Chapter 1: Antibiotics and commensal human gut bacteria

1.1 Antibiotics

Antibiotics are a cornerstone of modern medicine and are used extensively throughout the developed and developing worlds, in humans and for veterinary and agricultural purposes. They are drugs that either kill or inhibit the growth and replication of a bacterium[1] and are used to treat bacterial infections in, or on, the body. They are distinct from antiseptics (used to sterilise living tissue and reduce the risk of infection, rather than treat an infection) and disinfectants (non-selective antimicrobials that kill a range of microorganisms, not just bacteria, and are used on non-living surfaces)[1]. They are also not toxins, as toxins are defined as a poisonous substance produced by a microorganism, plant or animal that causes illness in the body; antibiotics do not cause direct harm to human cells. Antibiotics can be administered intravenously (into veins via syringe or catheter), intramuscular (into muscle via syringe), orally (in tablet, capsule or liquid form), or topically (e.g. creams, lotion, sprays or drops). Intravenous is considered the most effective route as it creates an immediate therapeutic blood level of the antibiotic, but oral routes are often preferred as they are less intrusive, do not require a hospital stay, and often achieve supra-inhibitory blood levels[2].

Antibiotics can now be synthesised in a laboratory, but are often based on compounds produced naturally by microorganisms to harm or kill bacteria in their environment. Not all microorganisms produce antibiotic compounds – in fact, there are only around 20 species that produce antibiotics that are now mass-produced and used in medicine[3]. Typically these are soil-dwelling microorganisms[3], although the search for new antibiotics is now beginning to shift to marine microbes[4]. Both of these environments feature high diversity and density of microorganism; antibiotic production is thought to be a mechanism of attack against

neighbouring bacteria or defence, enabling survival of the antibiotic-producer[5]. Antibiotic compounds can also act as signalling molecules between bacterial cells, regulating bacterial behaviour such as biofilm formation[6]. Therefore, they have important roles in nature that humans have co-opted for our benefit and integrated antibiotics into human and animal medicine. It is difficult to quantify exactly the overall impact of antibiotics on infection dynamics and distinguish between the impacts of other factors such as the introduction of vaccines[7] or improved sanitation[8]. Despite this, antibiotic use is associated with a decrease in deaths caused by communicable diseases per year[9] and increase in average life expectancy[9].

1.2 The history of antibiotics

It is only recently, and over a relatively short timeline, that antibiotics have become entrenched in our society to treat bacterial infections (Fig. 1.1). Just 110 years ago, Paul Ehrlich (the German biochemist, 1854-1915) coined the term "magic bullet" when theorising a chemical that could selectively target and kill disease-causing agents in the body without harming the patient[10]. The first "true" magic bullet antibiotic against bacteria that was used clinically against bacterial infections was in fact used a number of years before this term was used. Pyocyanase was an extract prepared from the Gram-negative bacterium *Pseudomonas aeruginosa* by Emmerich and Low in 1899[11]. It was active against a number of pathogenic bacteria but since its effectiveness was inconsistent and it was mildly toxic to humans it was abandoned[11]. Ehrlich, now considered the founding father of chemotherapy and large-scale, systematic drug screens, developed his own magic bullet ('Compound 606') against the causative bacterium of syphilis, *Treponema palladium*[12]. Compound 606, or Salvarsan, was the most frequently prescribed drug after its discovery in 1909[13]. Prontosil was another early antibiotic, a compound synthesised as part of a screen by Bayer chemists Josef Klarer and Fritz

Mietzsch and shown to have antibacterial properties against a number of diseases by Gerhard Domagk[14]. The active component sulphanilamide had been previously used in the dye industry and had already come off-patent, enabling sulphonamide derivatives to be produced by various companies[15]. Sulphonamides are the oldest class of synthetic antibiotics and new versions are still produced and used today.

