The impact of antibiotic exposure on the human microbiome and resistome: from laboratory to clinical studies

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List of Papers

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Characterization of a signaling system in *Streptococcus mitis* that mediates interspecies communication with *Streptococcus pneumoniae*.
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- III. Sturød K, Dhariwal A, Dahle UR, Vestrheim DF, Petersen FC. **Impact of narrow-spectrum penicillin V on the oral and fecal resistome in a young child treated for otitis media.**
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Candidate's contribution

Paper I

- Contributed in study design, methodology, carried out experiments, analyzed data and wrote the manuscript with input from all authors.

Paper II

- Contributed in methodology, experiments and analysis concerning antibiotic exposure. In addition, discussed the results with co-authors and contributed in drafting and commenting on the final manuscript.

Paper III

- Contributed in study design, methodology, applying for ethical approval, sample preparation, interpretation of the results and wrote the manuscript with input from all authors.

Paper IV

- Contributed in study design, methodology, sample preparation, interpretation of the results and wrote the manuscript with input from all authors.

List of Abbreviations

AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
Com	Competence
CSP	Competence stimulating peptide
DNA	Deoxyribonucleic acid
ESBL	Extended spectrum beta-lactamase
GAS	Group A <i>Streptococcus</i>
GBS	Group B <i>Streptococcus</i>
HGT	Horizontal gene transfer
HMP	Human microbiome project
IACG	Interagency Coordination Group
IBD	Inflammatory bowel disease
ICE	Integrative and conjugative elements
MIC	Minimum inhibitory concentration
MLS	Macrolide, lincosamide and streptogramin
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NGS	Next generation sequencing
PCR	Polymerase chain reaction
QS	Quorum sensing
Rgg	Regulator gene of glucosyltransferase (and proteins in the same family)
RNA	Ribonucleic acid
SHP	Short hydrophobic peptide
SigX	Alternative sigma factor
TSB	Tryptic Soya Broth
WHO	World Health Organization
XIP	<i>sigX</i> inducing peptide

Introduction

1. General Introduction to Antibiotics and Antimicrobial Resistance (AMR)

1.1 Historical Insight to Antibiotics and Antimicrobial Resistance

In 1677, Antoni von Leeuwenhoek discovered some tiny organisms in his self-constructed microscope. He called them “animalcules”, and this was probably the very first observation of bacteria ¹. At this point, there was no known connection between the observed animalcules and disease. About 200 years later, the theory of microorganisms causing disease, “The Germ Theory” gained increasing acceptance in the scientific community, much thanks to research by the famous scientists Robert Koch, Ignaz Semmelweis and Louis Pasteur ²⁻⁵. Their work and the germ theory opened up for new important research questions. As the origin of some diseases now were known, would it be possible to discover a cure?

In the search to find a substance or a remedy that could treat specific infection-causing agents in the early 20th century, scientists had different approaches. The German physician Paul Ehrlich had a systematic approach where he screened several hundred compounds in his search for a curative agent. In 1909, compound number 606 (arsphenamine) was found successful in targeting *Treponema pallidum*, which causes syphilis ⁶. Soon after the discovery, the drug was out on the market under the name Salvarsan ⁷. The same systematic approach also led the German pathologist Gerhard Domagk to the discovery of the first sulfa drug in 1932 ⁸. Other scientists, like the British physician and researcher Alexander Fleming, did a more accidentally discovery. In 1928, after returning from a summer vacation in Scotland to St. Mary’s Hospital in London, Fleming realized that he had forgotten to put one of his petri-dishes with *Staphylococcus aureus* culture in the incubator before he left. On this petri-dish, a moldy colony had contaminated the bacterial culture while he was gone. When he examined the plate more closely, he observed a bacteria-free zone around the mold ⁹. The mold was later isolated in pure culture by Fleming, and eventually identified as *Penicillium notatum* by the American microbiologist Charles Thom ¹⁰. By the help of several other scientists, including Howard Florey and Ernst Boris Chain, penicillin evolved from a moldy agar plate in 1928 to a lifesaving drug during the beginning of the 1940s ¹¹⁻¹³.

The discovery of penicillin by Fleming, together with the synthesis of sulfonamides in the 1930s, marked the beginning of the antibiotic era. In the following three decades, the

discovery and synthesis of several other antibiotic classes and drugs contributed to a revolution within modern medicine. This period is often referred to as the golden era of antibiotic discovery. Since then, there has been a discovery void and only a few new compounds have been approved for clinical use after this golden era ¹⁴.

For every new antibiotic discovery there has been a grey cloud hanging over it, namely the observation that soon after introduction of a new antibiotic agent, resistant bacteria have appeared ¹⁵. In 1948, three years after penicillin hit the market, resistant penicillinase producing staphylococci became a considerable problem ¹⁶. To combat penicillinase-producing bacteria, methicillin was introduced in 1959. However, less than two years later, the first reports of methicillin resistant *S. aureus* (MRSA) were published ^{17,18}. This pattern, with resistance rapidly following the introduction of new drugs, was unfortunately also the case for most other antibiotic agents introduced into the market. Interestingly, already in 1945, Fleming warned about how penicillin could change the nature of the microbe if not used correctly. In his Nobel Prize Lecture he stated: “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body” ¹⁹.

Today, 75 years and many new discoveries later, convincing scientific evidence shows that the extensive use of antibiotics in the last century have accelerated the evolution of resistant bacteria, leaving us soon without effective treatment options towards bacterial infections ²⁰. Recent reports from the World Health Organization (WHO) and the United Nations Interagency Coordination Group (IACG) have stated that a post-antibiotic-era is a real possibility for the twenty-first century, and that there is no time to wait; “unless the world acts urgently, antimicrobial resistance will have disastrous impact within a generation.” ^{21,22}.

1.2. Antibiotics – Classification and Mode of Action

Antibiotics are defined as drugs that kill or inhibit growth of bacteria. Prior to human intervention, microorganisms have produced antibiotic molecules for millions of years²³, though in concentrations much lower than those used in antibiotic therapy. The low concentrations have led to a debate about the primary function of these molecules, as it may be too low to kill competitors. Interestingly, it has been suggested that natural antibiotic molecules may function as signal molecules, having an effect on bacterial physiology²⁴⁻²⁷. Most antibiotic drugs used today are natural compounds or derivatives of compounds produced by microorganisms. Only a few antibiotic classes are completely synthetic (e.g. trimethoprim, sulfonamides and quinolones)²⁸.

1.2.1. Classification and Mode of Action

Different antibiotic classes are separated by their molecular structure, and most classes contain several compounds including similar or modified versions of the initial type. Further, antibiotic drugs can be classified using several different approaches²⁸.

Commonly, antibiotics are classified according to the range of species/groups of bacteria it affects. Narrow-spectrum antibiotics have a limited spectrum of activity that affects a specific subset of bacterial species, such as Gram-positive or Gram-negative bacteria. Broad-spectrum antibiotics have a wider range of activity, affecting both Gram-positive and Gram-negative bacteria. However, among narrow-spectrum antibiotics, some are narrower than others and among broad-spectrum antibiotics some are broader than others. Despite the common use of the terms “narrow” and “broad” spectrum activity, there is no international consensus on the definition of the two categories, and the terms are often presented differently in the literature²⁹⁻³¹.

A second mode of classification is based on the effect of the antibiotic drugs on the bacteria, either bacteriostatic or bactericidal. A bacteriostatic antibiotic inhibits growth while a bactericidal antibiotic aims to kill the bacteria. Notably, the definition is slightly vague as many antibiotic agents may potentially act both bactericidal and bacteriostatic depending on growth conditions and antibiotic concentrations³².

A third way of categorizing antibiotic drugs is by their target site ³³. A successful antibiotic agent targets bacteria-specific structures without being toxic to the individual receiving the treatment ³⁴. The number of different antibiotic agents on the market is large, however, the number of antibiotic targets in the bacteria is limited, with three major target sites used by most of the successful antibiotic agents ³³. The three most used targets for antibiotic agents are the cell wall ³⁵ (e.g. beta-lactams, glycopeptides), the protein synthesis apparatus ³⁶ (e.g. aminoglycosides, macrolides and tetracycline) and the nucleic acid metabolism and repair system ³⁷ (e.g. quinolones). In addition to these three groups, there are also antibiotic agents that affect membrane integrity^{38,39} (polymyxins, daptomycin), and folic acid metabolism (trimethoprim, sulfonamides) ⁴⁰.

Antibiotic agents used in Paper I – Paper IV of this thesis are described in more detail in table 1 ²⁸.

Table 1: Classifications and mechanisms of antibiotic agents included in this thesis

Paper	Antibiotic	Class	Origin	Mode of action	Spectrum
Paper I	Novobiocin	Aminocoumarin	Natural compound	Inhibition ATP hydrolysis and DNA supercoiling activity (GyrB)	Broad Spectrum
Paper I	Streptomycin	Aminoglycoside	Natural compound	Inhibition of protein synthesis by binding of 30S ribosomal unit.	Broad Spectrum
Paper I	Tetracycline	Tetracycline	Natural compound	Inhibition of protein synthesis by binding of 30S ribosomal unit.	Broad Spectrum
Paper I & II	Ampicillin	Beta-lactams (Aminopenicillin)	Semi-synthetic derivative of penicillin	Inhibition of cell wall biosynthesis	Broad Spectrum
Paper I & II	Ciprofloxacin	Quinolone (2 nd generation)	Synthetic	Interference with DNA replication and transcription (GyrA)	Broad Spectrum
Paper I & II	Erythromycin	Macrolide	Natural compound	Inhibition of protein synthesis by binding of 50S ribosomal unit.	Narrow Spectrum
Paper II	Nisin	Lantibiotic (bacteriocin)	Natural compound	Disruption of membrane function by pore formation	Narrow Spectrum
Paper III	Amoxicillin	Beta-lactams (Aminopenicillin)	Semi-synthetic derivative of penicillin	Inhibition of cell wall biosynthesis	Broad Spectrum
Paper IV	Penicillin V	Beta-lactams (Natural Penicillin)	Natural compound	Inhibition of cell wall biosynthesis	Narrow Spectrum

1.3. Antibiotic resistance

Antibiotic resistance is defined as the survival of bacteria when exposed to antibiotic concentrations that would normally stop their growth or kill them. Similarly to antibiotic molecules, antibiotic resistance genes (ARGs) can be found in natural environments free from human intervention ⁴¹. In fact, scientists have identified several ARGs from 30 000 year old Beringial permafrost sediments, showing that this is a natural and ancient phenomenon ⁴². However, the situation we are in today, with resistant bacteria leading to treatment failure, is not a natural phenomenon, but a consequence of overuse of antibiotics for a long time ²⁰. Now, the United Nations, including the WHO, states that antibiotic resistance is one of the biggest threats to global health, food security, and development today ^{21,43}.

1.3.1. Mechanisms of antibiotic resistance

Bacteria evolve and survive by developing strategies of resistance to avoid being killed by antimicrobial molecules. The most common strategies fall into three main categories depending on their mechanistic action, (I) - enzymatic breakdown or modifications of the antibiotic molecule, (II) - changing or protecting the target site or (III) - keeping the drug away by efflux mechanisms (extrusion) or preventing penetration into the bacteria (Figure 1) ⁴⁴. Resistance towards one antimicrobial class can be achieved through various strategies ⁴⁵, and one bacterial cell may be able to use several mechanisms to survive attacks from antibiotics ⁴⁶. Some mechanisms, e.g. multi-drug-efflux pumps, may affect many different classes of antibiotics⁴⁷, while mechanisms such as antibiotic inactivation usually are specific to one, or a similar group of antibiotic classes ^{44,48}.

Figure 1: Antibiotic resistance strategies in bacteria.

1.3.2. Emergence and Spread of Antimicrobial Resistance

The accelerating emergence of antibiotic resistance observed over the last decades is an example of a Darwinian selection process, complicated by several interlinking drivers of resistance (Figure 2) ⁴⁹. The main driver of resistance is use of antibiotic drugs, which directly or indirectly exposes bacteria to inhibitory and subinhibitory concentrations of antibiotics ⁵⁰⁻⁵⁴. This exposure leads to high selection pressure, which contributes in accelerating the emergence of resistant bacteria ²⁰. Antibiotic selection pressure occurs everywhere antibiotics are present, including in humans, animals, plants, and in the environment. This has led to the development of a One Health approach in tackling the challenge of antibiotic resistance.

Bacteria can develop resistance to antibiotics in two main ways. Either by mutating existing genes or by acquiring external genetic material via horizontal gene transfer (HGT) ⁴⁹.

Spontaneous mutations occur as a random process during DNA replication. The short generation time enables bacteria to evolve rapidly to changes in the environment such as

antibiotic selection pressure. Mutations leading to antibiotic resistance can include mutations affecting a gene product, such as a target site protein, or it can include changes in regulation of gene expression, leading to over- or under expression of a gene ⁵⁵. Recently, epigenetic modifications in relation to antibiotic stress have gained increased attention, suggesting that epigenetic mechanisms may increase mutation rate and contribute in resistance development ^{56,57}. The ability of taking up external genetic elements by HGT is the second way bacteria acquire resistance ⁵⁸. Transfer of genes between bacteria contributes greatly to the challenge of antibiotic resistance we experience today ^{59,60}. The mechanisms of HGT will be described in more detail in the following section.

In addition to spread of ARGs between bacteria, the resistant bacteria itself can spread in the environment with food ⁶¹ and water ⁶², between humans and animals ⁶³, among healthcare institutions and the community ⁶⁴, and they can spread all over the world through travel ⁶⁵. All these factors contribute to the current situation with increased prevalence of resistance worldwide and make it difficult to get under control.

Figure 2: Interlinking network of ARG spread. The figure is based on Davies and Davies, 2010 ²⁰.

1.3.3. Horizontal Gene Transfer

In contrast to vertical transmission, which happens from parent to offspring, HGT is the exchange of genetic material between organisms of the same or different species ⁶⁶, and even between kingdoms (bacteria to yeast or plants) ^{67,68}.

In prokaryotes, HGT is involved in acquisition of new genes and functions, such as virulence genes and antibiotic resistance genes. The recent development of sequence-based genomics has highlighted the tremendous impact HGT has on genome evolution in bacteria ^{69,70}. There are three main mechanisms of genetic exchange between bacteria: Conjugation, Transduction and Transformation (Figure 3).

Conjugation is a mechanism that depends on direct contact between bacterial cells. A pilus, or a hollow tube, is formed between the two cells and provides a channel for DNA to move from the donor cell to the recipient, as described already in 1946 by Lederberg & Tatum ⁷¹. Mobile genetic elements such as plasmids and integrative and conjugative elements (ICEs) are transferred using conjugation and are also mediators of the process itself ^{72,73}.

Transduction is the transfer of DNA from one cell to another by bacteriophages ⁷⁴.

Bacteriophages are viruses that incorporate genes from bacteria into its own genome. Further, injecting their genomes into another bacteria where it can either exploit the cell machinery for replication followed by cell lysis (lytic life cycle), or integrate into the bacterial genome and replicate as part of the host chromosome (lysogenic life cycle) ⁷⁵.

Transformation involves active uptake of free DNA from the bacteria's surroundings and incorporation of that DNA into its own genome. For transformation to occur, bacteria need to be in a competent state, and it requires a set of proteins for DNA uptake and the subsequent homologous recombination. The transformation machinery and its regulation varies between different bacterial genera and species. One of the most studied genera regarding natural transformation is the genus *Streptococcus*. Natural transformation is recognized as a major driver in streptococcal evolution. The mechanisms and regulation of streptococcal transformation are further discussed in section 4.4.1.

Figure 3: Transmission of DNA between bacteria via HGT.

