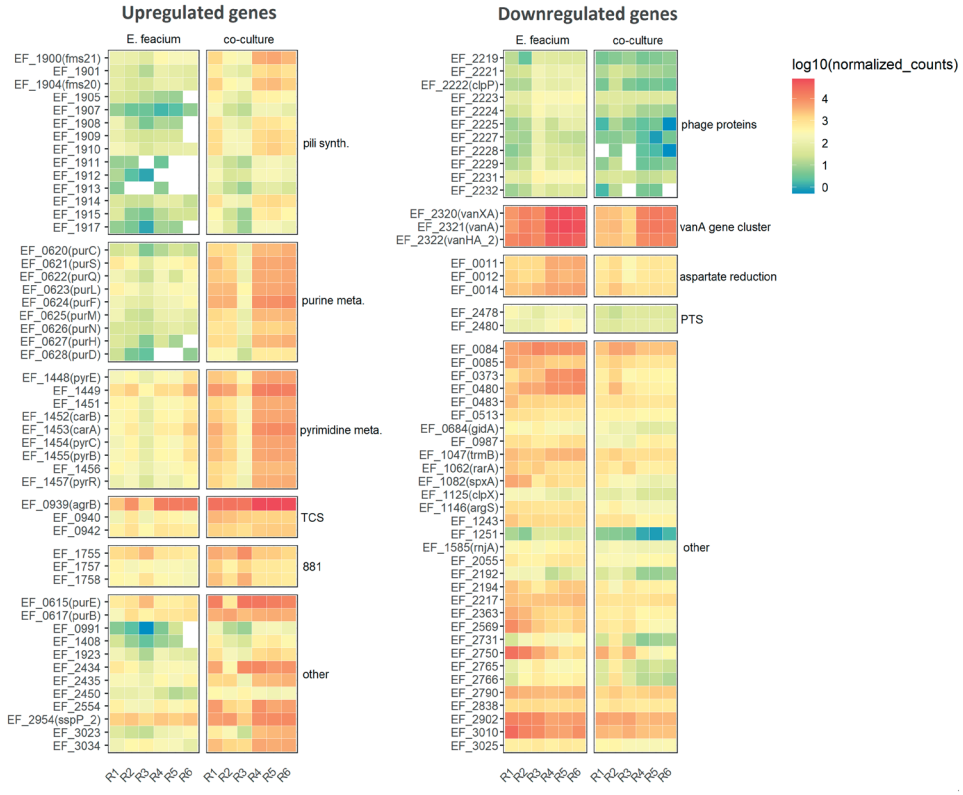

Figure 3 | Differentially transcribed genes in *E. faecium* E8202 upon co-culturing with human colonic epithelium. Top 50 significant differentially expressed, upregulated and downregulated, genes, comparing control condition with co-culturing with colonic epithelium. Read counts are normalized. Replicates are indicated by columns, while genes are grouped per gene operon.

The top 50 significantly downregulated genes are part of the following four operons: (1) genes EF_2219 – EF_2232, which are phage derived proteins (*EF_2219, EF_2221*, *clpP*, *EF_2223*, *EF_2224*, *EF_2225*, *EF_2227*, *EF_2228*, *EF_2229*, *EF_2231*, *EF_2232*) (figure 3); (2) genes EF_2320 – EF_2322, that are together part of the *vanA* gene operon (*vanH*, *vanA*, *vanX)*; (3) genes EF_11 – EF_14, that are putative aspartate reduction genes (*EF_0011*, *EF_0012*, *EF_0014*); (4) and genes *EF_2478 - EF_2480*, that are part of a putative phosphotransferase system. In addition, downregulated genes putatively encode a global transcriptional regulator (*EF_1082, spxA*), tRNA modification enzyme (*EF_0684*; *gidA*), tRNA synthetase (*EF_1146*; *argS*), replication-associated recombination (*EF_1062*; *rarA*), ATP-dependent protease (*EF_1125*; *clpX*), exoribonuclease (*EF_1585*; *rnjA*) and a methyltransferase (*EF_1047*; *trmB*). The remaining 22 significantly downregulated genes encode the following putative functions: amino acid permease (*EF_0084*), aspartate aminotransferase (*EF_0085*),

signal peptidase I (*EF_0373*), ABC transporter ATP-binding protein/permease (*EF_0480*), rhodanese-like domain-containing protein (*EF_0483*), amino acid permease (*EF_0513*), calcium-translocating P-type ATPase (*EF_0987*), Isochorismatase family protein (*EF_1243*), hypothetical protein (*EF_1251*), phospholipase/carboxylesterase (*EF_2055*), hypothetical protein (*EF_2192*), amino acid ABC transporter ATP-binding protein (*EF_2194*), HNH endonuclease (*EF_2217*), ferrous iron transport protein B (*EF_2363*), Integrase core domain protein (*EF_2569*), fructokinase (*EF_2750*), acetolactate synthase (*EF_2765*), alpha-acetolactate decarboxylase (*EF_2766*), hypothetical proteins (*EF_2790*, *EF_2902*), DNA replication protein (*EF_3010*), hypothetical protein (*EF_3025*).

Differentially expressed genes were selected for KEGG based pathway analysis using a less stringent cut-off (padj < 0.1, fold change > 1.5). From the resulting 681 differentially expressed genes, 335 could be annotated with a KEGG function (table s3). Pathway analysis revealed a significant role in different pathways, as shown in figure 4 and table s4, such as (1) alanine, aspartate and glutamate metabolism, converting aspartate into fumarate and n-carbamoyl-l-aspartate and, additionally converting glutamine into 5-phosphoribosylamine, or alternatively into carbamoyl phosphate (*carA*, *carB*, *purA*, *purB*, *purF*, *purQ*, *pyrB*); (2) arginine biosynthesis, which applies glutamine for conversion into ammonia, followed by carbamoyl phosphate, citrulline and lastly into arginine (*arcA*, *arcB*, *arc*, *purQ*); (3) purine metabolism, also converting glutamine, 5-phosphoribosylamine and possibly 5-phosphoribosyl diphosphate (PRPP) for the putative synthesis of inosinic acid (IMP) and consequently adenylic acid (AMP) (*arcC*, *purA*, *purB*, *purC*, *purD*, *purE*, *purF*, *purH*, *purL*, *purM*, *purN*, *purQ*, *purS*); (4) pyrimidine metabolism, utilizing glutamine, carbamoyl phosphate and n-carbamoyl-l-aspartate for the generation of PRPP and uridylic acid (UMP) (*carA*, *carB*, *EF_1449*, *EF_1451*, *pyrB*, *pyrC*, *pyrDB*, *pyrE*, *pyrR*), and 5) fructose and mannose metabolism, including the conversion of sorbitol to fructose-6P, and conversion of mannose to mannose-6P and fructose-1P to fructose-1,6P_2 (*EF_1276*, *EF_1821*, *EF_2637*, *EF_2740*, *EF_2743*, *EF_2744*). Expression of the genes belonging to these pathways was significantly upregulated when *E. faecium* was co-cultured with colonic epithelium compared to the control condition. Lastly, we found that expression of genes involved in biosynthesis of cofactors to be upregulated, converting 2-dehydropantoate into pantoate (*EF_1037*), menaquinone (vitamin k1) into menaquinol and phylloquinone (vitamin k2) into phylloquinol (*EF_2198*).

In contrast, KEGG based pathway analysis indicated downregulated expression of genes to be involved in (1) biosynthesis of amino acids, particularly the conversion of homocysteine into methionine, ornithine into proline and aspartate into lysine (*lysC*, *asd*, *dapA*, *dapB*, *dapH*, *patA*, *dapF*); (2) galactose metabolism, the conversion of galactose,

raffinose and stachyose (*galM*, *galK*, *galT*, *gale*, *sacA*); (4) pentose and glucuronate interconversions, the conversion of altronate and galacturonate (*uxaA*, *uxaB*, *uxaC*).

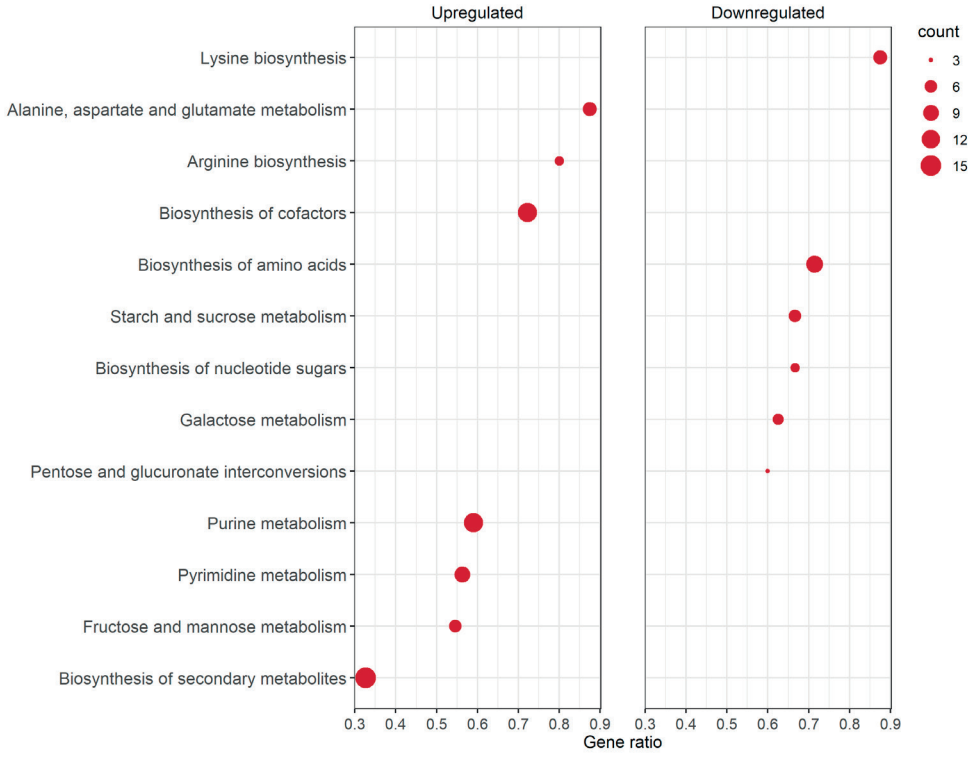

Figure 4 | Gene pathway annotation of *E. faecium* genes that are differentially expressed upon co-culturing with colonic epithelium. Genes that are significantly up- or downregulated clustered based on KEGG gene pathways analysis using clusterProfiler version 4.0 within R. Counts, displayed as circles, represents the number of differentially expressed genes that belong to a given pathway. Circle size is proportionate to counts. The x-axis represents the gene ratio; the proportion of differentially expressed genes to all the genes that are annotated in a specific pathway.