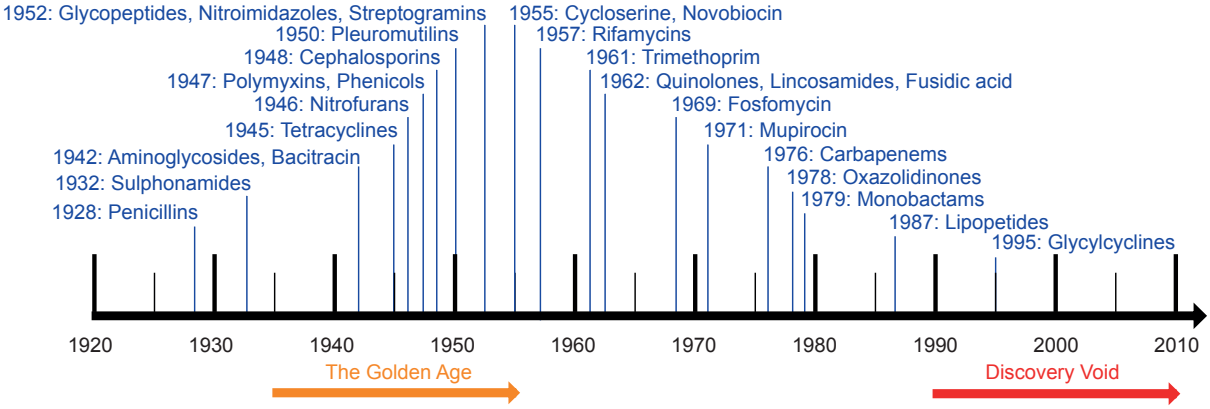

Figure 1.1. Timeline of antibiotic discovery. Adapted from Silver 2011[16].

Probably the most well-known antibiotic, and one of the oldest mass-produced antibiotics, is penicillin, "discovered" in 1928 in the famous tale of Alexander Fleming (Scottish bacteriologist, 1881-1955) and an open window. A mysterious fungus had blown through an open window and contaminated plates being used to study *Staphylococcus*, which was known to cause infections in humans, and the fungus had managed to halt the growth of this bacterium[17]. The antimicrobial properties of moulds were already known, but Fleming was remarkably dedicated in his efforts to purify the exact compound responsible for this effect. Penicillin was eventually brought into mass production in 1945, overtaking Salvarsan in usage[15]. Penicillin antibiotics are still the most prescribed drug globally to this day[18]. The mass production of penicillin coincided with the discovery of streptomycin in 1943, an antibiotic isolated from the soil bacterium *Streptomyces griseus* that could treat *Mycobacterium tuberculosis*, the cause of tuberculosis (TB)[19]. These events heralded the start of the "golden

age of antibiotics" (Fig. 1.1): eighteen new types of antibiotics were discovered between 1944 and 1970[16]. The rate of discovery then began to decline, with only four new classes in the 1970s and one in the 1980s. Since then, no new classes of antibiotics have been discovered[16] – only "re-discoveries" have occurred, involving modifications of already known antibiotics[16] (Table 1.1). The decline in discovery of novel classes of antibiotics is problematic due to the emergence and spread of antibiotic resistance, where bacterial infections can no longer be treated with the same drugs that were once so effective. Since antibiotics are inseparable from today's world, there are concerns a "post-antibiotic era" may be approaching[20]. In light of this, extraordinary research is taking place across the world to discover new antibiotics, develop alternatives to antibiotics, and understand the evolution and spread of antibiotic resistance.

Table 1.1. Sources of antibiotics. The year of discovery and source of current antibiotic classes are described. Adapted from Silver, 2011[16] and Chandra and Kumar, 2016[3].