2. The human microbiome

The human body is home to more than just human cells. Microorganisms are found in and on our bodies in vast amounts ⁷⁶. They inhabit the skin, mouth, nose, respiratory tract, genital tract and gastrointestinal tract, where they form site-specific eco-systems ⁷⁷ and contribute in a range of important functions, including metabolism ⁷⁸, nutrition ⁷⁹, endocrine functions ⁸⁰, protection against pathogenic overgrowth ⁸¹ and development and maturation of the host's immune system ⁸². The collection of all microorganisms in our bodies is known as the human microbiome, and the microbial genes in our microbiome outnumber the genes in our human genome by about 100 to one ⁸³. The human microbiome consists of mostly bacteria, but also includes viruses, archaea and eukaryote organisms.

The terminology in this field can be confusing as there are different definitions for the term “microbiome”. Some authors refer to the microbiome as the genetic content of the microorganisms in an environment, while others refer to the microbiome as the entire habitat of a niche, including the microorganisms and their genomes, and even the environmental conditions ⁸⁴. In this thesis, the term “microbiome” refers to the entire microbial habitat, including both the microorganisms and their genomes.

The last decades, advances in next generation sequencing technology has led to an enormous interest and research activity on the composition and functions of the microbiome. One of the first large-scale research initiatives within this field was the Human Microbiome Project (HMP), launched in 2007 and funded by the US National Institutes of Health ⁸⁵. The project sampled microbes from multiple sites from the mouth, nose, skin, colon and vagina. They included 300 healthy individuals, collected over 11000 samples in total, and contributed to a better understanding of the complexity of the microbial communities living in our bodies ⁸⁶.

2.1 Development of microbiome in young children

The composition of the human microbiome is influenced by the way we are born, where we are born, whom we live with, what we eat and medications we take, among several other variables ⁸⁷. The first major encounter a baby has with the microbial world is during birth. Depending on the mode of birth, vaginal or by cesarean section, the baby is covered with bacteria from its mother's vaginal tract (+ intestinal tract) and/or skin. This initial colonization

will affect the pattern of further colonization and microbial development, which may have lifetime implications⁸⁸. Other important factors for the development of the infant microbiome are diet (breast-feeding vs formula feeding)⁸⁹, host genetics⁹⁰, environment (siblings, pets), rural vs urban living, hospitalization and infrastructure⁹¹⁻⁹³ and antibiotic consumption (Figure 4)⁹⁴. Development and maturation of the microbiome differs between different body habitats. In the gut, there is a major shift in the microbial community around the time when solid food replaces breastmilk. From there, the gut microbiome will become more diverse until it stabilizes around 3 years of age⁹⁵. By contrast, the composition of the skin microbiome is restructured during puberty due to increased levels of hormones stimulating the sebaceous glands⁹⁶. In the mouth, there are constant changes due to tooth development in young children, and as recently reported, the oral microbiome is still distinct from that of their caregivers at 4 years old⁹⁷.

Figure 4: Microbiome development. Figure is based on Tamburini *et al.*, 2016, Nature Medicine⁸⁷.

2.2 Microbiome in health and disease

As mentioned in the first section, the understanding of microbes and their role in disease was recognized by famous scientists like Pasteur and Koch in the mid-19th century. Individual bacterial species were cultivated, isolated and then identified as causative agents of specific diseases. Later, scientists observed bacteria living in complex communities in the environment and in our bodies. Interestingly, most of these bacteria did not cause disease and were living in symbiosis with the host and are now known as commensals. Microbes within these communities interact with other microbes and their host, and this interplay has gained increased interest in the recent years ⁹⁸. Particularly, culture-independent ‘omic’-technologies, (genomics, transcriptomics, proteomics and metabolomics) have emphasized the role of the microbiome in human health and disease ⁹⁹. An imbalance of the microbiome is often referred to as a dysbiosis and it has been associated with several conditions and diseases. Conditions such as inflammatory bowel disease (IBD), obesity, type 2 diabetes and colon cancer have been associated with an unhealthy gut microbiome ¹⁰⁰. Interestingly, several conditions not typically linked with the gastrointestinal-tract, such as autism and Alzheimer disease, have also been associated with the composition and functions of the intestinal microbiome ¹⁰¹. In the mouth, the composition of the oral microbiome has been linked to diseases such as caries and periodontitis for a long time ¹⁰². Further, there is also increasing evidence that systemic diseases such as vascular disease and stroke can be associated with oral dysbiosis ^{103,104}.

Importantly, the mechanisms behind many microbiome-disease associations are not fully understood. The interpretation of associations between microbes and disease are challenging due to the multifactorial nature of most conditions, in addition to the individuality of the microbiome. However, the vast amount of research conducted in this field in the last decades, and the diverse functional repertoire of the microbiome, leave little doubt that there is an important connection between the human microbiome and our state of health.

2.3 Effect of antibiotics on the microbiome

Antibiotic therapy does not only target the infective bacteria but also affects the millions of microbes in the human body. This collateral effect will have at least two main consequences; alterations in the composition of the bacterial taxonomic profile and the emergence of antibiotic resistance ¹⁰⁵. Mechanisms and examples of antibiotic effects on emergence of resistance are presented later in section 3.2. Depending on the infectious agent and the anatomic location of the infection, factors such as antimicrobial spectrum, route of administration, absorption, elimination, dose and duration of treatment are of importance when choosing the most appropriate antibiotic agent for treatment. Importantly, these factors influence the drug concentrations at both target- and off-target-sites in the body, and consequently affect the antibiotic impact on the microbiome ¹⁰⁶⁻¹⁰⁸.

In 2015, Zaura *et al.* published a paper showing that different antibiotics affected the same ecological niche differently, and that different niches could have radically different responses to the same antibiotic agent. Treatment with clindamycin and ciprofloxacin reduced the diversity of the gut microbiome up to 4 and 12 months, respectively, while amoxicillin had no significant effect on microbiome diversity. Further, the authors reported that the salivary microbiome was far less affected by exposure to antibiotics than the fecal microbiome, irrespectively of the antibiotic used ¹⁰⁹.

Healthy adults have a relatively stable microbiome ⁷⁷. In general, exposure to antibiotics will have a temporary effect on the microbiome. However, some studies show long lasting effects that persist for years ¹¹⁰. During and soon after antibiotic exposure ecological disturbances such as reduced bacterial diversity and altered microbial composition are often observed. This leads to increased risk of overgrowth of pathogens, such as *Clostridium difficile* in the gut, or yeast infections in the genital tract ^{111,112}. Additionally, antibiotic exposure results in changes in the functionality of the microbiome. As described in section 2.2., the microbiome has an array of important functions, which might be disturbed by antibiotic treatment ¹¹³. In young children, with an immature microbiome, the consequences of antibiotic exposure are hypothesized to be of greater extent than in adults, and it is suggested that extensive use of antibiotics in a young age may be associated with conditions such as asthma, obesity and IBD ¹¹⁴⁻¹¹⁶. Worryingly, antibiotic use is particularly high in children aged <4 years ^{117,118}.

When treating an infection with antibiotics there will be a concentration gradient in the body, resulting in anatomical sites with subinhibitory concentrations of the drug ¹¹⁹. Also, the widespread and often inappropriate use of antibiotics worldwide have resulted in frequent human antibiotic exposure from the wider environment, via food, water and animals.

Antibiotic concentrations in the environment can vary, but are often in the subinhibitory range for most bacteria. Regardless of where the exposure happens, if it is via antibiotic drugs or from the environment, subinhibitory concentrations are a case of concern. It is suggested that antibiotics at subinhibitory concentrations may function as signaling molecules with consequences such as increased gene exchange, mutation rate and biofilm formation ¹²⁰. A study published in 2006 showed that a total of 386 genes (20 % of the genome) in *S. pneumoniae* changed their expression level due to sub-MIC concentrations of penicillin ¹²¹.

3. Resistome

The term *resistome* is defined as the collection of all ARGs found in the microbiome, including intrinsic and acquired genes, and genes found in commensal and pathogenic bacteria ¹²². Previously, ARGs found in clinically important pathogenic bacteria have been the focus of research. However, in the last 20 years, the presence of resistance in environmental bacteria and commensals have gained increased attention, particularly those in the human microbiome ¹²³. Advances in methodology, especially culture-independent methods, have revealed that ARGs in environmental and commensal bacteria may serve as a reservoir of resistance, as it can spread to more harmful species by HGT¹²⁴. In fact, several clinically important resistance genes, such as many extended spectrum beta-lactamases (ESBLs) and multidrug efflux pumps are thought to have originated from environmental bacteria ¹²⁵. Thus, the resistance observed in clinical settings is only the “tip of the resistome-iceberg”.

3.1 Effect of antibiotics on the resistome

As mentioned in section 1.3.2, the use of antibiotics is the main driver of emergence of ARGs, and emergence of resistance in bacteria can happen via mutations or by acquiring new genetic traits through HGT. The underlying mechanisms driving resistance emergence is partly due to

natural selection pressure, which selects pre-existing resistant bacteria, but also because antibiotic exposure itself increases the rate of mutations and HGT.

Increased mutation rates by exposure to subinhibitory concentrations of antibiotics have been reported in several studies, and different antibiotic agents may increase mutation rate by different mechanistic approaches. Fluoroquinolones act by inhibiting enzymes involved in DNA replication, which may activate an SOS response allowing for survival and continuous replication even if there is DNA damage ¹²⁶. Other antibiotic agents, such as ampicillin have been reported to increase the production of reactive oxygen species, which may cause DNA lesions and lead to the accumulation of mutations ¹²⁷. Further, subinhibitory concentrations of antibiotics have been reported to increase the rate of HGT ¹²⁸⁻¹³⁰. In 2006, Prudhomme *et al.* published a paper showing that aminoglycoside and fluoroquinolones as well as mitomycin C, a DNA-damaging agent with antitumour activity, induced competence and transformation in *Streptococcus pneumoniae*. *S. pneumoniae* lacks the classical SOS-system, and Prudhomme *et al.* suggested that competence might function as a general stress response in *S. pneumoniae* ¹²⁸. Increased conjugation frequency is also reported to be associated with subinhibitory exposure of antibiotics. For instance, subinhibitory concentrations of beta-lactam antibiotics increased the conjugative transfer of a plasmid containing tetracycline resistance in *S. aureus*, and exposure to several types of antibiotics increased conjugation of plasmids containing ESBLs in *Escherichia coli* ^{131,132}. Thus, it appears that low concentrations of antibiotics may act as a stressor on the bacteria, which respond by increasing mutation rate and gene exchange to maximize their chance of survival.

Antibiotic treatment promote resistance in both target and off-target microbes. This will increase the gene pool of resistance mechanisms in the community, and also promote resistance in bystanding opportunistic pathogens, which might be a source of future infections

¹¹⁹.

4. Streptococci

The *Streptococcus* genus represents a wide variety of pathogenic and commensal bacteria, colonizing both humans and animals. Streptococci belong to the phylum Firmicutes, are Gram-positive, have a coccoid or spherical shape, are facultative anaerobes and occur in pairs or chains, thus the name (streptos = chain, coccus =berries). Traditionally, streptococci were classified based on their ability to hemolyze erythrocytes in blood agar medium and on their cell wall polysaccharide composition (Lancefield grouping, A-G) ¹³³. However, not all streptococcal species, including most oral streptococci and *S. pneumoniae*, could be assigned to a Lancefield group. Improved DNA sequencing methods have introduced a new and more accurate approach in classification of streptococci based on 16S rRNA sequence phylogeny. The most recent classification have separated streptococci into eight major groups: Mitis, Sanguinis, Anginosus, Salivarius, Downei, Mutans, Pyogenic, and Bovis ¹³⁴. Bacteria within the genus *Streptococcus* are responsible for a high burden of morbidity and mortality worldwide. Among the most clinically important species are *Streptococcus pyogenes* and *Streptococcus agalactiae* belonging to the Pyogenic group, *S. pneumoniae* belonging to the Mitis group and *Streptococcus mutans* belonging to the Mutans group. *S. pyogenes* (often referred to as Group A *Streptococcus* - GAS) can cause a wide variety of acute infections, such as pharyngitis and necrotizing skin infections ¹³⁵, while *S. agalactiae* (often referred to as Group B *Streptococcus* - GBS) is known to cause severe infections in newborns¹³⁶. *S. pneumoniae* is an opportunistic bacterium with a high pathogenic potential, while *S. mutans* is associated with dental caries.

Although some streptococci may cause infections, most streptococci that colonize our bodies show a nonvirulent behavior. Streptococci are among the most dominating genus in the oral cavity and naso-pharynx, however, they can also be a part of the normal microbiome in the gut and vaginal tract ¹³⁷. Most oral streptococci are harmless, and seldom cause disease. In fact, they are most likely beneficial in keeping the microbiome balanced, and by protecting against more pathogenic bacteria ¹³⁸. Interestingly, previous reports have shown that oral commensals, such as *Streptococcus oralis*, *Streptococcus salivarius* and *Streptococcus mitis*, may protect against recurrent ear infections (otitis media) in otitis-prone children and *S. pneumoniae* lung infections in mice ^{139,140}. Hence, streptococci are a very diverse group of bacteria, involved in both health and disease. Streptococcal species used in laboratory experiments of this thesis are described in more detail in the next sections.

4.1 *Streptococcus pneumoniae*

As mentioned above, *S. pneumoniae* is one of the most clinically important species within the genus *Streptococcus*. It is often referred to as pneumococcus, and it can cause severe infections such as pneumonia, meningitis and sepsis ¹⁴¹. However, it is also a common inhabitant of the upper respiratory tract in humans (especially in young children), being part of the normal human microbiome. This two-faced behavior is what makes this species especially interesting regarding virulence factors, signaling systems and other regulation mechanisms¹⁴².

The polysaccharide capsule is a critical virulence factor. So far, over 90 different serotypes have been described based on differences in polysaccharide structure, and some of them seem to cause invasive disease more frequently than others ^{143,144}. High variability between *S. pneumoniae* strains may be attributed to the ability of natural genetic transformation ⁶⁰. *S. pneumoniae* reaches its competent state by recognizing one of two dominating competence stimulating pheromones (CSPs) within this species, leading to the uptake of external DNA. This will be further described in section 4.4. The ability to acquire new genetic traits through natural transformation makes both vaccine prophylaxis and antibiotic treatment challenging. Thus, pneumococcal diseases are still a major global health concern today.

Historically, *S. pneumoniae* has not only been an important cause of disease, it has also played a significant role in the research field of microbiology. In 1928, Frederick Griffith proposed the “transforming principle” by showing that information could somehow be transferred between different strains of bacteria using mice and encapsulated (Smooth) and unencapsulated (Rough) strains of *S. pneumoniae* ¹⁴⁵. Further, in 1944, Oswald Avery identified DNA as the substance of this 'transforming principle' ¹⁴⁶, leading to the later discovery of the DNA structure.

4.2 *Streptococcus mitis*

S. mitis is one of the first bacteria to colonize the human mouth after birth, and it remains in virtually all adults as a part of the oral microbiome throughout life ^{137,147}. It is usually harmless with a low pathogenic potential, despite being closely related to the more pathogenic *S. pneumoniae*. In 2014, Kilian *et al.* reported that *S. mitis* and *S. pneumoniae* probably have

evolved from a common ancestor and that *S. mitis* may have lost its virulence genes and adapted to a more mutualistic lifestyle ¹⁴⁸. In immunocompromised patients however, such as those with neutropenia, *S. mitis* is associated with septicemia ¹⁴⁹. The close relationship between *S. mitis* and *S. pneumoniae*, in addition to other species within the mitis group, has made taxonomic and phylogenetic characterization difficult. Still, even with methods such as 16s rRNA gene sequencing, correct identification remains a challenge as several species share >99% sequence homology ¹⁵⁰. Interestingly, interspecies gene transfer has been observed between *S. mitis* and *S. pneumoniae*. Thus *S. mitis* may function as a reservoir of antibiotic resistance genes for *S. pneumoniae* ¹⁵¹.