Transcriptome of colonic epithelium during colonization of *E. faecium* E8202

From the 5.4-29.1 million reads (10^{th}-90^{th} percentile) per sample, 0.9-13.2 million reads (10^{th}-90^{th} percentile) mapped to genes from the human genome indicating large variation in read counts between the six replicates (figure s1). PCA revealed that a large portion of the observed variance cannot be explained by culture condition (figure s3b). After batch-effect adjustment we observed a total of 219 differentially expressed genes ($p_{adj} < 0.05$, fold change > 1.5) of the colonic epithelium in response to enterococci relative to the control condition). From these, 117 genes were upregulated (53.4%) and

102 genes were downregulated (46.6%) (table s5). The top 50 significantly upregulated and downregulated genes are displayed in figure 5. From these genes we will highlight below gene sets that share similar functions.

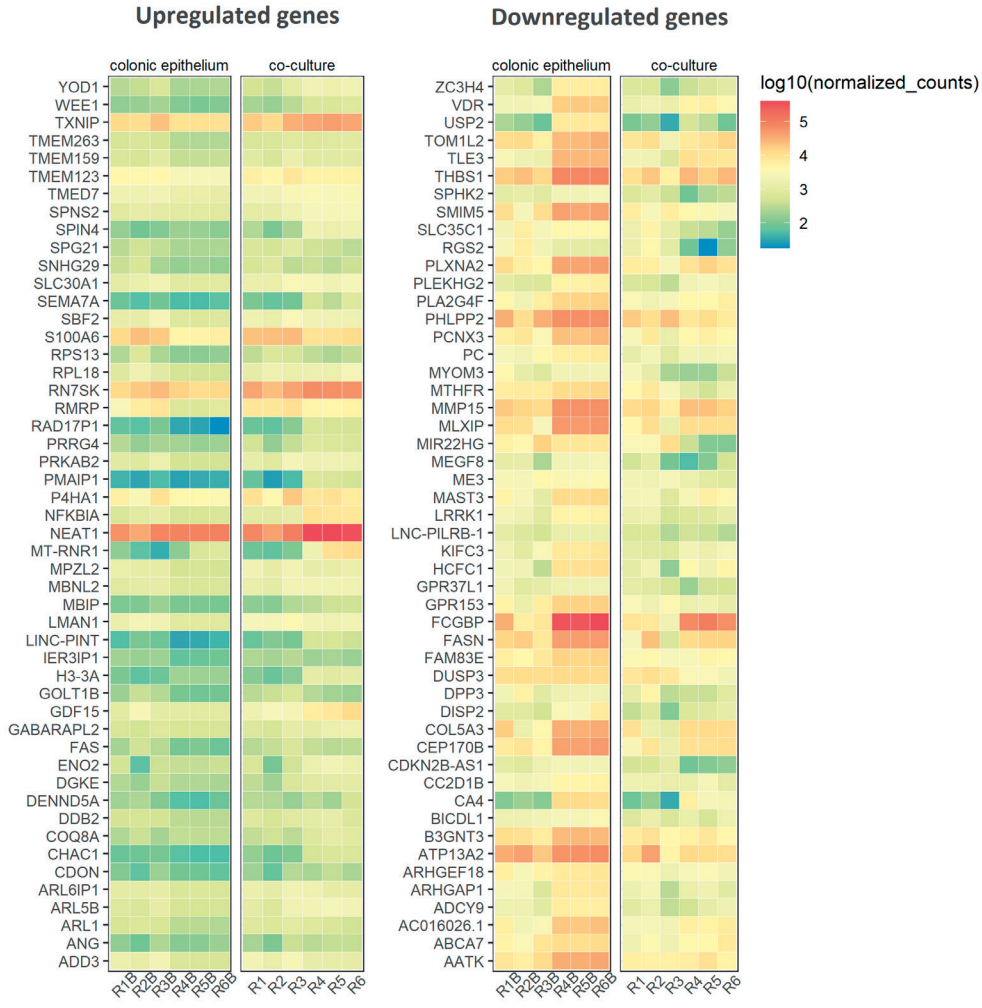

Figure 5 | Differentially transcribed genes in colonic epithelium upon co-culturing with *E. faecium* E8202. Top 50 significant differentially expressed, upregulated and downregulated, genes, comparing control condition with co-culturing with *E. faecium* E8202. Read counts are normalized and replicates are indicated by columns.

Significantly up- and downregulated genes, include genes putatively involved in:
(1) the activation of inflammation pathways, mediated by the upregulation of genes *GDF-15*, *NFKBIA*, *TXNIP* and downregulation of *VDR*; (2) cell death, mediated by the

upregulation of genes *PMAIP1*, *TMEM123*, *CDON* and downregulation of *THBS1*; (3) increased expression of GPT-binding proteins by an upregulation of genes *ARL1*, *ARL5B*, *ARL6IP1*. (4) increased expression of guanine nucleotide exchange factors by an upregulation of genes *SBF2*, *DENND5A*; (5) decreased expression of Rho GTPases by the downregulation of genes *PLEKHG2*, *ARHGEF18*, *ARHGAP1*; (6) downregulation of G protein signalling by the downregulation of genes *RGS2*, *GPR153*, *GPR37L1*.

In addition, individual upregulated genes include genes encoding Lysozyme (LYZ), Lectin Mannose-Binding 1 (LMAN1), Lipid Droplet Assembly Factor 1 (TMEM159) and four genes that encode long non-coding RNAs with yet unknown functions. Individual downregulated genes include genes encoding Matrix Metallopeptidase 15 (*MMP15*), Collagen Type V Alpha 3 Chain (COL5A3), fatty acid synthesis (FASN). Furthermore, genes encoding proteins involved in amino acid degradation and metabolism were downregulated, including malate dehydrogenase (*ME3*), aspartate synthesis dipeptidyl aminopeptidase (*DPP3*) and sugar metabolism (galactosyltransferase (*B3GNT3*) and pyruvate carboxylase (*PC*)). Lastly, four genes that encode long non-coding RNAs with yet unknown functions were significantly downregulated. Gene pathway analysis including the differentially expressed genes revealed no significantly impacted gene pathways.

Discussion

Colonization and subsequent overgrowth of vancomycin resistant *Enterococcus faecium* is an important step for subsequent infection in hospitalized patients. The impact of this intestinal overgrowth on host-enterococcal interactions is not well studied. In this study, we applied a dual RNA sequencing approach in order to unravel the transcriptional dynamics of both *E. faecium* and human derived colonic epithelium upon co-culturing. In our studies we mainly used the hospital-associated vancomycin (vanA-type) resistant *E. faecium* strain E8202, which was previously isolated from a patient during a Dutch hospital outbreak (19). Our results reveal that the main transcriptional response of *E. faecium* strain E8202 to colonic epithelium involves upregulation of genes involved in pilus biogenesis and downregulation of vancomycin resistance genes. In addition, we observed transcriptional rewiring of *E. faecium* by redirecting its metabolism towards amino acid scavenging and reduction.

Confocal live imaging of co-cultures of *E. faecium* E0745 and human colonic epithelium revealed that *E. faecium* was located on top of the colonic epithelium, while it was unclear if *E. faecium* and the colonic cells were in direct contact or separated by the mucus layer. Co-culturing of *E. faecium* E8202 with colonic epithelium for 24h resulted in the

differentially expression of a broad array of genes. The gene operon encompassing *fms21 (pilA)-fms20* pilus genes was significantly upregulated (20). This operon is located on plasmid 1, which is 189 Kb in size (table s1). This is in concordance with the findings of Freitas et al., where the *fms21 (pilA)-fms20* locus was found to be located megaplasmids up to 280 kb (21). Our results suggest that pilus production might be important during intestinal colonization, which is to our knowledge the first time that the production of *E. faecium* pili is associated with human intestinal colonization. Previously, Revtovich et al., studied *E. faecium* pili using an *in vivo* infection model and concluded that the *fms21 (pilA)-fms20* gene locus does not play a role in *C. elegans* killing in their killing assay (22). The genes *fms20* and *fms21* (*pilA*) are more frequently found in hospital-associated strains of *E. faecium*, suggesting that *fms21* (*pilA*)*-fms20* pili have an important role in intestinal colonization, specifically of hospital-acquired isolates (23).

In addition to the changes in the expression of the *fms21 (pilA)-fms20* operon, we also observed changes in gene expression of possible other virulence factors, such as proteases. Specifically, we observed upregulation of *sspP*, a putative staphopain peptidases C47. Studied peptidases from the C47 family include staphopain A in staphylococci. In *Staphylococcus aureus* staphopain A is a key mediators of *S. aureus* virulence and plays a putative role in the destruction of connective tissue, the inhibition of host immune response and the modulation of biofilm integrity (24, 25). It is currently unknown what the role of *sspP* in enterococci is. In contrast, expression of *clpP* and *clpX*, both ATP-dependent proteases, were downregulated (26)**.** *clpP* and *clpX* are important in bacterial growth and are part of the cellular protein quality control systems by refolding or degrading damaged proteins (27). Upregulation of *clpP* was observed in *E. faecalis*, as a result of cell stress and resulted in the downregulation of pyrimidine metabolism genes *pyrE*, *pyrC* and *pyrF* (28). In concordance with this finding, downregulation of *clpP* in *E. faecium* E8202 co-occurred with upregulation of genes implicated in pyrimidine metabolism upon co-culturing with colonic epithelium, including pyrimidine metabolism genes *pyrE* and *pyrC*. In addition, genes *pyrB* and *pyrR* were upregulated in our study, as well as purine metabolism genes *purC*, *purD*, *purF*, *purH*, *purL*, *purM*, *purN*, *purQ* and *purS*. Lim et al., compared gene expression during biofilm-formation with gene expression of cells in the planktonic phase using a vanA-type *E. faecium* strain (29). Although they observed a downregulation of clp protease in biofilm cells relative to planktonic cells, they did not report changes in the expression of pyrimidine metabolism genes. In *S. aureus*, the purine biosynthesis gene *purF* has been shown to regulate cell growth, biofilm formation and to play a key role in persistent methicillin-resistant *S. aureus* bacteraemia (30, 31). It remains to be investigated if *purF* could play a role in biofilm formation in enterococci as well and if this gene is also implicated in regulation of expression of pilli genes.