Year	Antibiotic	Source	Kingdom; Division (Fungi) or Phylum (Bacteria); Class; Order; Family)
1928	Penicillins	*Penicillium*	Fungi; Ascomycota; Eurotiomycetes; Eurotiales; Trichocomaceae
1932	Sulphonamides	Synthetic	
1942	Aminoglycosides	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1942	Bacitracin	*Bacillus subtilis*	Bacteria
1945	Tetracyclines	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1946	Nitrofurans	Synthetic	
1947	Polymyxins	*Paenibacillus polymyxa*	Bacteria
1947	Phenicols	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1948	Cephalosporins	*Acremonium*	Fungi; Ascomycota; Hypocreales; Hypocreaceae
1950	Pleuromutilins	*Clitopilus passecherianus*	Fungi; Basidiomycota; Agaricomyctes; Agaricales; Entolomataceae
1952	Glycopeptides	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1952	Nitroimidazoles	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1952	Streptogramins	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1955	Cycloserine	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1955	Novobiocin	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1957	Rifamycins	*Amycolatepsis rifamycinica*	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Pseudonocardiaceae
1961	Trimethoprim	Synthetic	
1962	Quinolones	Synthetic	
1962	Lincosamides	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1962	Fusidic acid	*Fusidium coccineum*	Fungi; Ascomycota; Hypocreales; Nectriaceae
1969	Fosfomycin	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1971	Mupirocin	*Pseudomonas fluorescens*	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae
1976	Carbapenems	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1978	Oxazolidinones	Synthetic (derived from cycloserine)	
1979	Monobactams	Synthetic (derived from *Chromobacterium violaceum*)	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae
1987	Lipopeptides	*Streptomyces* spp.	Bacteria; Actinobacteria; Actinomycetes; Actinomycetales; Actinomycetaceae
1995	Glycylcyclines	Synthetic	Synthetic (tetracycline derivative)

1.3 Clinically relevant antibiotics

Today, seventeen classes of antibiotics are considered essential by the World Health Organisation[21] (Table 1.2). There are three categories of essential antibiotics: Access (first or second line of defence options for common infections); Watch (higher resistance potential but still recommended as first or second line treatments); and Reserve ('last resort' options for serious antibiotic-resistant infections). Each antibiotic has a different mechanism of action (Fig. 1.2), mechanism of resistance and can target different types of bacterial pathogens (Table 1.2). This may include Gram-negative and/or Gram-positive bacteria, as determined by the Gram staining technique developed by Hans Christian Gram in 1884[22]. In addition, bacteria can be strictly aerobic or anaerobic, meaning that the absence or presence of oxygen respectively is toxic, or somewhere in between[23]. Antibiotics such as aminoglycosides rely on components of the aerobic respiration pathway, and as such their spectrum of activity is thought to only include aerobic bacteria and not anaerobes[24]. Essential antibiotics are discussed in more detail below, including their reported spectrum of activity.

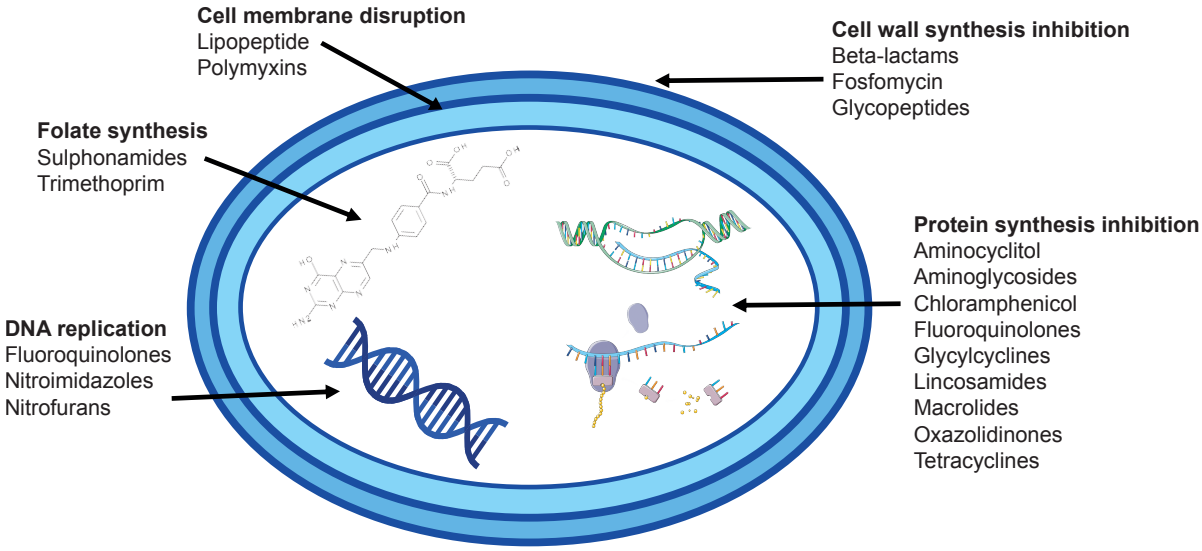

Figure 1.2. Mechanism of action for antibiotics on the WHO List of Essential Medicines 2017[21]. Adapted from Shaikh *et al.* 2015[25]. Vector images of DNA, folate and protein synthesis from Freepik.com and smart.servier.com.