4.3 *Streptococcus mutans*

S. mutans is commonly found in the oral cavity and is known as an important etiologic agent in dental caries ¹⁵². It colonizes the mouth of young children, usually around the time of tooth eruption, and is well adapted to a life in biofilm such as dental plaque. ^{153,154} In addition to the high cariogenic properties, it can also cause non-oral infections such as bacteremia and endocarditis ¹⁵⁵.

4.4 Signaling systems in streptococci

Bacteria use signaling systems for intra- and inter-species communication. By interacting with others and building social networks, bacteria can accomplish functions beyond what they can achieve as individuals. Over 50 years ago, two research groups independently discovered two different cell-cell-communication systems. Nealson *et al.* discovered that the luciferase enzyme in *Vibrio fischeri* was induced at a specific cell density ¹⁵⁶, while Tomasz *et al.* reported the discovery of a hormone-like cell product controlling competence in *S. pneumoniae* ¹⁵⁷. The phenomenon these two groups described is today known as Quorum sensing (QS). QS is defined as the regulation of gene expression in response to cell-population density by secretion and detection of signaling molecules. Signaling systems and signaling molecules vary between different bacteria. In general, Gram-negative bacteria use acylated homoserine lactones in their communication, while Gram-positive bacteria use oligopeptides, known as pheromones, as signaling molecules. Numerous important biological

functions are regulated by QS in both Gram-negative and Gram-positive bacteria, such as virulence, competence, conjugation, motility, antibiotic production (lantibiotics, bacteriocins) and biofilm formation ¹⁵⁸.

In streptococci, several QS pathways have been described, and in the next sections, two of these signaling systems will be described in more detail.

4.4.1 Competence – the Com-pathways

Competence is a temporary state in streptococci during which genes allowing for natural transformation and fratricide are upregulated in response to quorum-sensing signals. The induction of competence varies between the different groups of streptococci. In streptococci from the Mitis group (e.g. *S. pneumoniae* and *S. mitis*) and Anginosus group, the QS signaling system ComABCDE is activated by the accumulation of CSP in the extracellular environment ¹⁵⁹. When a threshold concentration is reached, CSP binds to and autophosphorylates the membrane-bound receptor ComD, which in turn transfers a phosphoryl group to the response regulator ComE ¹⁶⁰. This is considered as the early phase of competence. ComE stimulates transcription of the so-called early genes, by binding to their promoter region ¹⁶¹. Among the early genes activated is *comC* and *comAB*, which encode the pre-CSP and a transporting system for the processing and export of CSP out of the cell. Hence, the early activation of competence functions as a positive feedback loop, stimulating further activation of the system ¹⁶². The phosphorylated-ComE also initiates the expression of *sigX* and *comW*. The alternative sigma factor SigX is the master regulator of competence responsible for the link between the early and late responses, while ComW participates in SigX stabilization and activation ¹⁶³. In the late phase of competence, several genes, such as genes responsible for transformation, production of bacteriocins, and a cell lysis mechanism known as fratricide, are upregulated (Figure 5) ¹⁶⁴. Overall, a recent report on competence and gene regulation reveals that ComE and ComX directly control 4% of all annotated genes in *S. pneumoniae*, and 17% of all genes is affected, either directly or indirectly by competence regulators ¹⁶⁵.

Members of the Pyogenic, Salivarius and Bovis group have a different system for activation of their competence system. In these groups of streptococci a new quorum sensing system has been identified named ComRS, using XIP (*sigX* inducing peptide) as the stimulating signal^{166,167}. XIP is imported into the cell where it binds to ComR, a transcriptional regulator

belonging to the Rgg family (revisited in the next section), and initiates *sigX* transcription, followed by upregulation of specific genes¹⁶⁸. In the Mutans group, both these systems, ComABCDE and ComRS, have been identified, and competence may be directly induced by XIP, or indirectly by CSP via XIP in two different signaling pathways¹⁶⁹.

Figure 5: Model system of the competence circuit in *S. pneumoniae*.

4.4.2 Rgg regulators

Rgg (Regulator gene of glucosyltransferase) proteins are transcriptional regulators, first described in *Streptococcus gordonii* as a positive regulator of glucosyltransferase expression, hence the name¹⁷⁰. Later, several genes with sequence homology to the original Rgg regulator were detected in other bacteria, regulating transcription of genes with different physiological functions. Nonetheless, the name ‘Rgg’ was expanded to the whole family of proteins. Initially, the Rgg regulators were believed to be stand-alone regulators. However, in 2007, a small hydrophobic peptide (SHP) was found to be interacting with the Rgg regulator, and a new pheromone system was discovered¹⁷¹. Later, genome-wide surveys have shown that this pheromone system is present in most streptococci, regulating functions such as biofilm formation, bacteriocin production and virulence¹⁷²⁻¹⁷⁴. As described in the previous section, ComR is a transcriptional regulator belonging to the Rgg family, found in streptococci belonging to the Pyogenic, Salivarius, Bovis and Mutans group. ComR initiates *sigX* transcription, which activates the late phase of competence.

Aims of the research

General aim:

- The main goal of this work was to explore the impact of antibiotics on the human microbiome and resistome, and mechanisms related to spread of resistance.

Specific aims:

- To study the effect of antibiotic stress on quorum sensing systems leading to increased virulence in *S. pneumoniae*. (Paper I and II)
- To investigate the impact of antibiotics on competence and concurrent transformation in *S. pneumoniae*, *S. mitis* and *S. mutans*, which are all common residents in the human oral and respiratory microbiome. (Paper I)
- To explore the consequence of a 5-day course of narrow-spectrum antibiotics (Penicillin V) on the oral and fecal microbiome and resistome of a young child. (Paper III)
- To study the impact of long-term amoxicillin therapy on the human oral microbiome and resistome. (Paper IV)

Summary of results

Paper I

Antibiotics Alter the Window of Competence for Natural Transformation in *Streptococci*.

The effect of subinhibitory concentrations of six different antibiotic agents on the signaling system that leads to competence and transformation in *S. pneumoniae* was investigated. The impact on competence was determined by the level of *sigX* expression using a luciferase reporter, and these results were then correlated with transformation kinetics. The results showed that ciprofloxacin, novobiocin, erythromycin and tetracycline enhanced *sigX* expression, while ampicillin and streptomycin had an inhibitory effect. When comparing it to transformability, the induction of *sigX* expression by ciprofloxacin and novobiocin correlated with increased and prolonged transformability. The prolonged effect of ciprofloxacin on competence and transformation was also observed in the streptococcal relatives *S. mitis* and *S. mutans*. In contrast, tetracycline and erythromycin had either an inhibitory or an insignificant effect on transformation, whereas streptomycin and ampicillin inhibited transformation. Thus, the results show that antibiotics may vary in their effects on competence, ranging from inhibitory to stimulatory effects, and that responses affecting transcription of *sigX* do not always correlate with transformability.

Paper II

Characterization of a Signaling System in *Streptococcus mitis* That Mediates Interspecies Communication with *Streptococcus pneumoniae*

In paper II, the effect of subinhibitory concentrations of antibiotic agents was investigated in the Rgg/SHP signaling system. Here, this newly described signaling system was identified for the first time in *S. mitis* and shown to be able to cross-communicate with *S. pneumoniae*. The Rgg regulator of this system presented high similarity to a transcriptional repressor in *S. pyogenes*; however, with the employment of genetic mutagenesis and luciferase assays, it was shown that this Rgg regulator functioned as a transcriptional activator in *S. mitis*.

Transcriptome analysis showed that two downstream genes, in addition to the pheromone gene *shp*, were strongly upregulated in response to the synthetic SHP, a core characteristic of

the Rgg/SHP QS system. Closer analysis of these genes revealed that they were a part of a putative lantibiotic gene cluster found in a conjugative element locus in several members of the mitis group. However, when compared to the full gene cluster present in representative strains of *S. pneumoniae*, *S. mitis* lacked the full set of genes, potentially resulting in a futile cycle of autoinduction. To assess if the activation of these genes would affect viability under antibiotic stress, a growth assay was performed with *S. mitis* overexpressing the target cluster and with an *rgg* knockout mutant, but no effect on growth was observed. The SHP sequence in *S. mitis* differed with only one residue from other previously described streptococcal SHPs, and was functional in promoting cross-species communication with *S. pneumoniae*. This was shown by increased surface polysaccharide synthesis in *S. pneumoniae* when exposed to the native SHP from *S. mitis*. In conclusion, the Rgg/SHP system in *S. mitis* regulates its own *shp* expression and promotes cross-species-communication with *S. pneumoniae*, activating a potentially virulent phenotype with increased surface polysaccharide expression. Although a futile cycle of autoinduction would have the potential to affect *S. mitis* growth in response to antibiotics, no such effect was observed.

Paper III

Impact of Narrow Spectrum Penicillin V on the Oral and Fecal Resistome in a Young Child Treated for Acute Otitis Media

Antibiotics affect not only the causative infectious agents, but have the potential to disturb the entire human microbiome. Narrow-spectrum antibiotics like phenoxymethylpenicillin (penicillin V) are believed to have less off-target effects on the microbiome than alternatives with a broader spectrum. However, little is known about the impact of penicillin V on the microbiome and resistome. In this study we collected oral and fecal samples from a one-year old child before (day 0) and after (day 5 and day 30) receiving a 5-days course of penicillin V against acute otitis media. The samples were sequenced using deep shotgun metagenomic sequencing. Sequencing data was analyzed to determine taxonomic profiling, using Kraken and Bracken software, and resistance profiling, using KMA in combination with the ResFinder database. Our results showed that there was a remarkable difference in how antibiotics affected the oral microbiome in contrast to the fecal microbiome. In the oral sample, the taxonomic profile was relatively stable in the three time points. In contrast, the taxonomic alterations observed in the fecal samples were prominent, especially at day 5. In

the oral samples, 11 ARGs, belonging to four different classes, were identified at day 0. At day 5, the abundance of some ARGs was increased, some remained unchanged, while others disappeared. At day 30, most oral ARGs had returned to baseline levels, or lower. In the fecal samples, we observed seven ARGs at baseline and five at day 5, with only one gene observed at day 5 being present at baseline. At day 30, the number of ARGs increased to 21 ARGs from seven different classes. In conclusion, Penicillin V had a remarkable impact on the fecal microbiome and resistome indicating that even narrow-spectrum antibiotics may have important consequences in selecting for a more resistant microbiome.

Paper IV

Long-term treatment with amoxicillin – impact on the oral microbiome and resistome

Although the most common duration of an antibiotic treatment does not exceed 14 days, for some conditions, such as recurrent urinary tract infections, severe acne and chronic respiratory diseases, longer treatments are often indicated. In Paper IV, we explored the consequences of prolonged antibiotic exposure on the oral microbiome and resistome using deep shotgun metagenomic sequencing. Saliva samples were collected from seven patients receiving a 100 days amoxicillin treatment as part of a multicenter clinical trial on chronic back pain. Samples were collected before treatment started (baseline), last day of treatment (100 days) and 9 months post antibiotic exposure (1 year). Over 600 different bacterial species were identified in the samples, confirming the large diversity of the oral microbial community. We observed that exposure to long-term antibiotics was associated with decrease in salivary microbiome diversity from baseline to 100 days. However, the diversity returned to levels similar to baseline after one year. Further, antibiotic resistance genes were found in all saliva samples, and most patients showed an increase in abundance of beta-lactam resistance genes, Macrolide/Lincosamid/Streptogramin (MLS) and multidrug resistance genes, from baseline to end of treatment. However, the highest number of ARGs were observed at the 1-year time point. These results indicate that long-term amoxicillin treatment may not markedly shift the overall oral microbiome composition, but it may be associated with a larger reservoir of ARGs in the oral cavity.

Methodological considerations

In this section, methodological considerations in Paper I-Paper IV will be deliberated. Due to similarities of methodological choices and challenges in Paper I and Paper II, and in Paper III and Paper IV, this section will be divided in two main parts. The first part will discuss the choices of bacterial strains, growth media and other factors influencing signaling and transformation in streptococci, important in Paper I and II. The second part will discuss the challenges and considerations of using metagenomic sequencing in clinical research, as used in Paper III and IV.

Paper I and II

Strains and growth conditions in streptococcal competence and transformation

S. pneumoniae and its ability of natural transformation have been studied for almost a century. When Griffith described how unencapsulated (rough) harmless bacteria switched to encapsulated (smooth) harmful bacteria and proposed the transforming principle in 1928, he demonstrated the importance of investigating specific bacterial strains. His virulent and smooth variant was a serotype 2 strain, today known as D39. Since then, the D39 strain has been a leading model of natural transformation in streptococci, and most studies in this field have used either the original D39 strain, or one of its many derivatives such as R36, R36A, R6 or Rx-1. The R-derivatives have lost its capsule, and generally have a much higher transformability than the original strain. In Paper I, we used D39 (NCTC 7466), and not one of its derivatives, to mimic the clinical setting as much as possible. To show that the impact on competence was not strain-specific in *S. pneumoniae* we also included TIGR4, which is also a widely studied strain. TIGR4 was first isolated in a patient in Kongsvinger, Norway, and it has been used in several studies assessing virulence in *S. pneumoniae*^{175,176}. The strains of *S. mitis* (CCUG 31611) and *S. mutans* (UA159) were chosen in paper I as the function and genome sequence of these strains are previously well characterized. In paper II, the *S. mitis* type strain (CCUG 31611) was chosen also on the background of homology with the Rgg/SHP system in *S. pneumoniae*.

When studying competence and transformation in streptococci, using the appropriate growth medium is of great importance. Previous protocols for streptococcal transformation have suggested the use of rich media, such as Todd Hewitt Broth (THB) and Tryptic Soy Broth (TSB). However, most studies investigating transformation in *S. pneumoniae* have used semi-defined media such as CAT-medium or C-medium supplemented with yeast extract (C+Y). In 2011, Stevens *et al.* found that C+Y-medium with increased concentration of yeast extract and bovine serum albumin supported spontaneous competence in *S. pneumoniae*¹³⁰. This modified C+Y-medium was called C+Y_{YB}, and in 2016, colleagues from our lab published an article showing significantly higher *sigX*-expression in *S. mitis* cultures grown in C+Y_{YB} than in C+Y or TSB¹⁷⁷.

In Paper I, several rounds of testing, including both rich media and semi-defined media, concluded that semi-defined media were superior in supporting competence gene expression and transformation in D39. In Paper II, both a rich medium, TSB, and a semi-defined medium, C+Y, were included in the analyses. The rationale behind this was that the Rgg/SHP system in *S. mitis* was a new discovery, thus, it was unknown if peptide and nutrient content in the growth media affected the activation or function of the system.

In Paper II, two genes were upregulated by the Rgg/SHP system in *S. mitis*. These genes were annotated as an ABC transporter, and it showed some sequence homology with a lantibiotic transporter system in other bacteria. However, closer analysis revealed that these genes were a part of a larger gene locus in other streptococci, indicating that in *S. mitis* the system might not be functional. Hence, a growth assay was conducted to assess if the activation of this Rgg/SHP system could affect growth in response to antibiotics stress. In this assay, a rich media was used as this generally promotes bacterial growth and is usually used in minimum inhibitory concentration (MIC)-studies.