KEGG pathway analysis revealed a major shift in gene expression of *E. faecium*, in response to co-culturing with colonic epithelium, leading to a reconfiguration in metabolic pathways for energy supply. This included the upregulation of pathways involved in purine and pyrimidine metabolism and the downregulation of nucleotide sugars and amino acid biosynthesis, including lysine and methionine biosynthesis. Purine and pyrimidine metabolism pathways are connected to other pathways like alanine, aspartate and glutamate metabolism and arginine biosynthesis. Together, the combination of these results suggests a large-scale reduction of glutamine and aspartate into adenylic acid (AMP), inosinic acid (IMP) and uridylic acid (UMP). Since most of the easily accessible nutrients and carbon sources are absorbed in the small intestines, the microbiota of the colon is commonly composed of bacteria designed to salvage energy and nutrients from alternative sources. Some of these have evolved to salvage amino acids, purines and pyrimidines as primary sources of carbon, nitrogen, and energy, similar to the response of *E. faecium* in our study (32-35). Colonocytes are able to metabolize glutamine into glutamate, aspartate, alanine, and lactate, potentially explaining why these amino acids are available for *E. faecium* (36, 37). It is unclear if *E. faecium* competes for amino acids with the colonocytes in our model, or if *E. faecium* contributes to conversion pathways, for instance by the generation of arginine. Lim et al., observed a downregulation of the arginine deiminase system (*arcA*, *arcB*, *arcC*) in biofilm forming vanA-type vancomycin resistant *E. faecium* when compared to *E. faecium* grown in the planktonic phase (29). This is in contrast with our findings and also with their own expectations. They describe that an increase in arginine expression is expected during biofilm development, since it is an alternative energy source when sugars are depleted. While salvage pathways like that of amino acids can generally result in the production of beneficial short chain fatty acids, they are also known to create by-products that act as toxins to host cells, like indoles, phenols, and amines (38). The increased reduction of glutamine by *E. faecium* may result in the production toxic ammonia. Accumulation of ammonia is known to disrupt tight junctions of host cells and seems a possible way for pathogens to disrupt the barrier function of the intestines (39). However, in our study we also observed that during *E. faecium* and colonic epithelium co-culturing the arginine synthesis pathway of *E. faecium* is upregulated, which through the activation of the urea cycle, would result in the reduction of ammonia. Whether or not ammonia is accumulated during *E. faecium* and colonic epithelium co-culturing is therefore unknown, and remains to be investigated.

KEGG pathways analysis also revealed changes in expression of several *E. faecium* genes implicated in starch and sugar metabolism. Specifically, genes implicated in the reduction of galactose, raffinose and stachyose were downregulated. In contrast, genes implicated in mannose and fructose metabolism pathways were upregulated, increasing the conversions of sorbitol to fructose-6P, and conversion of mannose into mannose-6P

and fructose-1P into fructose-1,6P2. The uptake and utilization of fructose, mannose and amino sugars has been reported to be linked to *E. faecium* isolates belonging to the hospital clade (clade A1) and were shown to play an important role in colonization of the intestinal tract of mice, following antibiotic treatment (8, 11). The increased utilization of sorbitol and glycogen suggest sources of these compounds in our model. Sorbitol is generated from glucose by an aldose reductase across different cell types and generally plays a role in the osmotic regulation of epithelial cells. Glycogen is likely to be derived from secreted mucus, mainly MUC2, though *E. faecium* is not known as a mucus degrader (40). Maltose and sorbitol are furthermore examples of nutrients that are utilized by specialist bacteria in the colon (41-43). Together these metabolic changes suggest that they represent important functions during intestinal colonization.

Finally, we observed a downregulation of vancomycin resistance genes (*vanH*, *vanA*, *vanX*) and in lesser degree of *vanS*, upon co-culture with colonic epithelium, relative to the control condition. The *vanS* gene is a sensor histidine kinase that can detect vancomycin and forms a two-component system together with *vanR*. The *vanR* gene encodes the response regulator that regulates the expression of the *vanH*, *vanA* and *vanX* vancomycin resistance genes (44). The exact mechanism by which *vanS* detects vancomycin is still unknown. It is speculated that vancomycin is detected directly, or that downstream effect of vancomycin activity such as the accumulation of lipid II is being sensed (45). In our experiments, culture media was not supplemented with vancomycin and vancomycin can therefore not be responsible for changes in the expression of vancomycin resistance genes. These results suggest that an alternative mechanism might be responsible for the changes in the expression of vancomycin resistance genes. One possibility is that membrane stress is responsible for the observed changes in expression. In this case, *E. faecium* grown in the control condition should endure more membrane stress than the condition with colonic epithelium, causing the observed downregulation of vancomycin resistance genes during co-culturing. Nevertheless, to our knowledge, this is the first time that it is shown that upon interaction with human host cells representative of the human gut epithelium expression of vancomycin resistance genes is not induced in *E. faecium*. It is yet unknown if non-induction or downregulation of vancomycin resistance genes functions as a way to preserve energy, or if it has additional functions. Moreover, non-induction or downregulation of the *vanA* gene operon could be part of larger structural changes in the cell wall, which potentially affects host-pathogen interaction (46). Together, these results may imply that the vanA-type vancomycin resistant *E. faecium* strain E8202 used in this study, gained increased susceptibility to vancomycin upon colonization on colonic epithelium. Future studies involving vancomycin susceptibility assays are needed to determine potential changes in phenotypic susceptibility to vancomycin. Also, additional experiments in which media

is supplemented with vancomycin, might reveal *vanA* gene operon driven changes in host-pathogen interaction.

On the host side we observed a large number of genes (219 genes) to be differentially expressed upon co-culturing with *E. faecium* strain E8202. These 219 genes were not found to be part of gene clusters involved in specific pathways. One of the genes that was differentially expressed was the gene encoding GDF-15, which is often induced under stress conditions, and which consequently activates inflammation pathways (47). GDF-15 expression was found to be affected by calcitriol, downregulating its expression (48). We found a calcitriol receptor (VDR) to be downregulated, overall suggesting reduced calcitriol levels. The downregulation of VDR is associated with reduced integrity of tight junctions and the reduced suppression of bacteria-induced NF-κB activation (49, 50). In contrast, NF-κB inhibitor alpha (NFKBIA) was upregulated, thereby inhibiting the activation of pro-inflammatory pathways (51). We observed upregulation of TXNIP, which is an inhibitor of thioredoxin (TRX). TRX is a redox-active protein that possesses anti-oxidative, anti-apoptotic and anti-inflammatory properties. Therefore, the upregulation of TXNIP may result in the build-up of reactive oxygen species in cells and inflammation (52). In mice however, TXNIP was found to be a critical regulator of fructose metabolism (53), which could be in line with the changes in fructose metabolism that were observed in *E. faecium*. Furthermore, we observed differentially expressed genes involved in cell death; the upregulation of *PMAIP1* (NOXA), which was shown to induce apoptotic pathway of intestinal crypt stem/progenitor cells in IR-treated mice and which could be linked to the VDR downregulation that we observed (54). We also observed upregulation of the pro-oncosis surface receptor TMEM123 and the downregulation of THBS1 (55). In addition, we found CDON upregulated, which is a surface receptor and putative inducer of apoptosis as well (56). Because of the seemingly counteracting effects of genes involved in the activation of pro-inflammatory pathways and genes involved in cell death, it is unclear what the overall cell response is upon interaction with *E. faecium*. Furthermore, it is unknown if these responses take place in a single cell type or if it is dispersed over several cell types. In addition to these changes in gene expression, we observed the downregulation of genes encoding proteins involved in amino acid degradation and metabolism, potentially in response to the metabolic changes of enterococci. Together these results indicate a broad differential response of the colonic epithelium on *E. faecium*, which need to be further validated.

A limiting factor in the current analysis of differential expressed colonic epithelium genes is that the sequence depth of replicate 4-6 did not reach the previously recommended minimum of 10 million reads aligning with the eukaryotic part of the database (18). An additional limitation comes from the observed batch effect between replicates 1-3 and 4-6. While we correct for batch effects, this might impact the resolution of the analysis

and could explain why we did not find significant differences in the pathway analysis. The origin from these batch differences is currently unknown, although it has previously been observed that seemingly small changes in culture conditions and components such as Matrigel, can introduce variability between batches of human colonic epithelium (57). In our case, the organoid culture conditions for replicate 1-3 differed from replicate 4-6 during the Matrigel phase, which might contribute to batch effects.

To conclude, by using dual RNA sequencing of a confluent monolayer of human colonic epithelium co-cultured with vancomycin resistant *E. faecium*, we were able to reveal an elaborate and diverse bacterial response. When exposed to the colonic epithelium, colonization of *E. faecium* seems to be facilitated by pili expression and downregulation of vancomycin resistance operon, while metabolism switched to amino acid scavenging and reduction. Although changes in gene involved in inflammation and amino acid degradation suggest a specific response of host cells to *E. faecium* as well, we were unable to detect large pathway changes. Future studies involving additional *E. faecium* strains and isogenic mutants are required to confirm the results obtained here and to mechanistically study the role of the identified cell responses in host-pathogen-interaction. Increasing the knowledge on the mechanisms implicated in *E. faecium*-host interaction may help to design novel anti-infectivity strategies.

Methods

Organoid line and growth conditions

The clonal human-derived colonic organoid cell line Pt15-70206 was used to grow colonic epithelium according to the protocol described by Vonk et al. (58). In brief, a colonic organoid stock was thawed and organoids were cultured in domes of Matrigel covered by medium containing 15% Advanced DMEM/F12, 1x Glutamax, 100 U/mL Penicillin-Streptomycin, 10 mmol/L HEPES (all Invitrogen), 25% Rspo1 Conditioned Medium, 10% Noggin Conditioned Medium (conditioned medium was home-made), 2% B27 (Invotrogen), 1.25 mM N-acetylcysteine, 10 mM Nicotinamide, 10 μM p38 inhibitor SB202190 (all Sigma-Aldrich), 50 ng/mL mEGF (Invitrogen), 0.5 μM A83–01 (Tocris), 50% Wnt-3A Conditioned Medium (WCM). For replicate 4-6 the organoid culture conditions during the Matrigel phase were slightly different, by using 50% Wnt Surrogate-Fc Fusion Protein instead of 50% WCM. After on average 13 passages, organoids were disrupted and used to seed transwells and generate confluent colonic epithelium. During the first 24h in transwells, medium was supplemented with 10nM rock inhibitor Y-27632 (Selleck Chemicals). Once the TEER surpassed 100 ohm/cm2, the culture medium was replaced by culture medium lacking WCM, nicotinamide and p38 inhibitor, called differentiation medium (59). Differentiation

medium was refreshed 24 hours before the inoculation of co-cultures by differentiation medium without added Penicillin-Streptomycin, to prevent inhibition of bacterial growth. All experiments were performed in accordance with relevant ethical guidelines and regulations.