Table 1.2. The antibiotics included in the 20th Edition of the WHO List of Essential Medicines (2017)[21]. These antibiotics are considered essential by the WHO to treat bacterial infections. There are three categories: Access (first or second line options for common infections); Watch (higher resistance potential but still recommended as first or second line treatments); and Reserve ('last resort' options for serious antibiotic-resistant infections). Antibiotics can be considered both Access and Watch; here, they have been listed under the higher concern category of Watch for conciseness.

WHO Category	Antibiotic Class	Sub-class	Drug name	Mechanism of action	Mechanism of resistance	Used against
Access	Aminocyclitol		Spectinomycin	Protein synthesis inhibition: bind 30S ribosomal subunit	Ribosomal mutations; enzymes that modify either the antibiotic or target	Aerobic Gram-negative bacteria e.g. *Pseudomonas aeruginosa* and staphylococci
Access	Aminoglycoside		Amikacin Gentamicin	Protein synthesis inhibition: bind 30S ribosomal subunit	Ribosomal mutations; antibiotic- or target-modifying enzymes	Aerobic Gram-negative bacteria e.g. *Pseudomonas aeruginosa* and staphylococci
Access	Amphenicol		Chloramphenicol	Protein synthesis: interacts with 23S rRNA of 50S ribosomal subunit	Ribosomal mutations; antibiotic-modifying enzymes; efflux	Gram-negative bacteria e.g. *Haemophilus influenzae* and Gram-positives e.g. *Streptococcus pneumoniae*
Access	Beta-lactam	1st generation cephalosporin	Cefalexin Cefazolin	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives e.g. penicillin-resistant bacteria such as *Pseudomonas aeruginosa*
Access	Beta-lactam	Penicillin	Amoxicillin Amoxicillin + clavulanic acid Ampicillin Benzathine benzylpenicillin Benzylpenicillin Cloxacillin Phenylmethylpenicillin Procain benzylpenicillin	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives

WHO Category	Antibiotic Class	Sub-class	Drug name	Mechanism of action	Mechanism of resistance	Used against
Access	Folic acid metabolism inhibitors		Sulphamethoxazole + trimethoprim	Folate synthesis inhibition: inhibit enzymes in different stages of the folic acid biosynthesis pathway	Mutations to increase expression of trimethoprim targets, or in the targets themselves, or acquisition of genes encoding less sensitive targets	Gram-negative and Gram-positives including MRSA
Access	Nitroimidazole		Metronidazole	DNA replication inhibition: the prodrug is reduced and converted to the toxic drug that inhibits DNA synthesis	Decreased uptake; altered reduction efficiency; efflux; drug inactivation; acquisition of genes that encode alternative reductases and convert prodrug to non-toxic alternative	Anaerobic Gram-negative or Gram-positive bacteria e.g. *Prevotella*
Access	Nitrofuran		Nitrofurantoin	DNA replication inhibition: the prodrug is reduced and the intermediate metabolites bind enzymes involved in DNA and RNA synthesis	Mutations in reductases	Gram-negative and Gram-positives excluding intrinsically-resistant *Klebsiella* and *Pseudomonas* spp.
Access	Tetracycline		Doxycycline	Protein synthesis inhibitor: prevent translation by binding 16S rRNA in 30S subunit	Efflux; target-protection proteins that dislodge tetracycline	Gram-negative and Gram-positives including MRSA
Watch	Beta-lactam	Anti-pseudomonal penicillin	Piperacillin + tazobactam	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives including *Pseudomonas aeruginosa*
Watch	Beta-lactam	Carbapenem	Imipenem + cilostatin Meropenem	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives, especially multi-drug resistant infections