Choice of antibiotics

Antibiotics used to treat or prevent infections in the body have the potential to disturb the entire human microbiome. Therefore, not only antibiotics used to treat streptococcal infections were included in these studies. Antibiotic agents included in Paper I and II belong to different antibiotic classes, and consequently, impose different antibiotic stress on the bacteria. In paper II, we included Nisin as one of the antibiotic agents. Nisin is a bacteriocin with antibacterial activity produced by *Lactococcus lactis*, often referred to as lantibiotics¹⁷⁸.

Due to the possible association between the two upregulated genes and a lantibiotic gene cluster found in other members of the mitis group, we sought to investigate if Nisin affected growth in this bacteria.

Gene expression and transformation assays

In paper I, we investigated the impact of antibiotics on competence in *S. pneumoniae* by measuring *sigX* gene expression, often referred to as the master regulator of competence. Initially in this project, we tested two different methods for investigating gene expression, namely Real Time PCR based on RNA extraction with cDNA synthesis (unpublished results) and Luciferase Reporter Assays. Both methods showed an increase in *sigX* expression as a result of antibiotic exposure. However, with the luciferase reporter assay we were able to use more variables (different agents, concentrations) with continuous readings during the entire window of competence. A limitation of reporter assays is the possibility of accumulation of signal over time in comparison with Real Time PCR, which shows a snapshot of the situation at one time point. However, the bacterial luciferase enzyme has a relatively rapid turnover which reduce accumulation of the signal measured ¹⁷⁹. In addition, it is a high throughput and cost effective method. Thus, the luciferase reporter assay was the preferred method in the further investigations. The Luciferase reporter assay is a commonly used method to study gene expression at the transcriptional level, and it has been employed in several studies investigating competence ^{128,130}.

Although stimulation of competence by antibiotic stress has previously been reported, for most studies, actual transformation was not included as an end point. As antibiotics have the potential to affect competence in different ways, and in different stages of the competence circuit, we wanted to test if gene expression results correlated with transformation efficiency. Previous studies investigating the impact of antibiotics on competence and transformation in *S. pneumoniae* have generally used growth conditions that suppress spontaneous competence. We sought to investigate how antibiotics affected an already competent bacterial culture, and therefore grew the bacteria in permissive conditions for spontaneous competence.

Several laboratory conditions influence transformation efficiency, including the concentration and size of homologous donor DNA (PCR-product), pH of the culture medium, CSP concentration and the optimal bacterial density in the starting culture. Studies recently performed in our and cooperating laboratories have optimized a variety of conditions for

streptococcal transformation, thus, the transformation assays performed in Paper I followed the already established protocols ^{177,180}.

Paper III – Paper IV

Investigating the microbiome

Next-generation sequencing (NGS) has revolutionized the field of microbiology, with the human microbiome project in 2007 as an important milestone ¹⁸¹. The introduction of NGS has rapidly extended our understanding of the composition and function of bacterial populations in various environments. However, the many opportunities using NGS are not without challenges.

There are two main approaches when investigating the composition of the microbiome, 16S rRNA gene sequencing and shotgun metagenomic sequencing. 16S rRNA is a gene universally present in all prokaryotes. It consists of highly conserved nucleotide sequences containing phylogenetic information. Thus, this gene has been used in microbial classification for several years ¹⁸². The most important advantages of this method are low costs, and reduced workload compared to shotgun metagenomic sequencing. However, there are several disadvantages to this method, such as the need for PCR-amplification, copy-number variations of the 16S rRNA gene, no functional information and limited taxonomic resolution. The second method used to investigate the microbiome is shotgun metagenomic sequencing, an untargeted approach, sequencing DNA from all cell types in an entire community ¹⁸³. It can provide detailed information on taxonomic composition in addition to functional information such as virulence genes and ARGs. Shotgun metagenomic sequencing requires complex analyses by skilled bioinformaticians, which are both costly and time consuming. Nonetheless, with the benefits of metagenomic sequencing, especially the possibility of investigating the resistome, we decided to use shotgun metagenomic sequencing in Paper III and IV.

Shotgun Metagenomic Sequencing

Shotgun Metagenomic studies involve several steps, (i) collection and storage of samples, (ii) DNA extraction, (iii) library preparation and sequencing, (iv) preprocessing of the reads and (v) sequence analysis. In each step, the choice of protocol may influence the end result, and this is a major challenge in the reproducibility of metagenomic research, as standardized protocols are lacking¹⁸⁴.

(i) Collection and storage of samples:

Choice of protocol for collection and preservation of samples can affect the quality of DNA and the reliability of the metagenomic data¹⁸⁵. In our projects, we tested several methods for collection of oral samples, including different swabs, sponges, drooling and spitting, and we tested storage with and without preservatives. Because of the young age of the child participating in Paper III, a swab was used to collect the oral sample, as this was the most practical alternative. A detailed instruction of how to swab the different areas of the mouth was followed to assure comparability between samples from different time points. Oral samples in Paper III were directly put on ice, and DNA was extracted within 2 hours. Fecal samples were collected in sterile containers without preservatives, and immediately frozen. In Paper IV, saliva was collected by spitting into a sterile container without any preservatives, and directly frozen at -80°C. It is important to be aware that different collection and storage methods can affect the results, and in some circumstances, the effect of different protocols can be larger than the effects of the biological variables¹⁸⁶.

(ii) DNA extraction:

DNA extraction methods can significantly alter the results of sequence data, as reported in several studies¹⁸⁷. Extraction methods usually rely on chemical lysis, mechanical lysis or a combination of both, with each method affecting important factors such as DNA amount, purity, molecular weight and degradation differently. In the oral samples used in Paper III and IV, after much testing, we ended up using MasterPure Gram Positive DNA Purification Kit from Epicentre, as recommended by HOMINGS (Human Oral Microbe Identification using Next Generation Sequencing) at the Forsyth Institute. DNA from fecal samples (Paper III) was extracted using PowerLyzer™ PowerSoil® DNA Isolation Kit from MoBio, as also used in the HMP¹⁸⁸.

(iii) Library preparation and Sequencing:

The process of library construction generally includes fragmentation and tagmentation, or introduction of adapter sequences to the fragmented DNA. Several kits for library construction are available, and they use different fragmentation methods, such as enzymatic fragmentation, sonification or fragmentation and tagmentation by transposomes. As with previous steps in the metagenomic workflow, the choice of library preparation method may influence the result of sequence data ¹⁸⁹. In our projects, the choice of library preparation and sequencing platforms were done in collaboration with the Norwegian Sequencing Center, which recommended the most suitable protocol for our samples. Sample preparations in paper III were done using the TruSeq DNA PCR-free kit and sequenced on Illumina HiSeq2500, while library preparations of samples in Paper IV were done using Nextera Flex and sequenced on the Illumina 3000 platform.

(iv) Preprocessing of the reads

Preprocessing of sequence reads includes removing adapter sequences, low quality reads and host DNA. There are several preprocessing strategies available. In our metagenomics projects, we used the Trimmomatic tool, as this is a commonly used and efficient preprocessing tool, optimized for Illumina NGS data ¹⁹⁰. Contamination with host-DNA is a significant challenge in human samples, especially in oral samples. Data from the HMP shows that fecal samples usually have low levels of human DNA contamination (< 10 %). In contrast, over 90% of the sequenced DNA aligns to the human genome in oral samples ¹⁹¹. Commercial kits that remove host DNA from metagenomic DNA samples are available, and were tested in the beginning of the project for Paper III. Unfortunately, the amount and quality of the remaining prokaryotic DNA after host DNA removal was not good enough to continue with sequencing. Therefore, we sequenced all DNA in the sample and removed host-DNA by aligning sequencing reads to a human reference genome, using BowTie2 ¹⁹².

(v) Sequence analysis:

Organizing and analyzing the sequence data is the last part of the shotgun sequencing pipeline. Several approaches and tools are available for analyzing sequence data, and the choice of methods may impact the end results ¹⁹³. There are two main approaches when analyzing metagenomic data, read-based or assembly-based analysis. In assembly-based approaches, the reads are assembled by overlapping regions into longer sequences called

contigs. Further, contigs are grouped into genomes, and then analyzed either by comparing the assembled sequences with available databases, or by de novo assembly. One of the advantages of assembly-based analyses is the possibility of identifying new genes and species. However, the complex and difficult task to assemble short reads from many different species in one sample, often resulting in misassemblies, remains a problem. To combat the issue with misassemblies, it is possible to combine long-reads using for instance Nanopore technology to facilitate correct assembly with the accuracy of short reads from the Illumina platforms, but this increases the costs considerably. In read-based approaches, short reads are directly analyzed by mapping reads to existing databases, thus, analyses are considerably less time and labor demanding but does not provide information on new genes and species. Notably, read-based approaches are more accurate in samples from previously well characterized environments with updated databases, than if the samples originated from a poorly characterized habitat ¹⁹⁴.

The aim of the study, together with costs and technological limitations, should ultimately determine which method to use. In Paper III and IV, we sought to investigate the impact of antibiotics on taxonomic composition and the resistome of the previously well characterized human microbiome. Several large and updated databases were available for both taxonomy and ARGs. Hence, being both time saving and less labor demanding, read-based approaches were used in these studies.

Taxonomic classification in Paper III was done with the Kraken program. Kraken is a fast and accurate program mapping k-mers to a library of genomes and assigning it to the lowest common ancestor ¹⁹⁵. Because many reads are similar in closely related species, there will be an underestimation of certain species with this method, and many reads will be assigned only at genus level or higher. To overcome this limitation in Kraken, the Bracken program was used to increase the species-level identification. In paper IV, an updated version of MetaPhlAn, which was used by the HMP, was used for taxonomic classification. MetaPhlAn2 uses a marker gene approach, with approximately 1 million clade-specific marker genes, to assign the reads into appropriate taxonomic groups ¹⁹⁶. This approach is faster than most other methods because the reference database only consists of a subset of the genomes found in other databases. For ARG profiling, different approaches were used in the two studies. In Paper III we used the ResFinder 2.1 database, which was a widely used database containing over 2000 ARGs at the time of analyses for Paper III ¹⁹⁷. However, the field of metagenomics and AMR are quickly evolving, and when analyzing the resistome for Paper IV, we utilized

the novel DeepARG approach with an updated database, which has proved to be more accurate than previous methods ¹⁹⁸.

Choosing the most appropriate method for analyzing metagenomic samples is challenging. However, unlike the first steps of the shotgun sequencing pipeline, the choice of methods in this last step can be re-assessed using other methods. This almost endless potential for further investigations makes shotgun metagenomic sequencing a valuable resource in microbiome research.

Discussion

The rapid emergence of antibiotic resistance worldwide is an evolutionary response to antibiotic exposure. During antibiotic treatment, the entire bacterial community is exposed, not only the target pathogen of treatment ⁴⁹. High concentrations of antibiotics will kill or inhibit growth of target and off-target susceptible bacteria. Further, increasing evidence shows that also subinhibitory concentrations have a broad range of physiological effects in bacteria exposed to antibiotics ¹²⁰. Microbial communities, like those found in humans, are particularly subjected to the effect of subinhibitory concentrations as a result of antibiotic therapies ¹⁹⁹. In the work of this thesis, the overall goal was to evaluate the way individual bacteria and complex microbiomes respond to antibiotic stress. The initial aim was to investigate the impact of subinhibitory concentrations of antibiotics on gene expression and transformation in a well described QS system in *S. pneumoniae* (Paper I), and further, if the presence of the newly discovered Rgg/SHP system in *S. mitis* affected its viability when exposed to antibiotics in the laboratory (Paper II). Next, the aim was to investigate how antibiotics affect clinical microbial communities. We explored this by analyzing the microbiome and resistome in the mouth and gut of a young child during and after penicillin V treatment (Paper III), and finally, we investigated the impact of long-term amoxicillin treatment on the salivary microbiome and resistome in adults (Paper IV).

Microbes communicate with each other by using QS systems. Such systems enable bacteria to modulate their collective behavior in response to different environmental cues. Competence, a classical QS system, is well described in streptococci, and it regulates bacterial behavior such as natural transformation. During the activated competent state, several genes including those involved in DNA uptake and recombination are upregulated. This enables the bacteria to incorporate external DNA into their own genome. The benefits of DNA uptake by competent cells are not fully understood and traditionally there have been three main hypotheses for the evolutionary function of competence and transformation; (1) uptake of DNA as nutrient, (2) uptake of DNA to repair damaged regions of the chromosome, or (3) to favor genetic exchange. Although competence regulates the expression of a variety of genes and functions, only a small portion of these are involved in transformation ²⁰⁰. In 2006, Claverys *et al.* suggested a theory that the broad effects of competence, not only transformation, may act as a general stress response in *S. pneumoniae*²⁰¹. Engelmoer and Rozen strengthened this theory in 2011, when they showed that for some types of stress, transformation is not essential for cells

to benefit from the induction of competence²⁰². Another, more recently described QS system in streptococci is the Rgg/SHP system. Rgg regulators are transcriptional regulators shown to control a variety of functions in different bacterial species, including pathways that indirectly regulate the competence system in several streptococcal species²⁰³.

Several studies have reported that antibiotic exposure may affect the regulation of QS systems, and that QS systems may in turn affect how the bacteria tolerate antibiotic stress. This highlights the complexity of antibiotic microbial interactions, and the possibility that antibiotics may function as signaling molecules in subinhibitory concentrations. It also explains how antibiotic exposure can result in substantial transcriptional changes in bacteria. In Paper I, we investigated if antibiotics affected the competence system by measuring gene expression of *sigX* and the subsequent transformation efficiency in *S. pneumoniae*. In accordance with previous studies, fluoroquinolone antibiotics enhanced gene expression of *sigX* and led to increased transformation efficiency¹²⁹. Interestingly, the aminoglycoside Streptomycin, reduced gene expression and transformation in our study but activated competence in a study by Prudhomme *et al.* in 2006¹²⁸. An important difference between these two studies was the laboratory conditions under which the experiments were performed. Prudhomme *et al.* grew *S. pneumoniae* in non-permissive conditions for spontaneous competence, while our experiments were performed in permissive conditions. The effect of permissive vs. non-permissive conditions was also reported by Stevens *et al.* in 2011, which observed a competence-inducing effect of aminoglycosides only under non-permissive conditions¹³⁰.

Another interesting finding in Paper I was the effect of tetracycline on competence and transformation. Tetracycline strongly increased gene expression of *sigX* in our experiments. However, the transformation efficiency was lower than in the control samples. According to previous literature, tetracycline has been shown to reduce decoding errors in translation, thus resulting in less stress and reduced activation of competence¹³⁰. This is in line with our results showing reduced transformation efficiency upon exposure to tetracycline. However, the increase in *sigX* expression due to tetracycline exposure was unexpected. A possible reason for this observation might be that tetracycline affects mechanisms downstream of SigX in the competence cascade. (Paper I). Ampicillin reduced both the *sigX* gene expression and the transformability of *S. pneumoniae* in this study, which is in accordance with previous findings performed in non-permissive conditions^{128,204}. Noteworthy, non-permissive conditions as used in most previous studies, are unsuitable for examining reduced competence

activity. By using permissive conditions, we were able to address this limitation. The overall findings in Paper I indicated that antibiotic agents affected competence and transformation in *S. pneumoniae* in different ways, depending on the specific mechanism of the agent. This might be of clinical relevance when choosing antibiotic agent in treating disease.