Bacterial strain and growth conditions

The vancomycin (vanA-type, ST117) resistant *E. faecium* strain E8202 was previously isolated from a patient via a rectal swab during a Dutch hospital outbreak in 2015. This strain was fully sequenced and assembled and contains a total of 6 plasmids (table s1). *E. faecium* E8202 is used throughout this study except for the confocal live imaging experiment in which *E. faecium* strain E745 was used (9, 19). Sequences were annotated using Prokka version 1.10 and Operon-mapper, using standard settings (60, 61). Annotation was further expanded by blasting genes manually, applying a 98% identity cut-off (table s2). Bacteria were initially cultured at 37 °C and shaking at 150 rpm in Brain Heart Infusion broth (BHI) to an OD of 0.4. After a washing step in PBS, bacteria were resuspended in organoid culture media and concentrated to an artificial OD of 0.6. These cultures were subsequently used for co-cultures with colonic epithelium, applying a multiplicity of infection (MOI) of 6. Co-cultures were spun down at 250 x g for 5 minutes to ensure cell contact and used for 24 hours co-culture at 37°C and 5% CO2.

Confocal live imaging

Co-culture of a colonic epithelium and *E. faecium* were analysed for fluorescence using confocal live imaging with a confocal laser microscope (Leica SP5) and a Plan Apo 40x objective (numerical aperture of 0.85). Only for this experiment the vancomycin (vanA-type) resistant *E. faecium* strain E745 was used as we were not able to generate a GFP expressing strain E8202 (17). A constitutively GFP expressing plasmid was constructed by ordering a gblock containing a strong bacillus promotor (P*groE*) and the free-use GFP (Integrated DNA Technologies) (62, 63). This gblock was then digested and ligated with pWS3-msrC-Pbac-T7 using the *ApaI* and *SmaI* restriction sites located on both the plasmid as gblock (64). The resulting plasmid was then used to transform *E. faecium* strain E745 in order to constitutively express GFP in confocal microscopy. GFP was excited at 488 nm, Alexa Fluor 647-conjugated WGA was excited at 633 nm and Alexa Fluor 405-conjugated Phalloidin (ThermoFisher Scientific) at 405 nm. Pictures were analysed with LAS AF software (Leica).

RNA extraction and Dual RNA sequencing

RNA extraction was performed according to a modified protocol of the RNeasy Mini Kit (Qiagen). First, 80% of the media in the upper compartment of transwells was slowly removed. Cells were then resuspended in 200ul RLT buffer, transferred to 2ml bead beat tubes (0.5 ml glass 0.1 mm beads, Sigma-Aldrich) and snap frozen in liquid nitrogen.

Beat beating was applied at 3800 rpm for 1 minute, followed by 1 minute incubation on ice and two additional rounds of beat beating, using the Mini-beadbeater-24 (Biospec). The RNeasy Mini Kit protocol was then resumed at the ethanol addition step. The recommended DNases treatment was applied during RNA extraction, followed by an additional DNase treatment (TURBO DNA-free kit, Ambion). Total RNA was quantified by Qubit assay (Invitrogen). Samples were sent to Utrecht Sequencing Facility (USEQ) for rRNA removal using the TruSeq Stranded mRNAn kit (Illumina) for replicates 1-3 and the Stranded Total RNA Library Prep for replicates 4-6, due to discontinuation of the rRNA removal kit. The NextSeq500 (Illumina) was used, with high output and single-end sequencing of 1 x 75 bp (Illumina) and the company protocol/standard settings (USEQ).

Data processing and analysis

Sequence data was trimmed using Trim Galore version 0.6.4 with standard settings (50). The human genome (Ensembl release 99, chromosome 1-22, X, Y, MT) was merged to the genome of *E. faecium* E8202 and used to create a database with STAR version 2.7.9a using standard settings and *-sjdbOverhang 49* (65). Sequence data was subsequently aligned to STAR with standard settings and *-quantMode GeneCounts*. The resulting list of aligned genes was split into two parts; analysis of the host response and pathogen response. Analysis of sequencing data was performed in R version 4.0 and the functions of the packages ggplot2 (66, 67). DESeq2 version 1.30 was used for the analysis of differentially expressed genes, while applying batch correction and apeglm shrinkage estimator version 1.14 (68, 69). A cut-off of adjusted p-values 0.05 and foldchange of 1.5 was applied to distinguish differentially expressed genes. The top 50 up and down regulated genes were selected based on the lowest adjusted p-values. A cut-off of adjusted p-values 0.1 and foldchange of 1.5 was applied to select genes for pathway analysis. KEGG automatic annotation server was first applied to predict gene function (table s3) (70). Pathway analysis was subsequently performed using clusterProfiler version 4.0, with 999 permutations and gene set size limitations of 8 and 100, respectively (71). R scripts to reproduce the analysis reported in this study can be found at; https://gitlab.com/PB_Stege/RNA_seq_analysis.

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Supplementary data

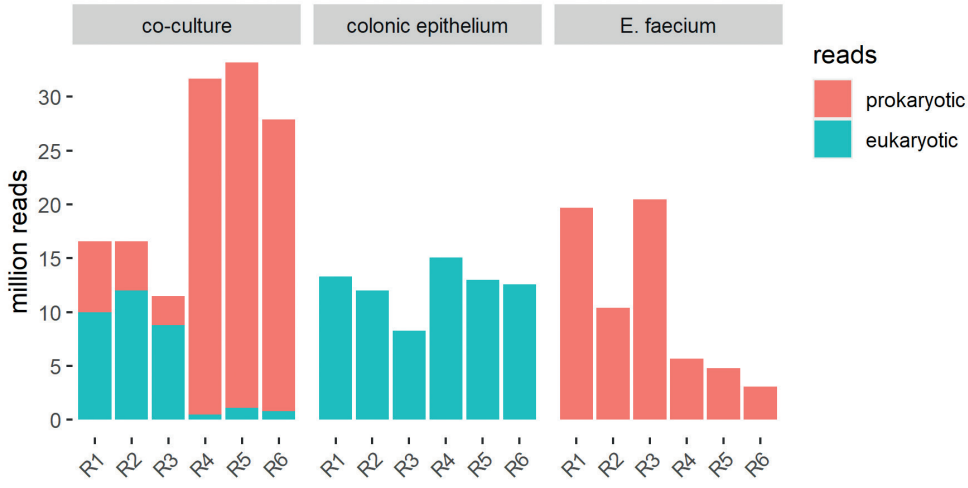

Figure s1 | Ratio sequenced reads per replicate. The number of million reads, aligning with either the eukaryotic or prokaryotic part of the database, grouped per condition.

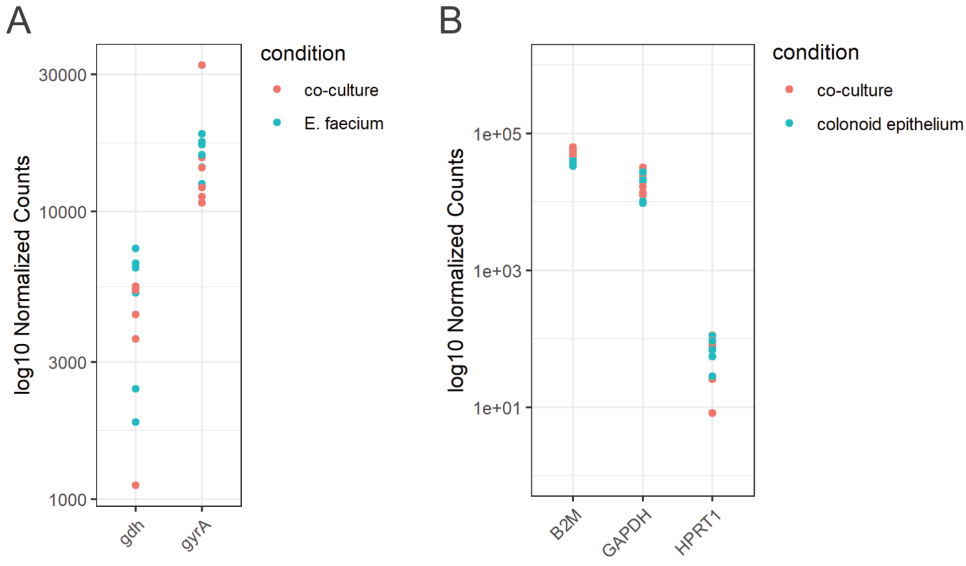

Figure s2 | Normalized read counts of housekeeping genes. Co-culture samples are represented in red, while the *E. faecium* and colonic epithelium samples are represented in blue. (A) *E. faecium* housekeeping genes *gdh* and *gyrA*. (B) Housekeeping genes of colonic epithelium B2M, GAPDH and HPRT1.

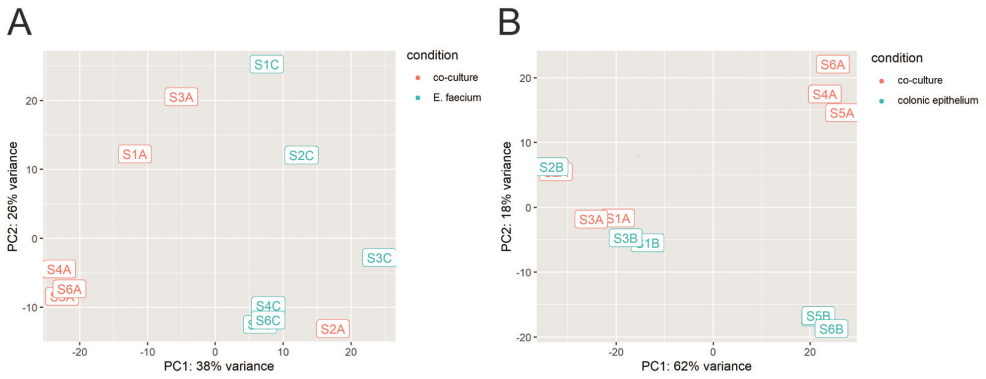

Figure s3 | PCA plot of replicates. Normalized reads per gene plotted by Principal Component Analysis. (A) Samples representing the different conditions are separated for the *E. faecium* analysis. (B) For the analysis of colonic epithelium, there is a small separation between conditions, which might indicate batch effects.