WHO Category	Antibiotic Class	Sub-class	Drug name	Mechanism of action	Mechanism of resistance	Used against
Watch	Beta-lactam	3rd generation cephalosporin	Cefixime Ceftriaxone Cefotaxime Ceftazidime	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives, especially for bacterial meningitis and sepsis
Watch	Beta-lactam	Penem	Faropenem	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives, especially multi-drug resistant infections
Watch	Fluoroquinolone		Ciprofloxacin Levofloxacin Moxifloxacin Norfloxacin	DNA replication inhibition: inhibit DNA gyrase	DNA gyrase mutations; efflux; Qnr production (protein that competes for fluoroquinolone binding site)	Gram-negative and Gram-positives, including anaerobes
Watch	Glycopeptide		Teicoplanin Vancomycin	Cell wall synthesis inhibition: bind D-Ala-D-Ala cell wall molecules	Acquisition of van gene cluster (replaces final D-Alanine in cell wall molecules to prevent glycopeptide binding and destroys normal D-Ala-D-Ala molecules)	Gram-positives e.g. *Enterococcus faecalis* and *Clostridioides difficile*
Watch	Lincosamide		Clindamycin	Protein synthesis inhibition: inhibit translocation by binding 23S rRNA in 50S ribosomal subunit	Ribosomal mutations or modifications; efflux	Gram-positives and anaerobes, but not typically Gram-negative aerobes
Watch	Macrolide		Azithromycin Clarithromycin	Protein synthesis inhibition: inhibit translocation by binding 23S rRNA in 50S ribosomal subunit	Ribosomal mutations or modifications; efflux	Gram-positives and anaerobes, but not typically Gram-negative aerobes
Reserve	Beta-lactam	Monobactam	Aztreonam	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative aerobes, including *Pseudomonas aeruginosa*

WHO Category	Antibiotic Class	Sub-class	Drug name	Mechanism of action	Mechanism of resistance	Used against
Reserve	Beta-lactam	4th generation cephalosporin	Cefepime	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives
Reserve	Beta-lactam	5th generation cephalosporin	Ceftaroline	Cell wall synthesis inhibition: interact with cell wall catalysis enzymes	Antibiotic-degrading enzymes, beta-lactamases; mutations in cell wall proteins	Gram-negative and Gram-positives including MRSA
Reserve	Peptide: cyclic lipopeptide		Daptomycin	Membrane function disruption: inserts into cytoplasmic membrane, disrupts integrity and triggers rapid cell death	Mutations in genes that alter the target or entry of daptomycin into cytoplasmic membrane	Gram-positives e.g. vancomycin-resistant enterococci and MRSA
Reserve	Fosfomycin		Fosfomycin	Cell wall synthesis inhibition: inhibits the first step of cell wall synthesis	Mutations in *murA*, the protein that initiates cell wall synthesis; mutations in membrane transporters, acquisition of fosfomycin-inactivating enzymes	Gram-negative and Gram-positive bacteria, especially for antibiotic-resistant UTIs
Reserve	Glycylcycline		Tigecycline	Protein synthesis inhibition: binds 30S ribosome, blocking tRNA entry	Efflux	Gram-negative and Gram-positive, including tetracycline-resistant bacteria
Reserve	Oxazolidinone		Linezolid	Protein synthesis inhibition: binds 23S rRNA in 50S ribosomal subunit and interacts with tRNAs to prevent protein synthesis initiation	Efflux (especially in Gram-negative aerobes); ribosomal mutations	Gram-positives e.g. vancomycin-resistant enterococci and MRSA, Gram-negative anaerobes
Reserve	Peptide: polymixins		Colistin Polymyxin B	Outer membrane disruption: bind lipopolysaccharide to destabilise OM and IM, induce osmotic imbalance and oxidative stress	Chromosomal mutations; acquisition of genes that alter the lipopolysaccharide	Multi-drug resistant Gram-negative aerobes