Other QS systems, besides competence, are also known to regulate a variety of cellular processes which can affect antibiotic susceptibility, such as biofilm formation, extracellular polysaccharide synthesis, lantibiotic production, and efflux mechanisms²⁰⁵. In Paper II we characterized a new signaling system in *S. mitis* and showed how it promoted cross-species communication. Moreover, we examined if the Rgg/SHP system affected the susceptibility towards antibiotics by using a mutant strain without the *rgg* gene. Our hypothesis was based on the findings that this new system seemed to trigger a futile cycle, as indicated by the transcriptome analyses, and that the up-regulated locus resembled those leading to protective responses towards bacteriocins in other streptococcal species²⁰⁶. No difference in MIC was observed in this *S. mitis* strain with or without *rgg*, indicating that the system did not initiate any mechanisms beneficial for survival under antibiotic exposure, at least under these experimental conditions.

In addition to the individual response of single strains to antibiotics, antibiotic stress can lead to functional and compositional disturbances in the microbiome, having consequences such as ARG enrichment and overgrowth of pathogenic bacteria. To begin investigating the connection between narrow-spectrum antibiotics and the impact on the microbiome and resistome, we started with a clinical case of a young child receiving penicillin V for treatment of acute otitis media, as described in Paper III. Despite only including one patient, the lack of knowledge on effects by narrow-spectrum penicillin on the microbiome, and the novel deep sequencing strategy performed in this study, made it scientifically relevant to publish the results. Globally, the use of narrow-spectrum penicillin is low. However, in Scandinavia it is the most used antibiotic drugs^{207,208}. Because of the narrow-spectrum, it is assumed that the collateral effect on the microbiome is lower compared with broad-spectrum antibiotics. However, there is not much evidence to support this assumption, and knowledge on the impact of penicillin V on the microbiome and resistome is limited. We decided to investigate the microbial communities in the oral and gut of this child. Previous studies have reported that the oral microbiome is more resilient to antibiotic perturbations than the gut microbiome¹⁰⁹, and this was also evident in this project. The mechanisms behind the resilience in the oral microbiome are still unknown. However, with the constant shifts in the oral environment

(temperature, nutrients, pH, oral hygiene procedures etc.) it is suggested that bacteria in the oral cavity have evolved to cope better with stress, and as a result, are more resilient towards other influences such as antibiotics ²⁰⁹. In addition, antibiotics taken orally will disturb the gut for longer time and with a higher concentration than the rest of the body, influencing the level of antibiotic effect ^{210,211}.

Paper IV focused on the investigation of the effect of amoxicillin, a beta-lactam antibiotic with broad-spectrum activity, on the oral microbiome and resistome in adult participants. Patients in this study were participants in a multicenter clinical trial, which evaluated the effect and possible side effects of a three months long treatment with amoxicillin on chronic low back pain with type 1 or 2 Modic changes ²¹². Long-term-treatment of antibiotics has been indicated for patients with Modic changes, and is also commonly used in several other conditions such as recurrent urinary tract infections, severe acne and chronic respiratory infections. However, there is to date limited knowledge on the off-target effects long-term antibiotic treatments have on the microbiome and resistome. As observed in Paper III, the overall changes in the oral microbiome were limited, indicating that the oral microbiome indeed exhibits an overall resilience towards antibiotics.

As expected, the baseline microbiomes in paper III and IV varied. In Paper III, an oral swab was used to collect the sample, while Paper IV used unstimulated saliva. In addition, the subject studied in Paper III was a one year old child with an immature microbiome, while in Paper IV, all participants included were adults. Despite such differences, microbial diversity decreased at the end of treatment in both studies. Interestingly, the average diversity at the last follow up sample was higher compared to the initial baseline samples in both studies, with the largest increase observed in the child in Paper III. These results showed that the oral microbiome recovered quite quickly following antibiotic treatment, which is line with another study investigating the effect of amoxicillin ¹⁰⁹. However, other studies, often associated with the use of broad-spectrum antibiotics, show alterations in microbial diversity for a long period after antibiotic treatment ^{110,213}. The abundance of Proteobacteria increased in the follow-up samples at the end of treatment in both Paper III and IV, most likely due to increase in abundance of the genera *Neisseria* and *Haemophilus*. These genera include important respiratory pathogens and naturally transforming species that are capable of intra and inter-species DNA-exchange ⁶⁶. This is of great importance since the oral microbiome, because of its proximity to the nasopharynx, may act as a reservoir of respiratory pathogens and ARGs ²¹⁴.

In the oral samples of paper III, an increase in abundance of some ARGs was observed after treatment, but no new ARGs appeared, and the abundance was generally back to baseline values after one month. Most patients in Paper IV had an increase in abundance of ARGs related to beta-lactam resistance, MLS resistance and multidrug resistance between baseline and end of treatment. Further, the increase of ARGs observed at the end of treatment was still observed at the follow-up sample 9 months post antibiotic exposure. The number of ARGs increased from an average of 33 at baseline, to 38 after 1 year. These results might imply that a longer treatment period requires a longer recreation time for the oral microbiome to stabilize. However, it is important to highlight that amoxicillin used in Paper IV is a broad-spectrum beta-lactam, which might have a larger effect on the resistome than Penicillin V used in Paper III.

In vast contrast to the oral samples in Paper III, antibiotics had an extensive influence on the fecal samples. The composition of the microbiome changed completely from phylum to species level, and it did not fully recover after one month. After Penicillin V treatment the fecal microbiome went from being dominated by the health associated genus *Bifidobacterium* to the potentially pathogenic genera *Escherichia*, *Klebsiella* and *Enterococcus*. The reduction in *Bifidobacterium* and concurrent increase in Enterobacteriaceae supports previous observations of the effect of broad-spectrum beta-lactam antibiotics, however, the considerable changes observed following treatment with the narrow-spectrum beta-lactam penicillin V were unexpected. At the last time point, 21 ARGs were observed in the fecal sample, which is threefold the amount in the baseline sample. An obvious limitation of this study is the fact that only one patient is included. However, the sequencing depth is a strength, allowing for higher accuracy when analyzing the results. These results indicate that narrow-spectrum penicillin may have a large impact on the microbiome and resistome in young children, and also highlights the importance of future studies to focus on narrow spectrum antibiotics.

Currently, it is not possible to directly assign the observed ARGs to the bacterial host with read based metagenomic sequencing approach, which is a limitation of this methodology. When looking at the resistome at the last follow up sample in paper III, it is possible that mutations and HGT played a role, as well as the dysbiosis leading to a large taxonomic shift, with newly appeared taxa containing new resistance genes. Interestingly, only one out of the 21 ARGs identified in the fecal sample at the last time point, conferred resistance toward beta-lactam antibiotics, which might indicate that the increase of ARGs at day 30 was most

likely due to new species colonizing the gut after penicillin V treatment opened up for recolonization.

Conclusions and Future Perspectives

The findings in this thesis have demonstrated that subinhibitory concentrations of antibiotics affected competence and transformation in *S. pneumoniae*. Further, this observed effect was dependent on the antibiotic agent. In *S. mitis*, the newly discovered Rgg/SHP signaling system was able to cross-communicate with *S. pneumoniae*. However, despite the indication of a futile cycle and a possible involvement of immunity mechanisms towards bacteriocins, the presence of this signaling system in *S. mitis* did not affect growth rate during exposure to subinhibitory concentrations of antibiotics. These results also indicated that a short-term treatment with narrow-spectrum Penicillin V altered the fecal microbiome, while the oral microbiome was relatively resilient towards antibiotic disturbances. The resilience of the oral microbiome was observed even after long-term treatment (3 months) with amoxicillin. Both short-term treatment with a narrow-spectrum beta lactam antibiotic and long-term treatment with a beta lactam of broader spectrum affected the resistome.

For a long time, research in the field of AMR has mainly focused on pathogens and how the pharmaceutical industry can win the battle against the forever evolving bacteria. Today, the focus has switched from “how we can win this battle”, to “how we can slow down the emergence and spread of resistance” so we can still be in the battle. In order to do that, a deeper understanding of how antibiotics function and how it influences the bacteria (and its hosts) is fundamental. In this context, the microbiome represents an important target and tool. Hopefully, the findings in this work may contribute with a piece of information in this massive puzzle, to help manage the global threat of AMR.

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ORIGINAL ARTICLE

WILEY molecular oral microbiology

Antibiotics alter the window of competence for natural transformation in streptococci

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Summary

Streptococcus pneumoniae transformation occurs within a short competence window, during which the alternative sigma factor X (SigX) is activated to orchestrate the expression of genes allowing extracellular DNA uptake and recombination. Importantly, antibiotic stress promotes transcriptional changes that may affect more than 20% of the *S. pneumoniae* genome, including competence genes. These can be activated or repressed, depending on the antibiotic agent. For most antibiotics, however, it remains unknown whether transcriptional effects on competence translate into altered transformability. Here we investigate the effect of antibiotic subinhibitory concentrations on *sigX* expression using a luciferase reporter, and correlate for the first time with transformation kinetics. Induction of *sigX* expression by ciprofloxacin and novobiocin correlated with increased and prolonged transformability in *S. pneumoniae*. The prolonged effect of ciprofloxacin on competence and transformation was also observed in the streptococcal relatives *Streptococcus mitis* and *Streptococcus mutans*. In contrast, tetracycline and erythromycin, which induced *S. pneumoniae sigX* expression, had either an inhibitory or a nonsignificant effect on transformation, whereas streptomycin and the β -lactam ampicillin, inhibited both *sigX* expression and transformation. Thus, the results show that antibiotics may vary in their effects on competence, ranging from inhibitory to stimulatory effects, and that responses affecting transcription of *sigX* do not always correlate with the transformation outcomes. Antibiotics that increase or decrease transformation are of particular clinical relevance, as they may alter the ability of *S. pneumoniae* to escape vaccines and antibiotics.

KEYWORDS

antibiotics, competence, natural transformation, quorum sensing, *Streptococcus*

1 | INTRODUCTION

Antibiotics are one of the most important pillars in modern medicine since they were introduced nearly eight decades ago.¹ However, their effectiveness is now threatened by the alarming spread of antibiotic-resistant bacteria.² Among the most important mechanisms for rapid spread of resistance are those involved in horizontal gene transfer, including conjugation, transduction and natural

transformation.³ Interestingly, for all three mechanisms there are examples of antibiotics that at subinhibitory concentrations stimulate DNA transfer.^{4–6} Such antibiotic effects contrast with the traditional view that antibiotics are specific in affecting particular target sites within the bacteria.⁷ Antibiotic stress affects the expression of a variety of genes, activating not only behaviours involved in DNA transfer,^{6,8} but also mutation frequencies,^{9,10} biofilm formation,¹¹ and virulence.^{12,13}

Streptococcus pneumoniae is a common inhabitant of the nasopharynx and it is responsible for a significant global burden of morbidity and mortality. *S. pneumoniae* can cause serious diseases such as pneumonia, meningitis and bacteremia.^{14–16} The genomic plasticity of *S. pneumoniae* is remarkable, and reflects its ability to develop competence.¹⁷ Competence is a physiological state during which genes allowing for killing of siblings, DNA uptake and recombination are upregulated in response to quorum-sensing signals.^{18,19} In *S. pneumoniae*, the competent state is activated by the accumulation of a competence signalling peptide (CSP) in the extracellular environment.²⁰ When the threshold concentration is reached, CSP binds to and autophosphorylates the membrane-bound receptor ComD, which in turn transfers a phosphoryl group to the response regulator ComE.²¹ ComE stimulates transcription of the so-called early genes, by binding to their promoter region. Among the early genes activated is *comC*, which encodes the pre-CSP and *comAB*. *ComAB* encodes a transporting system for the processing and export of CSP out of the cell. The promoter for the alternative sigma factor *sigX* is also recognized by phosphorylated-ComE. SigX is the master regulator of competence responsible for the link between the early and late responses. Transcription of the genes responsible for fratricide, DNA uptake and recombination, are all under the control of SigX.^{21–23} Natural transformation is considered the most important mechanism for the spread of resistance genes and emergence of vaccine escape variants in *S. pneumoniae*.¹⁸

Upper airway and ear infections by *S. pneumoniae* are some of the major reasons for antibiotic prescription in young children.²⁴ Importantly, subinhibitory concentrations of several antibiotics activate the expression of competence genes in *S. pneumoniae*^{6,25,26} including the protein synthesis inhibitors kanamycin and streptomycin,^{6,25,26} as well as fluoroquinolones, including norfloxacin, levofloxacin and moxifloxacin.⁶ Although such effects are generally interpreted as indicative of an increased ability for transformation, assessment of antibiotic effects on transformation has only been reported for streptomycin and norfloxacin at a single time-point during growth, and for two DNA damaging agents: mitomycin and 6-(*p*-hydroxyphenylazol)-uracil.^{6,25} Another constraint of current studies is that the correlation between the expression of competence genes and transformation has only been assessed under conditions that repress natural competence, hence precluding the identification of antibiotic effects that may inhibit transformation. Hence, there is currently limited knowledge regarding the actual effects of antibiotics on *S. pneumoniae* transformability.

Given the fact that antibiotics may regulate as much as 20% of the genes in *S. pneumoniae* genome,²⁷ and that a recent report shows disrupted coordination between transcriptional and phenotypic responses to antibiotics,²⁸ it is possible that altered expression of competence genes will not result in a corresponding effect on transformation. Of note, the machinery required for transformation is complex, and requires, among other mechanisms, a proper assembly of surface proteins that participate in the formation of pili and membrane pores for DNA import, and of several proteins that enable recombination of the incoming DNA within the genome.²⁹ In this study, subinhibitory concentrations of six different antibiotic

agents were investigated for their effects on *sigX* expression using a luciferase reporter, and correlated for the first time with detailed kinetic data on transformation. The results revealed that antibiotics may vary in their effects on competence, ranging from inhibitory to stimulatory effects, and that responses affecting transcription of *sigX* do not always correlate with kinetic changes in transformability.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and media

All bacterial strains used in this study are described in Table 1. Bacterial stocks were stored at -80°C in tryptic soy broth (Soybean-Casein Digest medium, Bacto™) supplemented with 30% glycerol. Blood agar base (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood (Oxoid) was used for plating. Selective plates were supplemented with either kanamycin ($500\text{ }\mu\text{g mL}^{-1}$) or erythromycin ($10\text{ }\mu\text{g mL}^{-1}$). Luciferase reporter and transformation assays were performed in casitone-yeast medium (C + Y) or modified casitone-yeast medium (C + Y_{VB}).

2.2 | Synthetic peptide and antibiotics

The sequence of the synthetic CSPs used in this study is listed in Table 1. Stock solutions were prepared by resuspending CSP-1 and CSP-*Streptococcus mitis* in distilled water to a concentration of 0.5 mM and 10 mM, respectively, and storing it at -20°C . For each experiment, antibiotics were diluted into correct concentrations in C + Y or C + Y_{VB}. Antibiotic stocks were prepared in distilled water (erythromycin was prepared in ethanol, ciprofloxacin in 0.1 M HCl) and stored at -20°C at the following concentrations: ampicillin (50 mg mL^{-1}), ciprofloxacin (25 mg mL^{-1}), erythromycin (20 mg mL^{-1}), kanamycin (100 mg mL^{-1}), novobiocin (2.5 mg mL^{-1}), streptomycin (25 mg mL^{-1}), tetracycline (12.5 mg mL^{-1}).