Table s1 | Genome sequencing information of *E. faecium* E8202

Molecule name	Molecule size (bp)
Chromosome	3014357
Plasmid 1	188833
Plasmid 2	40152
Plasmid 3	11924
Plasmid 4	11208
Plasmid 5	5778
Plasmid 6	4203

Table s2 | *Enterococcus faecium* differentially expressed genes.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter5-table_s2_-_E._faecium_DE_genes_.xlsx

Table s3 | *Enterococcus faecium* gene KEGG function.

Available online at: https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter5-table_s3_-_E._faecium_gene_KEGG_function.xlsx

Table s4 | *Enterococcus faecium* pathway analysis.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter5-table_s4_-_E._faecium_pathway_analysis.xlsx

Table s5 | Colonic epithelium DE genes.

Available online at:
https://gitlab.com/PB_Stege/supp_thesis/-/blob/main/Chapter5-table_s5_-_Colonic_epithelium_DE_genes.xlsx

Chapter 6

General discussion

Introduction

The human intestinal tract is colonized by hundreds of different species of bacteria and is therefore one of the most densely populated microbial habitats known on earth (1, 2). The bacteria in the gut are together called the gut microbiome and in healthy conditions the gut microbiome is in a relatively stable equilibrium state, where gut bacteria live in symbiosis with the host (3). Disruption of this equilibrium allows for proliferation of opportunistic pathogens such as extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC) and vancomycin-resistant enterococci (VRE) (4-12). While these opportunistic pathogens are able to proliferate in a dysbiotic gut microbiome, infections resulting from this proliferation are usually extra-intestinal. ESBL-EC can cause bacteremia, pneumonia and urinary tract infections, whereas VRE infections mainly involve line-associated bacteraemia (5, 9, 13, 14). Both ESBL-EC and VRE have become insensitive for common treatment procedures by rapidly acquire antibiotic resistance genes (ARGs) from the ecological niche that they reside in (15-17). This primary niche, the gut microbiome, represents an important reservoir of antibiotic resistance genes, called the resistome (18, 19). Therefore, studying environmental factors that shape the microbiome, such as diet or exposure to antibiotics, is not only important to understand how microbiome changes allow the proliferation of nosocomial pathogens but also how microbiome changes impact the prevalence and abundance of ARGs as part of the gut resistome.

Powerful next generation sequencing techniques allows studying the gut microbiome with unprecedented resolution. In addition, novel methods make use of capture-based sequencing to select for genes of interest out of the broad spectrum of genomic material that resides in the gut microbiome, thereby significantly increasing the sensitive of detecting targeted genes. With the use of these tools, we set out to investigate the role of the gut microbiome and resistome as reservoirs of potential pathogenic bacteria and ARGs (**part I** of this thesis). Since *Enterococcus faecium* was among the detected opportunistic pathogens in chapter 3 of this thesis and because it is clinically relevant for humans, we characterized the dynamics of host-pathogen cross talk during colonization. For this we first optimized tools to genetically modify *E. faecium* strains and then applied a model of gut colonization using human-derived colonic epithelium and a hospital-associated vancomycin resistant *E. faecium* strain to study host-pathogen cross talk (**part II** of this thesis).

The gut microbiome as reservoirs of potential pathogenic bacteria and ARGs

The human gut microbiome heavily fluctuates during the first years of life, after which it slowly reaches an equilibrium and becomes less prone to changes driven by environmental exposure (20). Nevertheless, the human gut microbiome can still be influenced during adulthood, by factors related to lifestyle such as diet, medication, smoking and consumption of alcohol (21-25). In chapter 2 of this thesis, we assessed whether long-term dietary habits impact the human gut resistome and microbiome in the general Dutch population. For this study, we analysed faecal samples from Dutch omnivores, pescatarians, vegetarians and vegans by Metagenomic shotgun sequencing (MSS) and ResCap targeted sequencing (ResCap). We found that the gut resistome among all diet groups forms a reservoir consisting of 119 and 145 unique antibiotic resistance genes (ARGs) as determined by MSS or ResCap, respectively. In concordance with literature, the most abundant antibiotic resistance genes are those that encode for tetracycline resistance, followed by macrolide and beta lactam resistance genes (26, 27). When comparing the relative abundance of antibiotic resistance genes between diet groups, we however did not detect associations of long-term dietary habits with a specific resistome signature. The recent large-scale study of Gacesa et al, found associations between a lifelines diet score and 79 taxa and 20 ARGs, when studying the gut microbiome of the Dutch population (28). However, like in our study, no associations were specified between a particular diet and ARG abundance. Furthermore, these 20 ARG associations do not only include acquired ARGs, but also include ARGs that are the result of mutations in chromosomal genes. In our study we restricted analysis to that of diet-induced effects on acquired ARGs. We additionally used the MSS data to compare for diet specific differences in the gut microbiome and found that mainly vegans have a distinct gut microbiome composition, compared to other diet groups.

The majority of diet studies that observed diet-induced effects on the gut microbiome either compare participants from different geographic areas or involve short term dietary intervention studies (29-31). Particularly studying diet effects in geographical distinct areas introduce an uncertainty of which of the observed effects in these studies can be specifically attributed to the different diets. Short-term diet intervention studies have found correlations between diets rich in protein and animal-fats and the increased relative abundance of *Bacteroides*, as opposed to carbohydrate-rich diets and the increased relative abundance of *Prevotella* (29, 32). Alternatively, the increased relative abundance of *Prevotella* was associated with fiber- and vegetable-rich diets (33). A long-term diet study confirmed the association between *Prevotella* and fiber- and vegetable-rich diets, but additionally observed an increase in *Lachnospira* as a result of fiber- and vegetable-rich diets and the association between an omnivore diet and increased abundance of

Ruminococcus (34). When studying the effect of long-term dietary habits within a single community on the gut microbiome of vegans, vegetarians and omnivores, Losasso et al. observed comparable diet induced effects in OTUs affiliated with *Bacteroides*, *Lachnospiraceae*, and *Ruminococcaceae*, but stresses that these are only present in low abundance (35). In concordance with these findings, De Angelis et al. only found minor differences in the abundance of bacterial families between diet groups and found *Ruminococcaceae* to be most abundant in omnivores, while *Lachnospira* were associated with vegans and vegetarians (36). Gacesa et al, studied the Dutch population and found associations between animal derived protein intake and the increased abundance of *Eubacterium eligens*, *Pseudoflavonifractor capillosus*, *Ruminococcaceae bacterium D16*, *Ruminococcus torques*, *Lachnospiraceae* sp. 8.1.57FAA and that of plant derived protein intake and the increased abundance of *Eubacterium eligens*, *Roseburia hominis*, *Subdoligranulum unclassified* and *Oxalobacter formigenes* (28).

Although the observations in these aforementioned studies are limited to the bacterial family level due to the use 16S rRNA profiling, they generally align with our results in chapter 2 of this thesis, where we assessed whether long-term dietary habits within a single geographical region (The Netherlands) impact the human gut microbiome and resistome in the general population. We observed that vegans had a lower relative abundance of *Ruminococcus torques* when compared to omnivores. We additionally found that, compared to omnivores, vegans had a lower abundance of *Streptococcus thermophilus*, *Clostridium sp.*, *Clostridium phoceensis* and *Clostridium saccharolyticum*. Similarly, *S. thermophilus*, *Lactococcus lactis* and *Firmicutes bacterium CAG:313* were less abundant in vegans compared to pescatarians. Finally, *Eubacterium eligens* was more abundant in the gut microbiome of pescatarians when compared to omnivores. These results suggest that only the vegan diet is associated with a distinct taxonomic composition. The study of Gacesa et al., associated a higher abundance of *R. torques* and *S. thermophilus* with animal derived protein intake and a higher abundance of *E. eligens* with both plant and animal derived protein intake in the Dutch population (28). These findings seem to partially align with the observations in our study in chapter 2. One contrasting result we observed relative to the aforementioned studies, is the observed differences in the higher relative abundance of lactic acid bacteria *S. thermophilus* and *L. lactis* in omnivores. These differences may be explained by the relatively high consumption of dairy products in the Netherlands, since previous studies on the Dutch population also found associations between the consumption of buttermilk and the abundance of *Leuconostoc mesenteroides* and *L. lactis* (37). This furthermore highlights that geographic differences are an important confounder when interpreting microbiome related studies.

It has been reported previously that, similar to the human gut microbiome, the microbiome of dogs will over time reach a state of equilibrium, where temporal variations within dogs is limited (38). Despite this observation of an apparent relatively stable dog gut microbiome it has also been shown that ESBL-EC can colonize the gut of the dogs and that this colonization status can fluctuate when monitored over time (39, 40). The reasons for this alleged intermittent ESBL-EC carriage are not well understood. In chapter 3 of this thesis, we investigated whether ESBL-EC carriage in dogs was associated with gut microbiome and resistome changes. For this, faecal samples were collected from 57 companion dogs in the Netherlands every two weeks for a total of six weeks. We confirmed by means of MALDITOF mass spectrometry, selective culturing and PCR that a large proportion of these dogs (68%) carried ESBL-EC during this time period. Faecal microbiome composition analysis by means of 16S rRNA gene sequencing revealed that ESBL-EC carriage was associated with an increased abundance of *Clostridium sensu stricto 1, Enterococcus, Lactococcus* and the shared genera of *Escherichia-Shigella*. Oxford Nanopore Technology, to distinguish between species using long-read metagenomic sequencing in a subset of the dogs, suggested that the highlighted genera may include the opportunistic pathogens *Clostridium perfringens* and *Lactococcus garvieae*. *C. perfringens* is a potential toxin producer that is associated with dog gastrointestinal diseases such as haemorrhagic diarrhoea, while the increased abundance of *E. coli* and enterococci has been associated with dogs that had chronic signs of gastrointestinal disease and confirmed inflammatory changes (41-45). *L. garvieae* is the only known pathogenic Lactococcus and a recognized human pathogen, although it is mainly found in dairy products and as a pathogen of fish (46, 47). Since dogs with known underlying health conditions were excluded from our study, the observed fluctuations in microbiome of dogs with detected carriage of ESBL-EC are unlikely to be directly associated with disease. In contrast to the associations of ESBL-EC carriage and increased abundance of bacteria in the microbiome, we found that ESBL-EC carriage was associated with a decreased abundance of *Colidextribacter*, *Faecalibacterium*, *Fournierella*, *Holdemanella*, *Muribaculaceae*, *Negativibacillus*, *Peptococcus* and *Prevotella*. Species of *Faecalibacterium*, *Holdemanella* and *Prevotella* are considered commensals, or even symbionts (48-51). Future studies involving these potential symbionts are needed to determine their role in the dog gut microbiome and if these could potentially counteract carriage of opportunistic pathogens like ESBL-EC. Similar associations between the prevalence of ESBL-EC and a reduction in commensal bacteria have been found when studying gut microbiome of residents of Dutch nursing homes. Ducarmon et al., found that 44% of the studied residents were colonized with a multidrug-resistant organisms (MDRO) at at least one time point throughout the 6-month study. ESBL-producing *E. coli* sequence type 131 had the highest prevalence from all detected MDROs. In addition, an association was found between the increased abundance of *Dorea*, *Atopobiaceae* and *Lachnospiraceae* ND3007 group in residents that were never colonised with an MDRO, including *E.*

coli. The authors speculate that these bacteria may be associated with protection against MDRO colonization (52). In a subsequent study of Ducarmon et al, the microbiome of the general population was studied in relation to ESBL-EC status, but no associations were detected (53). Important differences between this study and the before mentioned study include the relatively high prevalence of ESBL-EC among residents of nursing homes (10,8% of all samples), compared to a relatively low prevalence in the general population (1.7% of all samples). The population wide study furthermore determined microbiome compositions at OTU level instead of species level, and did not include a longitudinal analysis in contrast to the study in nursing homes, which might have affected the resolution to detect associations between ESBL-EC colonization and microbiome composition. While it is still under debate which commensals in humans and dogs contribute to colonization resistance against *E. coli* intestinal carriage studies in mice seem more conclusive. In mouse models, commensal strains of *E. coli* were found to prevent colonization of toxin-producing *E. coli*, via competition for the same nutrients, while bifidobacterial strains were shown to successfully prevent mice against death as a result of toxin-producing *E. coli* (54, 55).

When studying the dog gut resistome in relation to ESBL-EC carriage by using ResCap targeted sequencing, we observed that dogs colonized with ESBL-EC have a higher abundance of *cmlA*, *dfrA*, *dhfR*, *floR* and *sul3* genes resistant genes. These resistance genes are commonly found in *Enterobacterales* and are likely to originate directly from the shared genera of *Escherichia-Shigella* and are less likely to originate from the genera that were found to be associated with ESBL-EC carriage, namely *Clostridium sensu stricto 1* and *Enterococcus*, *Lactococcus* (56-60). Furthermore, co-resistance to trimethoprim and sulfamethoxazole is commonly observed in ESBL-EC via plasmids that carry a combination of *sul1*, *sul2*, *sul3* sulphonamide resistance genes with either *dfrA* or *dhfr* trimethoprim resistance genes (61-63). Metagenomic Nanopore sequencing reveals that the gut microbiome of dogS111 contained genes *dfrA1*, *sat2_gen*, *aadA1*, $bla_{\text{TEM-10}}$, *sul2*, that are located on a single genetic element. For dogS128 ARGs *floR*, *dfrA36*, *sul2* and the class 1 integron specific recombinase *intI1* are also located on a single genetic element, which is in concordance with previous findings, and may result in the increased circulation of ARGs in the environment through co-selection (56, 64).

Implementation of novel techniques to study the dynamics of host-pathogen interactions during colonization

Generally, gut colonization precedes infection and spread by multi-drug resistant nosocomial pathogens. This is also true for VRE, an important nosocomial pathogen known for causing hospital associated infections and hospital outbreaks. Where enterococci normally represent low minority species in the gut microbiome, hospital admission and subsequent antibiotic use can result in outgrowth and turn enterococci

into one of the most abundant bacterial species in the gut microbiome (8). This outgrowth can result in infections in hospitalized patients and patients to patients spread (5, 8, 9, 15). It is not entirely understood which enterococcal determinants facilitate colonization or outgrowth and whether and how the human host responds to a situation when a member of the gut microbiome that normally represent a minority species starts dominating the gut microbiome.

Before elucidating *E. faecium*-human host cross-talk it was first needed to expand the molecular toolbox to be able to molecularly manipulate *E. faecium*. While a wide array of genome editing tools exist for well-studied bacteria like *E. coli*, the existing tools to genetically alter *E. faecium* are labour-intensive and limited in use (65). Current genome editing tools rely on rare homologues recombination events that result in the introduction and subsequent removal of antibiotic resistant genes in the genome of *E. faecium* (66). In chapter 4 of this thesis, we demonstrated how a hospital-associated vancomycin resistant *E. faecium* strain was genetically modified, using CRISPR-Cas9. This resulted in the generation of a gene deletion mutant without genomic scarring, that only required half the time compared to traditional methods. Furthermore, this system was applied for the efficient generation of a chromosome integrated GFP expressing *E. faecium* strain E745. Although proven powerful and efficient, this tool can be further optimization to facilitate its application. While the current design applies two plasmids, one for the expression of Cas9 and the other for that of CRISPR and a repair template, in future setups this can be reduced to a single functional plasmid, thereby introducing the complete system in a single transformation step, and furthermore reducing the change of plasmid incompatibility with plasmids that are naturally carried by enterococcal strains.

In chapter 5 of this thesis, we then applied *E. faecium* strain E745 producing GFP and *E. faecium* strain E8202 to study VRE colonization and enterococcus-host talk in a recently developed gut organoid model. Traditional gut models such as human cancer cell lines and mouse models introduce several limitations when studying host-response (67). While mouse models offer the advantage that treatment-induced effects are studied in a complete organism, including all different cell and tissue types and a fully developed immune system, limitations include scalability in terms of replicates and the variability that is introduced between replicates when studying such a complex system (68, 69). Even more importantly, while a mouse model has many similarities with the human body, important differences in the intestinal tract include dissimilarities in cell types, cell structure and microbiome composition (70). In contrast, human cancer cell lines represent human cells types more accurately and have the advantage of increased accessibility to the cells, compared to a mouse model. However, human cancer cell lines are composed of a limited number of cell types, that do not display a normal cell growth phenotype and sometimes express unique gene patterns (71). Although the organoid

model resolves some of these problems, it comes with its own set of limitations. When using organoids to generate human-derived colonic epithelium, the resulting cell layer is composed of different cell types such as colonocytes and mucus-producing goblet cells (72). These cells are connected via important cell structures like tight junctions and can produce components of the innate immune system, such as antimicrobial peptides (73-75). However, the organoid model lacks an adaptive immune system and currently, the culturing media is not yet fully standardized, which increases the risk of variations between replicate experiments (72, 76).

Co-culturing of *E. faecium* strain E745-GFP with colonic epithelium revealed close interaction between the colonic epithelium and the bacterial cells. We next applied a dual RNA sequencing approach in order to unravel the transcriptional dynamics of both *E. faecium* strain E8202 and human-derived colonic epithelium after 24 hours of co-culturing. In total 213 *E. faecium* genes were upregulated and 186 were downregulated. Of these genes several stood out, such as the gene operon encompassing *fms21* (*pilA*)-*fms20* pilus genes, which was upregulated (77). This is, to our knowledge, the first time that enterococcal pilus production is shown to be possibly associated with interactions with human-derived cells. We furthermore observed the upregulation of potential virulence factors, including the *sspP* gene, that encodes a putative staphopain peptidase C47. Although the role of *sspP* is unknown in enterococci, staphopain A in *Staphylococcus aureus* is part of the staphopain peptidase C47 family and a known key mediator of *S. aureus* virulence (78, 79). In contrast, we observed that two ATP-dependent proteases, *clpP* and *clpX*, were downregulated when *E. faecium* was exposed to colonic epithelium. In *E. faecalis* these genes influence the virulence. Cell stress was furthermore observed to upregulate *clpP* expression and consequently resulted in a downregulation of pyrimidine metabolism genes (80, 81). In our case downregulation of *clpP* coincided with upregulation of *pyrE* and *pyrC*, which seem to corroborate the findings in *E. faecalis*, that *clpP* expression is linked with pyrimidine metabolism. When studying gene expression during biofilm formation in a vanA-type vancomycin-resistant *E. faecium*, Lim et al., also found downregulation of the *clp* protease gene in biofilm cells relative to planktonic cells, however this did not seem to coincide with changes in the expression of pyrimidine metabolism genes (82).

When performing KEGG pathway analysis, we found in *E. faecium* an overall reconfiguration in metabolic pathways for energy supply when exposed to the colonic epithelium. This included the upregulation of pathways involved in purine and pyrimidine metabolism and the downregulation of nucleotide sugars and amino acid biosynthesis, including lysine and methionine biosynthesis. The shifts in expression suggest a large-scale reduction of glutamine and aspartate into adenylic acid (AMP), inosinic acid (IMP) and uridylic acid (UMP). Since most of the easily accessible

nutrients and carbon sources are absorbed in the small intestines, the microbiome of the colon is commonly composed of specialist bacteria, that have evolved to salvage energy from remaining sources like amino acids, purines pyrimidines as a primary source of carbon, nitrogen, and energy (83-86). Our results suggest that *E. faecium* might apply a similar strategy. Since colonocytes are able to metabolize glutamine as well, this specific finding is of interest as it may suggest competition over glutamine between *E. faecium* and colonocytes (87, 88). The increased reduction of glutamine by *E. faecium* may result in the accumulation of ammonia, known to disrupt tight junctions of host cells and a possible way for pathogens to disrupt the barrier function of the intestines (89). Whether or not *E. faecium* competes over glutamine and if ammonia is accumulated during *E. faecium* and colonic epithelium co-culturing is topic of future investigations.

Finally, we observed a downregulation of vancomycin resistance genes (*vanH*, *vanA*, *vanX*) and in lesser degree of *vanS*, upon co-culture with colonic epithelium. The *vanS* sensor histidine kinase can detect vancomycin and in turn activates the *vanR* response regulator, which then upregulates transcription of the vancomycin resistance genes *vanH*, *vanA*, *vanX*. In our experiments, culture media was not supplemented with vancomycin and vancomycin can therefore not be responsible for changes in the expression of vancomycin resistance genes. These results suggest that an alternative mechanism might be responsible for the changes in the expression of vancomycin resistance genes. Possibly growth of *E. faecium* in cell culture medium, which was the control condition caused more membrane stress than the condition with colonic epithelium, causing the observed downregulation of vancomycin resistance genes during co-culturing. Nevertheless, to our knowledge, this is the first time it has been shown that co-culturing *E. faecium* with human colonic epithelium does not induce expression of vancomycin resistance genes. Together, these results may imply that the vanA-type vancomycin resistant *E. faecium* strain E8202, used in this study, is still susceptible to vancomycin upon colonization of colonic epithelium, despite carrying the *vanA* operon. Future experiments involving vancomycin susceptibility assays are needed to determine if there are compound present in the co-cultures that can reduce the expression of vancomycin resistance genes, potentially by reducing membrane stress. This could be a very relevant finding as compounds that inhibit the expression of resistance could function as antibiotic adjuvants, enhancing vancomycin's potency and restoring antibiotic sensitivity to VRE.