2.3 | Construction of mutants

For construction of SP020 (*comC*-luciferase reporter) and SP032 (*ldh*-luciferase reporter), the primer pairs FP423/FP424 and FP516/FP517, respectively, were used to amplify the specific promoter regions from *S. pneumoniae* TIGR4 (ATCC BAA334). The amplicon was further ligated to the pFW5-*luc* plasmid (Spec^R) and cloned into *Escherichia coli* as previously described.³⁰ Plasmids with the correct insert were then purified and transformed into the TIGR4 strain. SP045 (Δ *shp0939*)³¹ was constructed using a polymerase chain reaction (PCR) mutagenesis strategy.³² Briefly, fragments in the upstream and downstream region of *shp0939* were amplified with primer pairs FP952/FP953 and FP954/FP955, respectively. A third fragment containing a kanamycin resistance cassette was amplified from plasmid pR410. The PCR fragments were then purified (QIAquick PCR purification kit), restriction sites were digested with *AscI* and *FseI* enzymes (New England Biolabs, Ipswich, MA), and the fragments were ligated with T4 DNA ligase

TABLE 1 Strains, primers and synthetic pheromones used in this study

Strains	Description	Source or references
NCTC 7466	<i>Streptococcus pneumoniae</i> D39	NCTC
ATCC BAA334	<i>S. pneumoniae</i> TIGR4	ATCC
SP047	D39 but p _{sigX} -luc: Spt ^R , DNA from SP039	This study
SP020	BAA334, but p _{comC} -luc: Spt ^R	This study
SP039	BAA334 but p _{sigX} -luc: Spt ^R , DNA from MI055	This study
SP032	BAA334, but p _{ldh} -luc: Spt ^R	This study
MI055	CCUG 31611, p _{sigX} -luc: Spt ^R	Salvadori et al. ^a
SM068	UA159, p _{sigX} -luc: Spt ^R	Khan et al. ^b
SP045	D39, Δshp0939, Kan ^R	This study
MI009	CCUG 31611, ΔsrtA, Ery ^R	Salvadori et al. ^a
SM045	UA159, but ΔdexA, Kan ^R	Morrison et al. ^c
Primers for PCR amplicons (donor DNA)	Sequence (5' to 3')	Description
FP906	ATTCACCCCAAAAAGTGCTG	<i>Streptococcus mutans</i> : aRJ02 ^c (6,3 kb, Kan ^R , from SM045)
FP907	ATAATATGCGGACGCTGAGG	
FP914	TCTCGCGGAAGATAAAGTCG	<i>Streptococcus mitis</i> : aRJ20 ^a (6,9 kb, Ery ^R , from MI009)
FP915	GGGTAGCAGCAACAAGGAA	
FP1175	TCTGGTTGTCGCAAGAGAAA	<i>S. pneumoniae</i> : aKS01 (6,0 kb, Kan ^R , from SP045)
FP1176	TGGAACCTCATATCCCTCCA	
Primers for mutant construction	Sequence (5' to 3')	Restriction site
SP045		
FP952	AAAATTTACGAGAATGCCTTA	
FP953	aggcgccgctTGTGGGAAAATGGGAAAA	–
FP954	aggccggccCGTAGAAAGTTTAACATACGATGA	Ascl
FP955	TCCCTCAGCATAGGTAACCTC	FseI
FP001	aggcgccgctGTTTGATTTTAAATG	–
FP068	aggccggccTAGGTACTAAAACAATTCATCCAGTA	Ascl (kanamycin cassette)
FP037	TCATTTTCTCCCACCAGCTT	FseI (kanamycin cassette)
FP109	CAAGCCTGATTGGGAGAAAA	–
SP020		
FP423	aaagtcgacGGCCAGTGAATCAGAAAATCA	Sall
FP424	aggatccAATCCTCATCTCCCCACCTT	BamHI
SP032		
FP516	agctagcTCCATGACCAACTTCACTGC	NheI
FP517	aggatccCACCATCACCGACAAGGATA	BamHI
Competence stimulating peptide (CSP)	Sequence	Source of reference
CSP-1 (<i>S. pneumoniae</i> D39)	EMRLSKFFRDFILQRKK	Håvarstein et al. ^d
CSP- <i>S. mitis</i> (CCUG 31611)	EIRQTHNIFNFKRR	Håvarstein et al. ^e

References: ^aSalvadori G, Junges R, Morrison DA, Petersen FC. *Front Microbiol.* 2016;7:1009; ^bKhan R, Rukke HV, Ricomini Filho AP, et al. *J Bacteriol.* 2012;194:3781-3788; ^cMorrison DA, Khan R, Junges R, Amdal HA, Petersen FC. *J Microbiol Methods.* 2015;119:134-141; ^dHåvarstein LS, Coomaraswamy G, Morrison DA. *Proc Natl Acad Sci U S A.* 1995;92:11140-11144; ^eHåvarstein LS, Hakenbeck R, Gaustad P. *J Bacteriol.* 1997;179:6589-6594.

(Fermentas, St.Leon, Germany). Confirmation of the insertion was performed by phenotypic characterization (kanamycin resistance) and genotyping using primer pairs FP952/FP037 and FP955/FP109. For the reporter strain SP047, the p_{sigX}-luc locus was transformed

into *S. pneumoniae* D39 (NCTC 7466) with DNA from SP039. This strain (SP039) was constructed using transforming DNA from the previously reported *S. mitis* strain MI055 into the TIGR4 strain ATCC BAA334 by natural transformation.

2.4 | Donor DNA amplicons

Bacterial DNA (from strains SP045, MI009 and SM045) and the PCR primers to construct the 6-kb, Kan^R-amplicon used as donor in the transformation assays are listed in Table 1. The DNA fragment was amplified by PCR using Q5 DNA polymerase (New England Biolabs) following the manufacturer's instructions. Optimal annealing temperature was calculated using the NEB Tm Calculator (online software provided by New England BioLabs).

2.5 | Luciferase reporter assays

Antibiotics were diluted to different concentrations and divided into aliquots (100 μ L per well) in a white 96-well plate

with clear bottom (Nunc™ MicroWell™ 96-Well Optical-Bottom Plate). All experiments contained a control without antibiotics in addition to a blank medium control. Bacterial cultures were prepared by inoculating fresh colonies grown overnight in fresh medium until an optical density at 600 nm (OD_{600}) of 0.08 ($2\times$). Cultures were incubated for 15 minutes at 37°C, 5% CO_2 before addition of CSP and/or luciferin (100 ng mL^{-1}) (Synchem, Felsberg-Altenberg, Germany). Next, 100 μ L aliquots of the culture were added to wells containing the antibiotic solutions ($2\times$). The final concentration of CSP-1 was 50 nM, and for CSP *S. mitis* the final concentration was 300 nM. OD_{600} and luciferase activity were measured every 30 minutes for 9 hours in a spectrophotometer plate reader (Synergy HT; BioTek, Winooski, VT). The 96-well-plate was incubated at 37°C, 5% CO_2 in between readings.

FIGURE 1 Effect of antibiotic agents on *sigX* expression in *Streptococcus pneumoniae* D39. Fresh colonies of the *sigX* reporter strain (SP047 p_{sigX} luc) were grown in C+Y_{VB} supplemented with CSP-1 to a working concentration of 50 nmol L^{-1} and aliquots were placed into a 96-well plate with different concentrations of antibiotics. Expression of *sigX* was measured every 30 minute during growth at 37°C in 5% CO_2 . The results are presented as relative luminescence units per culture optical density at 600 nm (RLU/OD, solid line) on the left y-axis and growth curves (OD_{600} , dashed line) are presented on the right y-axis. The results are averages from three replicates and representative of three independent experiments

2.6 | Transformation assays

The antibiotics and bacterial cultures were prepared as described for the luciferase reporter assay. Transformation rates were assessed at different time-points during growth. At each time-point, DNA was added into the wells at a concentration of 100 ng mL^{-1} . After 30 minutes, DNase (DNase I recombinant grade I; Roche, Basel, Switzerland) was added at a final concentration of 100 U mL^{-1} . This was followed by another period of 30 minutes of incubation, after which $25 \mu\text{L}$ of culture from each well was diluted and plated on selective and nonselective agar plates. Two parallels were included at each time-point and plates were incubated at 37°C , $5\% \text{ CO}_2$. After 24 hours, colony-forming units were counted and transformation efficiency was calculated. Fold change was measured by dividing the transformation efficiency of the treated samples by the transformation efficiency of the nontreated controls.

2.7 | Statistical analysis

Statistical comparison of the transformation data was performed with unpaired *t*-test. *P*-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Effect of subinhibitory concentrations of different antibiotics on *sigX* expression

Antibiotics are known to induce the expression of competence genes in *S. pneumoniae* under suppressive conditions for spontaneous competence.^{6,25,26} To investigate whether an effect is observed under nonsuppressive conditions of competence, a D39-*sigX* luciferase reporter was grown with CSP and various

FIGURE 2 Effect of different antibiotics on *sigX* expression in *Streptococcus pneumoniae* D39 grown without exogenous competence-stimulating peptide (CSP). Fresh colonies of the *sigX* reporter strain (SP047 p_{sigX} luc) were grown in C+Y_{VB} and aliquots were placed on a 96-well plate with different concentrations of antibiotics. Expression of *sigX* was measured every 30 minute during growth at 37°C in $5\% \text{ CO}_2$. The results are presented as relative luminescence units per culture optical density at 600 nm (RLU/OD, solid line) on the left y-axis and growth curves (OD_{600} , dashed line) are presented on the right y-axis. The results are averages from three replicates and representative of three independent experiments

antibiotics in a semidefined medium. Expression of *sigX* reached maximal levels by 160 to 200 minutes. Cultures treated with ciprofloxacin, novobiocin, erythromycin, or tetracycline enhanced the level of *sigX* expression compared with the nontreated samples and also prolonged the time during which expression of *sigX* occurred. Contrastingly, in cultures treated with ampicillin or streptomycin, *sigX* expression was decreased for most of the period (Figure 1; and see Supplementary material, Figure S1). Under conditions relying exclusively on endogenous CSP, similar effects to those observed in the presence of synthetic CSP were observed, although the expression level of *sigX* was lower (Figure 2; and see Supplementary material, Figure S2).

FIGURE 3 Effect of different antibiotics on *comC*-expression in *Streptococcus pneumoniae* TIGR4 grown without exogenous competence-stimulating peptide (CSP). Fresh colonies of the TIGR4 *comC* reporter strain (SP020 *p_{comC}* luc) were grown in C+Y_{VB} and aliquots were placed on a 96-well plate with different concentrations of antibiotics. Expression of *comC* was measured every 15 minutes during growth at 37°C in 5% CO₂. The results are presented as relative luminescence units per culture optical density at 600 nm (RLU/OD, solid line) on the left y-axis and growth curves (OD₆₀₀, dashed line) are presented on the right y-axis. The results are averages from three replicates and representative of two independent experiments

We also examined the effect of different antibiotics in the *S. pneumoniae* TIGR4 strain, using a different reporter for early competence gene expression. Similar to D39, *S. pneumoniae* TIGR4 showed both increased and prolonged expression of early competence when stressed with ciprofloxacin and tetracycline, and reduced expression when stressed with ampicillin (Figure 3; and see Supplementary material, Figure S3). To rule out a general effect of antibiotics on translation and activity of the luciferase enzyme, a luciferase reporter for the constitutive gene *ldh* in TIGR4 was included. The results showed that antibiotics had no observable effect on expression of *ldh* (see Supplementary material, Figure S4).

3.2 | Antibiotic effects on *sigX* expression do not always correlate with transformability outcomes

To gain insight into the kinetics of transformation in the presence of antibiotics, we measured the ability of D39 to take up and incorporate exogenous DNA in cultures treated with and without antibiotics, using six different time-points. A protocol for high efficiency of transformation was used.³³ Here we hypothesized that enhanced *sigX* expression would lead to increased transformation efficiency. As expected due to similar *sigX* expression levels, transformation efficiency at the initial time-point in cultures grown with most antibiotics did not differ significantly from the control (Figure 4). The exception was bacteria treated with erythromycin, which had a decrease in transformation efficiency in the first time-point compared with the nontreated control. Cultures grown with ciprofloxacin and novobiocin showed a higher transformation efficiency than the nontreated control after 150 minutes (Figure 4), which corresponds with the appearance of difference in *sigX* expression observed in Figure 1.

Tetracycline and erythromycin had inhibitory and no significant effects on *S. pneumoniae* transformability, respectively, despite being the antibiotic agents that induced the highest expression of *sigX* (Figures 1 and 4). Hence, the results indicate that subinhibitory concentrations of antibiotics that increase and prolong *sigX* expression do not always lead to an increase in transformability. Cultures grown with ampicillin and streptomycin had an overall decreased transformability compared with the control, which correlated with a decrease in *sigX* expression (Figure 4).

Furthermore, we also tested the effect of ciprofloxacin on *sigX* expression and transformation in *S. mitis*, a close relative of *S. pneumoniae* and *Streptococcus mutans* an oral streptococcus with an early competence-stimulating machinery that is different from those of *S. pneumoniae* and *S. mitis*. Interestingly, in *S. mitis*, as for *S. pneumoniae*, ciprofloxacin enhanced and prolonged *sigX* expression. Transformation in *S. mitis* correlated with an enhanced effect showing increased transformation at 240 minutes. (Figure 5). A prolonged effect on *sigX* expression was also observed in ciprofloxacin-treated *S. mutans*, with an increased transformability at 730 minutes (Figure 5; and see Supplementary material, Figure S5).³⁴

FIGURE 4 Fold changes in transformation efficiency between *Streptococcus pneumoniae* D39 grown with and without antibiotics. Fresh colonies of the *sigX*-reporter strain (SP047 p_{*sigX*} luc) were transferred to liquid medium supplemented with competence-stimulating peptide 1 (CSP-1) to a final concentration of 50 nmol L⁻¹ and aliquots were placed in wells with and without antibiotics (ciprofloxacin 0.4 µg mL⁻¹, novobiocin 0.15 µg mL⁻¹, erythromycin 0.01 µg mL⁻¹, tetracycline 0.04 µg mL⁻¹, ampicillin 0.05 µg mL⁻¹, streptomycin 5.0 µg mL⁻¹). Donor DNA (6 kb, Kan^R PCR-amplicon) was added at different time-points, followed by DNase supplementation after 30 minutes (symbols). One hour after DNA addition, bacterial culture from each replicate was diluted and plated on blood agar plates with and without supplemented kanamycin. Transformation efficiency was calculated by dividing the number of colonies growing on the selective agar plate by the total number of colonies growing on the nonselective agar plate multiplying it by 100. Fold change was calculated by dividing transformation efficiency of the treated sample on the transformation efficiency of the control. Bars represent fold change (log₂) between the treated sample and the control at each time-point and results are averages of a minimum of two to three individual experiments with two replicates in each experiment. (* $P < 0.05$)

4 | DISCUSSION

Antibiotics represent one of the most important discoveries in human history. However, overuse and misuse of antibiotics have led to widespread resistance. Of particular importance is the accumulated evidence that low concentrations of antibiotics have a non-negligible effect on bacterial behaviours associated with emergence and spread of resistance.³⁵ Such behaviours include induction of cell-to-cell communication, biofilm formation and horizontal gene transfer, all of them inter-related, and induced by a range of antibiotic classes in *S. pneumoniae*.^{6,10} Horizontal gene transfer by natural transformation in *S. pneumoniae* can indeed be pervasive

under crowded conditions and high antibiotic exposure, as recently suggested in a large scale study of humans living in a refugee camp.³⁶

Although several studies have shown that antibiotics induce competence in *S. pneumoniae*, either by microarray, RNA sequencing, or luciferase reporter assays, only a few have directly accessed their effects on phenotypic transformation.^{6,25} For those studies in which transformation data are present, the results are limited to a single time-point during growth (Table 2).^{6,25} Here we investigated transformability during growth and showed that the effect of antibiotics on competence and transformation do not always correlate, and that different antibiotic agents may either increase or decrease the ability of *S. pneumoniae* to transform.