On the host side we observed a large number of genes (219 genes) to be differentially expressed upon co-culturing with *E. faecium* strain E8202. This involved among others, the activation of genes in inflammation pathways and pathways implicated in cell death, increased expression of GPT-binding proteins, increased expression of guanine nucleotide exchange factors, decreased expression of Rho GTPases, and downregulation of G-protein signalling. However, critical assessment of the sequence depth of the host

(human) reads indicated that not all replicates reached the recommended sequencing depth of 10 million reads and revealed clear batch effects when comparing batch 1-3 with batch 4-6. This might explain why we do detect changes in gene expression that are involved in for instance inflammation and amino acid degradation, but that these changes in gene expression cannot be linked to large-scale changes in the expression of pathways. Future studies are required to delineate in more detail the host response, preferably using single-cell RNA-sequencing in order to unravel the cell-type specific response.

Future perspectives

We studied the effects of long-term dietary habits on the gut microbiome in a geographically confined region, the Netherlands. Using a cohort of 149 participants we observed a largely comparable microbiome composition in the different diet groups. However, the differences we observed were in concordance with the results found in a recently published much larger cohort of 8208 participants (28). With this cohort, Gacesa et al., were not only able to determine diet-induced effects, but also showed that other factors like genetics, exposome, and lifestyle affected the gut microbiome most and then continued to include these as confounding factors in models for subsequent analysis. By reducing the proportion of unexplained variance observed in the gut microbiome, the development of such models can help to increase the resolution when studying effectors of the gut microbiome.

Since there is a direct link between the gut microbiome and the gut resistome, the resolution of resistome analyse could also benefit from these advanced models, but it is important to acquire sufficient metadata about the studied cohort in order to do so. However, a limitation of studying the gut resistome is the fact that only a subset of the gut microbiome carries ARGs (90). Therefore, we applied ResCap in chapter 2 and 3 in order to enrich for ARGs and were able to find associations in the gut resistome of dogs (chapter 3) that matched those observed in the gut microbiome. When we first applied ResCap in chapter 2 alongside with MSS, we included a side-by-side comparison for the ability of each method to capture the resistome. The advantage of ResCap is the probe-based capture system, applied to enrich for ARGs prior to sequencing. By selectively sequencing the resistome, ResCap was expected to surpass the detection limit of MSS and detect ARGs that are present in low abundance (91). Indeed, when comparing the resistome sequencing results of 64 identical DNA samples by using a rarefaction curve, we confirmed that ResCap is able to detect a higher number of ARGs than MSS. Even when the sequencing depth of MSS is increased to 70M reads, it does not reach the level of sensitivity of ResCap.

However, ResCap does come with a set of limitations that are important to consider for future experiments. When applying ResCap, around 1.0 μg of DNA as input for the capture process is needed, while MSS sequencing can be performed with as much as 30 ng of input DNA. It is not always possible to extract high amounts of DNA from samples, and using less than 1.0 μg input DNA can significantly reduce the sensitivity of ResCap. The specific version of ResCap that was applied in our studies was designed using ARG databases of 2017. The probe set therefore lacks the most recently discovered ARGs, posing additional limitations. This means that the ResCap system can only capture DNA of known ARGs or putative ARGs, while this is not the case for MSS. Moreover, the designed probe set of the ResCap set-up that we used did not only contain probes for ARGs, but also probes for relaxases, heavy metal and biocide resistance genes (91). While this might add to the possibility of capturing other biologically important genes, it also increases the risk of capturing high abundance genes that will therefore reduce the sensitivity of confirmed ARGs. The high costs of the probe capture step of the ResCap system of €1000,- per sample or sample pool, introduces a final limitation when studying the gut resistome, resulting in limiting sample sizes of studies due to these financial constraints.

Regardless these limitations, we clearly showed that the enrichment of ARGs through capture-based sequencing is a promising tool with more resolution than MSS to determine the dynamics of the gut resistome. By adjusting the probe set and design of capture-based sequencing, competitors such as the myBaits platform of Arbor Biosciences, allow for alternative designs to capture the resistome at a price of €180,- per sample or sample pool. This increase in cost-effectiveness and applicability of capture-based sequencing allows future studies to reach an increased sequencing depth and to apply larger sample sizes, thereby resolving some of the current limitations observed with ResCap (92, 93).

In chapter 3 we were able to determine associations between the dog microbiome composition and ESBL-EC carriage using 16S rRNA gene sequencing. We did find genera that were found in higher or lower relative abundance comparing ESBL-EC positive and negative dogs but we could not reliably determine which species or subspecies accounted for these differences in relative abundance. MSS is known to be able to detect differences at bacterial species level in the human gut microbiome, as we also showed in chapter 2 of this thesis, and should therefore be applied to e.g. identify species that may act as antagonists to ESBL-EC in the gut microbiome of dogs and potentially also in that of humans. The interplay between these species can additionally be determined when MSS is combined with metatranscriptomics. While performing metatranscriptomics on a complex community like the gut microbiome is still challenging, its resolution might be sufficient to reveal part of the inter-species crosstalk, which may be an important

factor in ESBL-EC colonization (94). We furthermore pointed out that the application of long-read metagenomic sequencing allows to distinguish between bacterial species and simultaneously reveals large genomic fragments that can harbour multiple ARGs. Long-read metagenomic sequencing is therefore a promising tool to acquire information about ARG context and possibly species origin, when implemented on a larger scale than done in the study in chapter 3. It can be utilized to compare for shared species in the gut microbiome between humans and dogs, or alternatively for shared genomic fragments with ARGs, thereby gaining more insight in the gene flow between companion animals and their owners. The continuous developments of long-read sequencing will help to increase the cost-effectiveness making it possible to increase the number of samples and sequencing depth and therefore allows for realising an increased resolution in the detection of bacterial species and ARGs. Developments that could be combined with long-read sequencing include metagenomic chromosome conformation capture (meta3C or Hi-C), where two DNA fragments (e.g. chromosome and plasmids) are linked in 3D space to retain the associations of plasmids and bacterial species, thereby further facilitating the contextual information of biological important genes such as ARGs (95, 96).

To further investigate the interaction between clinically important members of the gut microbiome and their host we applied a model using colonic epithelium to study *E. faecium*-host cross-talk. This revealed that *E. faecium* was found to rewire its transcriptome to adapt to this new environment. These findings should be validated using other models, to counteract the potential limitations of the colonic epithelium model. However, the initial results of this study suggest that colonic epithelium can be applied to study aspects of bacterial colonization. Because of large scale genomic differences between *E. faecium* strains from hospitalized patients, called clade A1 and *E. faecium* isolates colonizing healthy humans primarily clustering outside this clade, it could be of interest to compare bacterial-host crosstalk between the different *E. faecium* clades. Furthermore, this model can be applied to study the effect of small communities on the colonization resistance against opportunistic pathogens. Alternatively, the effectiveness of antagonists to counteract overgrowth by opportunistic pathogens can be studied in a more complex community in order to assess their importance in the gut microbiome or even as potential probiotics.

Summary

The results presented in this PhD thesis highlight the importance of the gut microbiome as a reservoir of potential opportunistic pathogens as well as of antibiotic resistance genes carried by the microbiome. The results of these studies show that humans and dogs without known underlaying diseases, can be carrier of clinically relevant antibiotic resistance genes. We determined that the gut microbiome of humans from the general population contains bacteria that carry resistance genes against several classes of antibiotics. While we did not find strong diet-induced associations with ARGs, we did observe diet-induced associations with particular members of the gut microbiome. In the case of the dog microbiome, we established that these are frequent carriers of ESBL-EC, and that this carriage is associated with several differentially abundant bacterial genera. Among the bacteria that were increased in abundance in dogs carrying ESBL-EC positively, we detected species that are opportunistic pathogens and additional studies can provide more insight if dogs carrying ESBL-EC from a reservoir from which these and other potential pathogens are transferred to humans. Enterococci were among the bacteria that were found associated with ESBL-EC carriage. Since *E. faecium* was among the detected opportunistic pathogens and because it is clinically relevant for humans, we characterized the adaptive response during colonic epithelium colonization. This revealed that over the course of 24 hours, *E. faecium* rewires its transcriptome to adapt to this new environment. These findings should be validated using alternative methods, for instance by generating knock-outs using the developed CRISPR-Cas9 system, in order to substantiate the importance of some of these adaptations during colonization. More in-depth knowledge about the role of specific adaptive elements in gut colonization and/or intestinal overgrowth may provide new targets for new anti-infective therapies to curtail ecological dominance of *E. faecium* in the gut of hospitalized patients and prevent subsequent infections and clonal spread.

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Appendices

Nederlandse samenvatting

In tegenstelling tot andere organen in ons lichaam zijn de darmen één van de weinige organen waar een rijke variatie aan bacteriën leeft. Doorgaans bevinden er zich honderden verschillende soorten bacteriën, die samen onderdeel uitmaken van de darmmicrobiota. Als gevolg van onderlinge competitie van bacteriën voor zowel voedingsstoffen als ruimte, vormt er zich uiteindelijk een balans in de samenstelling van de darmmicrobiota. In deze staat is de darmmicrobiota minder gevoelig voor kolonisatie van nieuwe, externe bacteriën, zoals pathogenen. Hierdoor draagt de darmmicrobiota, samen met het immuunsysteem van de gastheer, bij aan de afweer tegen pathogene bacteriën. Daarnaast speelt de darmmicrobiota een belangrijke rol bij de acquisitie van voedingsstoffen en zijn specifieke darmbacteriën geassocieerd met darmaandoeningen en ziektes.