Aminoglycosides, as well as quinolones that target topoisomerases, and mitomycin have all been shown to induce competence in *S. pneumoniae*.^{6,25,26} Common to them is their ability to damage

DNA. One of the mechanisms of DNA damage by aminoglycosides and quinolones is related to their effects in promoting bacterial production of hydroxyl radicals,^{37,38} whereas mitomycin acts mainly by

FIGURE 5 Effect of ciprofloxacin on *sigX* expression and transformability in *Streptococcus mutans* and *Streptococcus mitis*. (A, C) Fresh colonies of the *S. mitis sigX* reporter strain (MI055) were grown in C+Y_{VB} supplemented with CSP-*S. mitis* at a final concentration of 300 nM. (B, D) Fresh colonies of the *S. mutans sigX* reporter strain (SM068) were grown in chemically defined medium (CDM) without exogenous competence-inducing peptide. (A, B) The strains were divided into aliquots in a 96-well plate with different concentrations of ciprofloxacin. Expression of *sigX*, presented on the left y-axis as relative luminescence units (RLU/OD, solid line) and optical density at 600 nm (OD₆₀₀), presented on the right y-axis (dashed line), were measured periodically during growth at 37°C in 5% CO₂. (C, D) Samples were grown with and without ciprofloxacin (*S. mitis*: 1.0 µg mL⁻¹ and *S. mutans*: 0.5 µg mL⁻¹). Donor DNA was added at different time-points, followed by DNase supplementation after 30 minutes. One hour after DNA addition, aliquots of each replicate were diluted and plated on blood agar plates with and without selective antibiotics. Transformation efficiency was calculated by dividing the number of colonies growing on the selective agar plate by the total number of colonies growing on the nonselective agar plate multiplying it by 100. Bars represent fold change (log₂) at the each time-point. Fold change was calculated by dividing the transformation efficiency of the sample grown with antibiotics by the transformation efficiency of the control sample. Results are averages of a minimum of two independent experiments with two replicates in each experiment

TABLE 2 Antibiotic effects on competence and transformation in this study compared with previous studies

Antibiotic	Classification	This study		Prudhomme et al. (6) ^a		Slager et al. (25) ^b	Stevens et al. (26)	
Ciprofloxacin	Fluoroquinolone	+	+	nt	nt	+	nt	nt
Novobiocin	Aminocoumarin	+	+	x	nt	x	nt	nt
Erythromycin	Macrolide	+	ns	x	nt	nt	nt	nt
Tetracycline	Tetracycline	+	-	x	nt	nt	-	nt
Ampicillin	β-lactam	-	-	x	nt	nt	nt	nt
Streptomycin	Aminoglycoside	-	ns	+	+	+	nt	+/- ^c

White columns show increased (+) or reduced (-) expression of a competence luciferase reporter gene. Grey columns show increased (+) or reduced (-) transformation rates.

ns, not significant; nt, not tested; x, not induced (suppressed conditions).

^aCulture treated with norfloxacin and mitomycin C induced transformation; one-time-point experiment.

^bCulture treated with 6-(p-hydrophenylazo)-uracil (HPUra) induced transformation; one-time-point experiment.

^cStreptomycin induced competence under nonpermissive conditions for competence development, but not under permissive conditions.

creating DNA cross-links.³⁹ Induction of *S. pneumoniae* competence by such agents has been used to support the hypothesis that competence in *S. pneumoniae*, which lacks a classical SOS-system, has evolved as a mechanism to repair DNA damage.⁴⁰ More recently, target-specific mechanisms by which different antibiotics induce competence have been proposed. Aminoglycosides such as kanamycin and streptomycin, for instance, may promote decoding errors during protein synthesis,²⁶ which in turn can lead to the production of misfolded proteins that are targeted to degradation by the HtrA protease, so protecting CSP from HtrA cleavage. Notably, the effect of aminoglycosides in inducing competence was observed under nonpermissive conditions for development of competence, but not under permissive conditions. Such findings were confirmed by our results showing that streptomycin under permissive conditions had a slightly reducing effect on the expression of *sigX*.

In the case of antibiotics that target DNA replication, including ciprofloxacin, the effect on competence induction has been attributed to stalling of the replication fork close to the origin of replication.²⁵ This leads to increased transcription dosage of the regulatory genes in the competence cascade, which are located close to the origin of replication. Our results confirmed the effects on the expression of competence genes, and showed a positive effect of ciprofloxacin on transformability. Furthermore, novobiocin, an antibiotic that targets the *gyrB* subunit of DNA gyrase, had also an increasing effect on both competence and transformability in our study. This effect on competence was associated with a delay in the decline of transformation efficiency observed during competence, as differences between antibiotic-treated and nontreated cultures were more pronounced close to the exit phase of the competence period.

Two antibiotics targeting ribosomes, erythromycin and tetracycline, induced competence gene expression in this study. Both agents were among the six tested antibiotics that had the strongest inducing effect on *sigX* expression. Notably, both antibiotics failed in a previous study to show any inducing effect on competence.⁶ Here, erythromycin led to a reduced transformability in the first time-points compared with the control, and at the latest time-points we observed no significant difference from the control. For tetracycline, however, despite a strong inducing effect on *sigX* expression, reduced transformation rates were observed in all time-points compared with the nontreated control. From a mechanistic perspective, induction of *sigX* by tetracycline was unexpected, since tetracycline has been shown to reduce decoding errors in translation, in contrast to antibiotics that seem to induce competence by the increase in decoding errors. It is possible, however, that the effect of tetracycline in inducing competence gene expression may be related to other stress mechanisms. The effect of aminoglycosides in enhancing production of free radicals, as described above, is in fact a common feature to several antibiotics belonging to other classes, including tetracycline and β -lactams.⁴¹ Hence, although the current findings cannot exclude a more general mechanism of free-radical-induced stress or growth-rate reduction as mechanisms behind the triggering of competence by antibiotics, the results indicate that mechanisms

specific to the different antibiotic agents are important. Of note, our results show that the β -lactam ampicillin decreases transformability, and supports previous findings that β -lactams have an inhibitory effect on expression of competence gene expression.^{1,27,42} This is of particular relevance, as β -lactams are one of the most important therapeutic resources against pneumococcal infections.

In conclusion, specific antibiotic agents affect competence and transformability in different ways, and antibiotics that stimulate competence gene expression may either increase or reduce *S. pneumoniae* transformability, depending on antibiotic type. Antibiotics that increase or decrease transformability are of particular clinical relevance, as they may confer survival advantages to *S. pneumoniae*, such as those resulting in capsule exchange or antimicrobial resistance, allowing vaccine and/or antibiotic escape.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Impact of narrow-spectrum penicillin V on the oral and faecal resistome in a young child treated for otitis media

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ABSTRACT

Objectives: Antibiotic overuse has led to the global emergence of antimicrobial-resistant bacteria, and children are among the most frequent users of antibiotics. Most studies with broad-spectrum antibiotics show a severe impact on resistome development in patients. Although narrow-spectrum antibiotics are believed to have fewer side effects, their impact on the microbiome and resistome is mostly unknown. The aim of this study was to investigate the impact of the narrow-spectrum antibiotic phenoxymethylpenicillin (penicillin V) on the microbiome and resistome of a child treated for acute otitis media. **Methods:** Oral and faecal samples were collected from a 1-year-old child before (Day 0) and after (Days 5 and 30) receiving penicillin V for otitis media. Metagenomic sequencing data were analysed to determine taxonomic profiling using Kraken and Bracken software, and resistance profiling using KMA in combination with the ResFinder database.

Results: In the oral samples, antimicrobial resistance genes (ARGs) belonging to four classes were identified at baseline. At Day 5, the abundance of some ARGs was increased, whereas some remained unchanged and others could no longer be detected. At Day 30, most ARGs had returned to baseline levels or lower. In the faecal samples, seven ARGs were observed at baseline and five at Day 5. At Day 30, the number of ARGs had increased to 21.

Conclusions: Following penicillin V, we observed a remarkable enrichment of the aecal resistome, indicating that even narrow-spectrum antibiotics may have important consequences in selecting for a more resistant microbiome.

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1. Introduction

The increasing prevalence of antimicrobial-resistant bacteria is now threatening what has been modern medicine's security blanket for over 70 years, namely effective antibiotics [1]. Antibiotics are among the most essential and prescribed drugs in the world, but extensive antibiotic use has led to the global emergence of antimicrobial-resistant bacteria, making bacterial infections once again difficult to treat. Antibiotic use is particularly high in children aged <4 years [2,3]. The leading reason for antibiotic prescription in these children is upper airway infection, including otitis media [3,4]. In Scandinavia, the treatment guidelines for otitis media are much more restrictive than they were some years ago [3,5] and doctors are encouraged to use as few antibiotics as possible in these cases. However, when

recommended, the first-line antibiotic is phenoxymethylpenicillin, also known as penicillin V [6,7]. Penicillin V has activity against Gram-positive bacteria but is less active against Gram-negative bacteria. This drug is among the narrowest-spectrum antibiotics in use.

Use of broad-spectrum antibiotics is associated with a reduction in gut microbiome diversity, as demonstrated in several studies [8,9]. The impact on the microbiota in other body sites is less known, but the microbiome in saliva appears to be more resilient to antibiotics than the gut microbiome, at least in healthy volunteers exposed to broad-spectrum antibiotics [10]. In children, loss of diversity is of particular importance given the relevance of the microbiome on immune system maturation [11]. Whilst such antibiotic effects on microbial composition have been the focus of several investigations, less is known about the effects on the overall antimicrobial resistance gene (ARG) load in the human microbiome, known as the resistome [12]. The resistome constitutes an important reservoir of ARGs that can be transferred to pathogens [13]. Emerging data indicate that broad-spectrum

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antibiotics can lead not only to a general increase in resistance genes related to the antibiotic target, but also to other groups of antibiotics [14]. Such resistance genes can in some cases persist for long periods and increase upon cumulative exposure to antibiotics [15]. For narrow-spectrum antibiotics such as penicillin V, the impact on the resistome remains largely unknown.

In the present study, the effect of the narrow-spectrum antibiotic penicillin V on the oral and gut resistome of a young child treated for otitis media was followed. Deep metagenomic sequencing was used to investigate its possible relevance in promoting enrichment of pathogens and ARGs. The results indicate that penicillin V may affect both the taxonomic and resistome profiles of the microbiome, as observed at 25 days after therapy termination.

2. Materials and methods

2.1. Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics—South East ('REK sør-øst'). Written informed consent was obtained from the child's parents. The participants received no compensation.

2.2. Sample collection

Faecal and oral samples from a 1-year-old child presenting with otitis media at the Accident and Emergency Department, Municipal Hospital, South Norway, were collected for shotgun metagenomic sequencing. The child was of Caucasian origin, had no history of travel outside the Nordic Countries, and had previously received antibiotic treatment on one occasion (>1 month ago). Samples were collected at three time points: (i) the same day as the visit to the emergency department (baseline); (ii) immediately after the antibiotic treatment course was completed (Day 5); and (iii) after 25 days post-antibiotic treatment (Day 30). Oral samples were collected using FLOQSwab® (Copan Diagnostics, Murrieta, CA, USA). The swab was stored in 2 mL of TE buffer, was placed directly on ice and DNA was extracted within 2 h of sample collection. Faecal samples were collected in sterile containers and were placed directly in a –20 °C freezer. Faecal DNA was extracted after all samples had been collected.

2.3. DNA extraction and sequencing

DNA from oral samples was extracted using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA). Sample material in TE buffer was pelleted by centrifugation (10 000 rpm for 10 min) and was extracted following the manufacturer's protocol. Faecal DNA extraction was performed using a PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Minor adjustments to the protocol were implemented according to the Human Microbiome Project (Core Microbiome Sampling Protocol A, HMP Protocol #07-001) [16]. The amount of extracted DNA was measured using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was evaluated by agarose gel electrophoresis. Metagenomic shotgun sequencing was conducted at the Norwegian Sequencing Centre (Oslo, Norway) on an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) using a paired-end sequencing approach with a targeted read length of 125 bp in high-output mode.

2.4. Quality control and pre-processing of metagenomic data

The quality of raw sequencing reads was assessed using the FastQC tool [17], with all samples passing commonly applied

quality criteria including base quality, GC content, nucleotide distribution and duplication rate (mean Phred score >20; read length >100 bp; nucleotide distribution %A = %T and %G = %C with a constant proportion over the length of the read; duplicated sequences should make up <50% of the total reads). Low-quality reads and adapter sequences were trimmed out using Trimmomatic [18], followed by removal of human DNA sequences by extracting all reads mapping to an assembly of the human genome (version GRCh38, downloaded from NCBI GenBank) using BowTie2 [19]. The remaining reads were subsequently used to characterise the microbiome and resistome in oral and faecal samples.

2.5. Taxonomic profiling

Paired non-host reads were classified and assigned taxonomic labels using Kraken v.1 software [20]. Reads (k-mers) were aligned against the prebuilt MiniKraken DB_8GB database, which encompasses complete archaeal, bacterial and viral genomes in NCBI's RefSeq (18 October 2017). Kraken assigns the most specific taxonomic label to each read in a given set of metagenomic reads for classification. However, certain times the label assignment is not at the species level. Especially in the case of nearly identical reads that are found in more than one species, Kraken will assign it to the lowest common ancestor, which could be genus level or higher. Therefore, the number of species-specific reads would be underestimated in samples containing one or few highly similar species. To overcome this issue, the species abundance after classification with Kraken was re-estimated using Bracken (Bayesian Reestimation of Abundance after Classification with Kraken) [21]. Furthermore, Shannon and Chao1 diversity indexes were calculated at species level for oral and faecal samples. The samples were rarefied to even sequencing depth (per million reads) for comparative analysis. Only reads that were taxonomically classified were used in downstream analyses.

2.6. Identification and quantification of antimicrobial resistance genes

The KMA program [22] was used in combination with the ResFinder 2.1 database [23] to identify ARGs in the metagenomic data set. The ResFinder database consists of ~2200 resistance genes and is curated from existing databases and published literature. Resistant phenotypes attributed to mutations within an antibiotic target gene [target resistance alleles (TRAs)] were not included in the analysis since read-based approaches using short reads are still limited in providing reliable identifications of TRAs [24]. KMA was selected over other alignment tools (SRST2, MGmapper etc.) because of its accuracy in aligning short reads against redundant databases. In addition, this method is able to resolve the issue of multiple read matches by evaluating and statistically testing the global alignment scores. Quality-controlled reads were mapped against the ResFinder database using KMA, with 80% identity threshold for resistance gene identification (see Supplementary Table S1 for detailed information). To reduce the number of false positives, only genes present at significant levels ($P < 0.05$) with a coverage >50% were used for further analysis. For co-occurrence analyses, the source (organism) of genes (identified template) was manually searched through their accession ID in NCBI and the Comprehensive Antibiotic Resistance Database (CARD). On the background of this search, genes described in multiple bacterial species or defined by CARD as present in mobile elements were not included in the co-occurrence analysis.

Furthermore, the given depth/abundance of each resistance gene and the classes they belong to were normalised using RPKM (reads per kilobase million mapped reads) for comparison across samples. The abundance profiles are represented in the form of a

heatmap both for oral and faecal samples. Heatmaps were generated using 'pheatmap' package in R. Likewise, all of the co-occurrence or correlation line plots between the ARGs and potential microbial taxa were created in R.

3. Results

3.1. Sequencing results

A total of six samples were sequenced and analysed. The samples were collected from two body sites (O = oral and F = faecal) and from three different time points (Day 0 = d0, Day 5 = d5 and Day 30 = d30). For all six samples, a total of 260 million paired-reads were generated from shotgun metagenomic sequencing. The mean Phred quality score of raw reads across all samples was 37.3. Host DNA contamination was higher in oral compared with faecal samples. On average, 66% (28M) and 8% (3M) reads were identified as human DNA in oral and faecal samples, respectively. Human DNA was filtered from the sequencing data during analysis as described in Section 2.4.

3.2. Oral microbiome composition

On average, 48% reads in oral samples remained unclassified. The abundance of the remaining taxonomically classified reads are illustrated as a stacked bar chart in Fig. 1. The results suggest that the taxonomic composition of the oral metagenomes following antibiotic treatment varied slightly with respect to genus and species, but less with respect to phylum. All of the oral samples were dominated by Gram-positive bacteria, most of them belonging to the phylum Firmicutes. The baseline (O-d0) sample contained around 67% Firmicutes, with strong dominance of the genus *Streptococcus* (64%). The majority of reads were assigned to *Streptococcus mitis* (24%) at species level. In addition, 15%, 12% and 5% of the reads were also assigned to bacteria from the phyla Proteobacteria, Actinobacteria and Bacteroidetes, respectively. The second most abundant species in the baseline sample was *Rothia mucilaginosa* (11%), belonging to the phylum Actinobacteria. The sample taken at Day 5 (O-d5), corresponding to the day of therapy termination, shows a pattern that was slightly different from O-d0. Firmicutes was still the dominating phylum. However, the relative proportion of Proteobacteria was doubled compared with O-d0. Actinobacteria and Bacteroidetes were both reduced in their relative abundance compared with baseline (O-d0). The most abundant species was still *S. mitis*, but the proportion of some of the Gram-negative species such as *Haemophilus parainfluenzae* increased from 5% in O-d0 to 12% in the O-d5-sample. The sample collected 25 days after the end of antibiotic exposure (O-d30) revealed a taxonomic composition that was more similar to O-d0 than O-d5. Whilst the fraction of reads belonging to Proteobacteria was still high as in O-d5, the relative proportion of Bacteroidetes was restored to the level shown in O-d0. Within the Proteobacteria, the highest proportion of reads were assigned to the *Neisseria* and *Haemophilus* genera. Also, the relative abundance of *Neisseria* was found to be highest at O-d30 (12%). Furthermore, the Chao1 (richness) and Shannon (evenness) species diversity measurements decreased drastically at O-d5 compared with O-d0 and increased again at O-d30 (Fig. 2). The statistical significance of such changes could not be measured due to only one child (case) in the study.

3.3. Faecal microbiome composition

Large differences in microbiota composition were observed in the faecal samples before antibiotic treatment (F-d0), at the termination of antibiotic treatment (F-d5) and 25 days after

antibiotic termination (F-d30) (Fig. 1). The faecal baseline sample (F-d0) was strongly dominated by the phyla Actinobacteria (62%) and Firmicutes (36%). However, after 5 days of antibiotic treatment the taxonomic profile showed a shift towards the phylum Proteobacteria completely dominating the sample with a contribution of >76% in total reads compared with <1% at baseline. A massive increase in abundance of *Escherichia coli* (49%) contributed particularly to the high proportion of Proteobacteria. In the F-d0 sample, species belonging to the Firmicutes were mainly from the genus *Streptococcus* or *Anaerostipes*. *Enterococcus* (especially *Enterococcus faecalis*) was the leading genus in the Firmicutes in the F-d5 sample. In the follow-up sample from Day 30 (F-d30), the relative abundance of Proteobacteria decreased to levels that approached those at baseline, with Actinobacteria again dominating the sample. The Firmicutes showed also a tendency of returning to baseline values, except for *Streptococcus thermophilus*, whilst the phylum Bacteroidetes, with its most abundant species *Bacteroides fragilis* (17%), became a substantial part of the gut microbiome. Interestingly, *B. fragilis* was not detected in F-d0 or F-d5 samples. Furthermore, a considerably larger portion of unmapped reads was observed for F-d30 than for F-d0 and F-d05. For F-d05, only 10% of the reads were not mapped to a known reference compared with 58% for F-d30 and 40% for F-d0. Similar to the oral samples, the species richness and evenness in the faecal samples were reduced at F-d5 compared with F-d0 (Fig. 2). Both oral and faecal samples had the highest species diversity at d30.

3.4. Oral resistome

For oral metagenomes, seven ARGs were found that were present at all time points, i.e. O-d0, O-d05 and O-d30 (Fig. 3B). At baseline, a total of 11 annotated ARGs were identified. These genes encoded resistance to antibiotics belonging to four different classes, representing three different mechanisms of resistance. The largest group of ARGs at baseline were those conferring resistance to β -lactams. In total, five different genes belonging to the β -lactamase group (*bla*_{TEM-1B}, *cfxA3*, *bla*_{OXA-85}, *bla*_{SPU-1} and *penA*) were detected before antibiotic treatment started. The TEM-1 enzyme was described already in the early 1960s [25] and is one of the most prevalent β -lactamase enzymes in Gram-negative bacteria. It is found on transferable genetic elements and is able to inactivate penicillins and narrow-spectrum cephalosporins [26]. In addition to the β -lactamases, the efflux pump *mef(A)* conferring resistance to macrolides was detected at O-d0, together with *msr(D)*, which is a macrolide efflux protein expressed on the same operon as *mef(A)* [27]. The *lsa(C)* gene confers resistance to lincosamides, streptogramins and pleuromutilins, whilst *erm(F)* confers resistance to the macrolide–lincosamide–streptogramin B (MLS_B) group. Two genes conferring tetracycline resistance [*tet(M)* and *tet(Q)*] were also detected at O-d0. Most ARGs discovered at O-d0 were also identified at O-d5. Levels of *bla*_{OXA-85}, *lsa(C)* and *tet(Q)* were similar to O-d0, whereas the abundance of *bla*_{TEM-1B}, *cfxA3*, *mef(A)* and *msr(D)* were increased at O-d5. Of the four genes with an increased abundance after 5 days, different responses were observed at O-d30: *mef(A)* and *msr(D)* both decreased in abundance below their baseline level, whilst *cfxA3* increased even more at O-d30. *bla*_{TEM-1B} decreased from the high abundance at O-d5, but not to the low levels observed at Day 0. Thus, in oral samples most of the observed changes at O-d5 showed a tendency to return to baseline levels at O-d30 (Fig. 3B).

3.5. Faecal resistome

In the faecal samples, in total 25 different ARGs were detected. At baseline, seven different ARGs were identified, conferring resistance to four different antibiotic classes (Fig. 3A). *ant(6)-la*

Fig. 1. Taxonomic profile of the oral (left) and faecal (right) metagenomes. Relative abundance of taxa from three time points, namely baseline (d0), immediately after treatment completion at Day 5 (d5) and 25 days post-antibiotic treatment (d30), presented at phylum (top), genus (middle) and species (bottom) levels. Those found at <1% are grouped as 'Others'.

Fig. 2. Alpha diversity measured by Chao1 (richness) and Shannon (evenness) diversity indexes plotted for oral and faecal samples at three time points, namely baseline (0), immediately after treatment completion at Day 5 (05) and 25 days post-antibiotic treatment (30). Both oral and faecal samples have been rarefied to even sequencing depth for diversity estimation.

confers aminoglycoside resistance, *mef(A)* encodes a macrolide efflux pump, *msr(D)* is associated with resistance to the MLS_B group, and *tet(M)*, *tet(W)*, *tet(O)* and *tet(32)* encode ribosomal protection proteins preventing damage from tetracyclines. Only one of these genes [*tet(M)*] was identified again after 5 days (F-d5), thus showing a shift in the faecal resistome at Day 5. The abundance of *tet(M)* was increased at F-d5 compared with the baseline sample. In addition to *tet(M)*, four ARGs not present at baseline were identified at F-d5. One β -lactamase (*bla_{OXY-2}*), a fosfomycin resistance gene (*fosA*), and two efflux pumps [*lsa(A)* and *oqx(B)*] conferring resistance to macrolides and fluoroquinolones, respectively. In the last sample, 21 ARGs were identified in total, showing a considerably increase from the first two faecal samples. At F-d30, ARGs from seven different antibiotic classes were identified, including genes encoding resistance to vancomycin that were not detected at F-d0 or F-d5. *tet(M)* was the only gene detected at all timepoints, with its highest abundance at F-d5. Interestingly, 14 ARGs were solely identified at F-d30, 25 days after the last antibiotic exposure. Hence, the faecal resistome showed an extensive shift in composition and increase in diversity following antibiotic treatment (Fig. 3A).

3.6. Relationship between the resistome and microbiome

Some of the observed ARGs, such as *bla_{BRO-1}*, *bla_{OXA-85}* and *penA* in oral samples, and *bla_{OXY-2-7}* and the *van* genes in faecal samples, are found intrinsically in specific species. Others, such as *bla_{TEM-1}*, *tet* genes and most *erm* genes, are found either intrinsically in several bacteria or on mobile DNA elements. A correlation analysis was performed between intrinsic resistance genes and the bacterial species in which the resistance genes have been identified. For this, their abundances (co-occurrence) were plotted across the three different time points in oral and faecal samples (Fig. 4). In the oral samples, the rise in abundance of *bla_{BRO-1}*, a resistance gene found in the chromosome of *Moraxella catarrhalis*, correlated with the increase in *M. catarrhalis* abundance on Day 5. The same is true for *bla_{OXY-2-7}* and the *van* genes that are intrinsic to *Klebsiella oxytoca* and *Enterococcus casseliflavus*, respectively.

Enterococcus casseliflavus is known to harbour intrinsic, non-transferable resistance to vancomycin owing to a chromosomally encoded *vanC* gene cluster [28,29]. All five vancomycin resistance genes found in this study are gene variants found in this cluster. *Enterococcus casseliflavus* is not as well described as *E. faecalis* and *Enterococcus faecium*, however it can cause serious infections and, due to its high level of intrinsic resistance to several antibiotics, it is a difficult bacteria to treat [30].

Not all genes followed the expected source of origin. The resistance genes *penA* and *bla_{OXA-85}* (also named *bla_{FUS-1}*), which usually are found in *Neisseria meningitidis* and *Fusobacterium nucleatum*, respectively, did not follow the abundance profile of their expected host, suggesting that these genes may also be intrinsic in other bacteria or that the genes can be found on mobile elements. For genes that are found intrinsically in several bacteria or on mobile DNA elements, it is not possible to correlate them with bacterial species with the methods applied in this study.

4. Discussion

Young children are frequent users of antibiotics. Antibiotic use in Scandinavian countries is still lower than in many other European countries and the low usage most likely contributes to the low prevalence of antibiotic resistance that is observed [31]. Penicillin V is recommended in Norwegian treatment guidelines as a first-line drug for empirical treatment of otitis media [6].

In this study, a sequence-based metagenomic approach was used to elucidate the impact of penicillin V on the oral and faecal microbiome and resistome of a child living in Norway. Supporting previous findings [10], the results of the current study showed that the oral microbiota was more resilient to the effects of antibiotics than the faecal microbiota. However, previous observations have generally been done with broader-spectrum antibiotics, in healthy adults, and not in young children with an infection such as in the present study.

Microbial diversity both in oral and faecal samples decreased after 5 days of treatment. After 30 days there was a tendency for higher diversity than at baseline. This is an observation that is in line with recent results showing that in children the faecal

Fig. 3. Antimicrobial resistance genes (ARGs) identified in (A) faecal and (B) oral samples. Heatmap showing distribution of ARGs detected within the metagenomic samples at three time points, namely baseline (0), immediately after treatment completion at Day 5 (05) and 25 days post-antibiotic treatment (30). Genes are grouped by antibiotic class and mechanism of action. Colours shown in the legend (right) indicated the abundance of ARGs scaled from 'blue' (minimum) to 'red' (maximum). The scale 0–7 are normalised levels for the abundance of genes based on sequencing depth and gene length as determined by RPKM (reads per kilobase million mapped reads). Genes that have not been detected or have been excluded based on cut-off criteria are coloured grey.

microbial diversity continues to increase until it stabilises at around 3 years of age independent of antibiotic use [32]. Hence, the increase in diversity at Day 30 compared with baseline is not necessarily a result of antibiotic exposure.

The taxonomic profile of the oral microbiota at the three different time points was relatively stable despite the fact that penicillin V has antimicrobial activity against Gram-positive bacteria, which predominate in the oral cavity. In contrast, the taxonomic alterations observed in the faecal samples were prominent, especially at Day 5. In the faecal baseline sample, *Bifidobacterium* spp. predominated. After 5 days of treatment the potentially pathogenic Enterobacteriaceae were the dominating

family and the abundance of *Bifidobacterium* spp. was reduced. This is likely an undesirable outcome, since high levels of *Bifidobacterium* are associated with several health benefits [33], including reduced levels of antimicrobial resistance in early life [34]. Conversely, the Enterobacteriaceae are a group of bacteria known to harbour high levels of ARGs and they have been assigned the highest priority by the World Health Organization (WHO) in the fight against antibiotic-resistant bacteria [35]. The observed reduction of Bifidobacteriaceae and the increase in Enterobacteriaceae during penicillin V treatment supports previous studies investigating the effect of other β -lactams of broader spectrum on the faecal microbiome [36,37].

Fig. 4. Line plots showing the abundance of antimicrobial resistance genes (ARGs) (reads per kilobase million mapped reads) in the oral and faecal metagenome samples (black), together with the relative abundance of selected species potentially carrying the ARGs (based on annotation by KMA) (red).

Five ARGs [*mef(A)*, *isa(C)*, *msr(D)*, *tetM* and *tet(Q)*] were observed both in the oral and faecal microbiome. Four of these [*mef(A)*, *msr(D)*, *tetM* and *tet(Q)*] can be found in several bacteria on mobile elements and are therefore of particular significance for spreading via horizontal gene transfer [38]. The impact of penicillin V on the resistome was greater in faecal samples than in oral samples. Whilst in the oral samples the 10 ARGs detected at O-d30 were also observed at baseline (Fig. 3B), in the faecal samples 14 genes were observed that were exclusive to d30 samples (Fig. 3A). Interestingly, only one of the faecal ARGs identified at Day 30 conferred resistance to β -lactam antibiotics, which was used to treat the patient. ARGs conferring resistance to β -lactam antibiotics were also observed in oral samples, but these were not enriched following antibiotic therapy. The extensive impact on the faecal resistome at Day 30 compared with the modest effects on the oral sample might be explained by a larger taxonomic shift in the faecal samples. Newly appeared taxa or taxa with higher abundance at the last time point, such as *Clostridium* spp., *B. fragilis* and *E. casseliflavus* (Supplementary Table S2), may be contributing to new genes such as *ant(6)-Ia* and *aph(3')-III*, *cepA-44* and the *van* genes, respectively, in the resistome. Also, some of the enriched ARGs are known to cluster together, such as *mef(A)* and *msr(D)*, and will consequently be co-

selected. However, the large difference in the resistome of the faecal samples may also be a result of higher antibiotic selection pressure and horizontal gene transfer within the already existing microbiota [39].

Although definitive conclusions require a larger and ideally randomised study, this case report indicates that even narrow-spectrum antibiotics may have a significant effect on the faecal microbiome and resistome, thus warranting further investigations. Overall, acute and recurrent otitis media is among the most predominant conditions associated with antibiotic prescription and thus studies on these patients should be encouraged.

Data availability

The metagenome sequences generated in this work were deposited in the European Nucleotide Archive (ENA) (<https://www.ebi.ac.uk/ena>) under accession no. PRJEB31957.

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Competing interests

None declared.

Ethical approval

This study was approved by the Regional Committee for Medical and Health Research Ethics [2014/1946/REK sør-øst C]. Written informed consent was obtained from the participating child's parents.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jgar.2019.08.004>.

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