In **hoofdstuk 1** wordt beschreven welke factoren invloed kunnen hebben op de samenstelling van de darmmicrobiota. Dit betreft zowel interne factoren (e.g. leeftijd, geslacht en genetische factoren) als externe factoren (e.g. medicatie, roken, alcoholconsumptie en dieet). De darmmicrobiota is niet alleen een belangrijk reservoir voor bacteriën, maar ook voor de antibioticaresistentiegenen (ARGs) die ze bij zich dragen. Deze ARGs vormen tezamen het resistoom. Zowel commensale als pathogene bacteriën kunnen ARGs bij zich dragen en zijn bovendien in staat om deze onderling uit te wisselen in het darmmilieu. Om deze reden kan het bestuderen van externe factoren, bijdragen aan onze kennis van consequente veranderingen in de prevalentie van zowel opportunistische pathogene bacteriën als van ARGs. Hoewel voeding een geleidelijke verandering aanbrengt in de darmmicrobiota, is het gebruik van antibiotica een voorbeeld van een externe factor die voor een abrupte verandering kan zorgen in de darmmicrobiota. Dit kan ertoe leiden dat de balans wordt verstoord en dat de proliferatie van pathogene bacteriën wordt toegelaten. Dit proces treedt op bij zowel extended-spectrum beta-lactamase-producerende *Escherichia coli* (ESBL-EC) als bij vancomycine-resistente enterococcen (VRE). ESBL-EC en VRE zijn moeilijk te behandelen omdat ze efficiënt antibiotica resistentie genen (ARGs) kunnen opnemen uit de omgeving en kunnen integreren in hun genoom. Ook zijn deze bacteriën dusdanig aangepast aan een ziekenhuisomgeving, dat ze zich efficiënt kunnen verspreiden onder patiënten. Hoewel ESBL-EC en VRE in staat zijn om te prolifereren in de darmen, vinden de consequente infecties meestal buiten de darmen (extra-intestinaal) plaats. Waar ESBL-EC in staat is om bacteriëmie, pneumoniae en urineweginfecties te veroorzaken, geeft VRE voornamelijk risico op katheter-gerelateerde infecties. Deze extra-intestinale infecties berusten echter op de initiële proliferatie in de darmen en de darmkolonisatie die hieraan voorafgaat. Op deze darmkolonisatie wordt daarom onder andere de focus gelegd in dit proefschrift.

In **hoofdstuk 2** staat de samenstelling van de darmmicrobiota en het bijbehorende resistoom van deelnemers uit de Nederlandse bevolking beschreven. Om te bepalen of dieet invloed kan hebben op de samenstelling van de darmmicrobiota en het darmresistoom, zijn de volgende dieetgroepen onderzocht: omnivoren, pescotariërs, vegetariërs en veganisten. Wanneer deze groepen worden vergeleken op onderlinge verschillen in de darmmicrobiota, blijkt dat voornamelijk de darmmicrobiota van veganisten verschilt van die van omnivoren. Zo bevat de darmmicrobiota van veganisten doorgaans minder *Streptococcus thermophilus* en *Lactococcus lactis*. Dit zijn beide melkzuurbacteriën en deze staan mogelijk in verband met de consumptie van melkproducten, aangezien omnivoren wel melkproducten consumeren en veganisten niet. Voor het bepalen van het resistoom is gebruik gemaakt van een nieuwe techniek genaamd ResCap. ResCap bleek met hoge resolutie ARGs in kaart te kunnen brengen. Echter werden er geen verschillen gevonden bij het vergelijken van het darmresistoom tussen de dieetgroepen.

In **hoofdstuk 3** wordt het verloop van de darmmicrobiota van honden in huishoudens beschreven, over een periode van 6 maanden. Voorheen is vastgesteld dat de darmmicrobiota van honden gevoelig is voor kolonisatie met ESBL-EC. Uit ons onderzoek blijkt dat over een periode van 6 maanden, 68% van de honden in Nederlandse huishoudens drager zijn van ESBL-EC. Opmerkelijk is de associatie van ESBL-EC dragerschap en een toename van de hoeveelheid *Clostridium sensu stricto 1*, *Enterococcus*, *Lactococcus* en *Escherichia-Shigella*. Vervolgonderzoek indiceerde dat de opportunistische pathogenen *Clostridium perfringens* en *Lactococcus garvieae* hierbij mogelijk betrokken zijn. Zowel *C. perfringens*, *Enterococcus* als *E. coli* zijn opportunistische pathogenen, die geassocieerd worden met darmaandoeningen bij honden. Daarnaast wordt ESBL-EC dragerschap geassocieerd met een afname van een tiental andere bacteriën, waaronder *Faecalibacterium*, *Holdemanella* en *Prevotella*, die beschouwd worden als commensalen of zelfs symbionten. Vervolgonderzoek moet vaststellen wat de rol is van deze potentiële symbionten en of deze gebruikt kunnen worden om dragerschap van opportunistische pathogenen tegen te gaan. Op een vergelijkbare manier zijn er associaties gevonden tussen ESBL-EC dragerschap en een verhoogde hoeveelheid ARGs in het darmresistoom, in het specifiek van de genen *cmlA*, *dfrA*, *dhfR*, *floR* en *sul3*. Waarschijnlijk bevindt een deel van deze genen zich op één enkel stuk DNA, wat de verspreiding van antibioticaresistentie kan bespoedigen.

In **hoofdstuk 4** en **hoofdstuk 5** worden nieuwe technieken besproken, die gebruikt kunnen worden om het mechanisme van darmkolonisatie van *Enterococcus faecium* te kunnen achterhalen. CRISPR-Cas9 is een eiwit dat gebruikt kan worden om specifiek modificaties in het DNA aan te brengen. Deze eigenschap is verwerkt in een systeem om deze modificaties efficiënt te introduceren in *E. faecium*, ten behoeve van onder

andere kolonisatiestudies. Om vast te stellen hoe *E. faecium* in staat is om efficiënt de darmen te koloniseren, is er gebruik gemaakt van een colon organoid model. Dit organoid model bestaat uit stamcellen, geïsoleerd uit darmweefsel van een donor, die met behulp van specifieke groeicondities opgekweekt en kunnen worden tot colon weefsel. Deze bevatten verschillende celtypes en reflecteren daarmee grotendeels het weefsel in de humane colon. Met behulp van een pathogene vancomycine-resistente *E. faecium* stam, die oorspronkelijk geïsoleerd is uit een patiënt, kon vervolgens de interactie tussen colon organoids en *E. faecium* worden onderzocht. Wanneer *E. faecium* voor 24 uur is blootgesteld aan organoids, vindt er een verhoogde expressie plaats van genen die betrokken zijn bij de aanhechting van cellen (*fms21-fms20 pilA* operon). Daarentegen is de expressie van vancomycine resistentiegenen verlaagd (*vanH*, *vanA*, *vanX*). Tot slot vindt er een grootschalige verandering plaats in de expressie van metabolische processen die betrokken zijn bij de energiehuishouding. Deze verandering suggereert dat *E. faecium* zich richt op de reductie van twee aminozuren (glutamine en aspartaat). De reductie van glutamine speelt mogelijk een belangrijke rol in de interactie tussen *E. faecium* en darmcellen, omdat glutamine ook wordt gebruikt door specifieke darmcellen (colonocyten). De eventuele veranderingen in genexpressie van de colon cellen door de interactie met *E. faecium* was minder duidelijk. Dit kan hoogstwaarschijnlijk deels verholpen worden door een nog te optimaliseren stap in de gebruikte methodes.

In **hoofdstuk 6** worden de bevindingen uitgebreid bediscussieerd. De toegepaste technieken blijken belangrijke instrumenten te zijn om complexe processen te ontdekken en te bestuderen. Dit geldt zowel voor de ResCap techniek, als voor CRISPR-Cas9 en het organoid model. Met behulp van deze technieken is aangetoond dat de darmmicrobiota een reservoir vormt voor zowel opportunistische pathogenen als voor ARGs. Daarnaast laat dit proefschrift zien hoe het organoid model gebruikt kan worden om de interactie te bepalen tussen opportunistische pathogenen en darmcellen en dat het tot nieuwe bevindingen kan leiden.

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Curriculum Vitae

Paul Stege was born on April 6th in Groningen, the Netherlands. He obtained his secondary school diploma (Havo) at the Aletta Jacobs college in Hoogezand in 2010 and enrolled into the Biology & Medical Laboratory Research program at the Hanze University of Applied Sciences in that same year. Here, he completed his bachelor's degree and continued to enrol into Microbial Biotechnology and Health program at the Leiden University in 2014. In 2016, Paul obtained his master's degree. During his bachelor study, Paul performed an internship at the Laboratory of Gene Technology, part of KU Leuven (Belgium). Here, he worked on the characterization and domain shuffling of endolysins under the supervision of dr. Yves Briers and prof. dr. Rob Lavigne.

During the master study, Paul followed his first internship at the department of Molecular Microbiology and Biotechnology at Leiden University. During this time, he worked on CRISPR/Cas9-based technology for rapid generation of gene knock out in *Aspergillus niger*, under the supervision of dr. Jean Paul Ouedraogo and dr. Arthur F.J. Ram. During his second internship he studied the virulence of *Streptococcus pneumoniae* as a function of stochastic fluctuations. This internship took place at the department of Molecular Genetics at the University of Groningen, under the supervision of dr. Lance E. Keller and prof. dr. Jan-Willem Veening.

In 2017, Paul joined the group of prof. dr. Rob J. L. Willems as a PhD-candidate at the department of Medical Microbiology at the University Medical Center Utrecht (UMCU). This PhD project was part of the Netherlands centre of one health (NCOH) program and was supervised by prof. dr. Rob J. L. Willems and dr. Fernanda L. Paganelli. Here he studied the dynamics in the human and dog intestinal microbiota and resistome. He additionally worked on pathogen-organoid co-cultures to disclose host pathogen cross-talk. The results of his research are presented in this thesis.

Paul lives in IJsselstein with Amber and is currently working as a bioinformatician, in the group of multi-omics analytics and bioinformatics with group leader prof. dr. Alex Bossers at Wageningen Bioveterinary Research (WBVR).

List of publications

Impact of long-term dietary habits on the human gut resistome in the Dutch population
Paul B. Stege, Joost Hordijk, Sudarshan A. Shetty, Michael Visser, Marco C. Viveen, Malbert R. C. Rogeres, Esther Gijsbers, Cindy M. Dierikx, Rozemarijn Q. J. van der Plaats, Engeline van Duijkeren, Eelco Franz, Rob J. L. Willems, Susana Fuentes, Fernanda L. Paganelli.
Scientific Reports, 12:1892 2022

Ablation of liver *Fxr* results in an increased colonic mucus barrier in mice
Noortje Ijssennagger, Kristel S. van Rooijen, Stefanía Magnúsdóttir, José M. Ramos Pittol, Ellen C L Willemsen, Marcel R. de Zoete, Matthijs J. D. Baars, Paul B. Stege, Carolina Colliva, Roberto Pellicciari, Sameh A. Youssef, Alain de Bruin, Yvonne Vercoulen, Folkert Kuipers, Saskia W. C van Mil.
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