1.3.1 Access antibiotics: first or second line of defence

Access antibiotics are those that, according to the WHO, are first or second line drugs for common infectious diseases and should be: widely available, affordable, appropriately formulated and quality controlled[18]. First line antibiotics are typically narrow spectrum: they only target a limited range of bacteria, such as the intended pathogenic bacterium and possibly some close relatives[18]. They should also have low resistance potential and positive benefit-to-risk ratios[18]; i.e., the risk of harm to the patient is low compared to the potential treatment of their infection. Broad spectrum antibiotics, that target a wider range of bacteria, are generally used as second line options, as are drugs with higher resistance potentials or less positive benefit-to-risk ratios[21] (e.g. they might have more common or more serious side effects).

Aminocyclitol antibiotics, such as spectinomycin, bind the 30S subunit of the bacterial ribosome, disrupting protein synthesis. **Aminoglycosides**, such as amikacin and gentamicin, act via the same mechanism. To do this, they must enter the cell through its cytoplasmic membrane – a process that requires energy dependent active bacterial transport[24]. This in turn requires oxygen and an active proton motive force; therefore these drugs are only typically effective for bacteria capable of aerobic respiration[24]. Aminocyclitols and aminoglycosides are considered inactive against anaerobic bacteria, which have adapted to survive without oxygen and do not have the usual required components to transport these drugs into their cells[24]. Therefore, these drugs are considered broad-spectrum but only for a range of aerobic bacteria. Although these antibiotics are known to be toxic to humans and can cause hearing loss, they are considered first line treatments for respiratory infections in cystic fibrosis patients[26]. They are also used to treat multiple-drug resistant (MDR) infections, often as part of combination therapy with beta-lactams, since these classes of antibiotics have a

synergistic effect when used together[26]. Aminoglycoside resistance is mediated by ribosomal mutations, enzymes that modify the antibiotic, or enzymes that modify the target[27].

Chloramphenicol, an **amphenicol**, is considered a broad-spectrum antibiotic and interacts with the 23S rRNA of the 50S ribosomal subunit, preventing protein synthesis[24]. It is a first line option for eye infections such as conjunctivitis, which can be caused by bacteria including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*[28]. Chloramphenicol resistance is mediated by ribosomal mutations or chloramphenicol-modifying enzymes. It can also be due to efflux-mediated resistance[29]. Mutations in components of efflux systems can increase the affinity for certain molecules including antibiotics, resulting in increased resistance[29].

Beta-lactams are a diverse group of antibiotics with several sub-classes. This includes penicillins, cephalosporins, carbapenems and monobactams. Beta-lactam antibiotics have a chemical structure that is similar to that of D-Alanyl-D-Alanine (D-Ala-D-Ala), a 'building block' of bacterial cell walls[30]. This means the beta-lactams can interact with the enzymes that catalyse the cell wall synthesis, preventing proper cell wall synthesis and resulting in osmotic instability and bacterial cell death[30]. The different sub-classes of beta-lactams are categorised by the modification to the standard beta-lactam structure that defines that particular group. **Penicillins** and **1st generation cephalosporins** are on the Access list of antibiotics. Both these sub-classes of beta-lactam are typically considered narrow spectrum. However, the spectrum varies from drug to drug: for example, amoxicillin is considered broad-spectrum or extended-spectrum compared to the original penicillin[31]. Penicillins, especially amoxicillin, are the first line drug of choice for common bacterial infections, such as dental, ear, respiratory and throat infections[32], hence their position as the most used drugs in the world[33]. Genes that encode beta-lactamase enzymes, which degrade beta-lactam antibiotics, are some of the most

common antibiotic resistance genes (ARGs): over 1000 different beta-lactamase genes are described[29]. Beta-lactams can be given alongside a beta-lactamase inhibitor, such as amoxicillin with clavulanate, to prevent a beta-lactamase enzyme from functioning properly[24]. This combination increases the efficiency of the amoxicillin. First generation cephalosporins, such as cefalexin and cefazolin, have often been used as second-line options as an alternative to penicillin, in the case of resistance or allergies[21]. Beta-lactam resistance can also be mediated by mutations or modifications in the antibiotic target (penicillin-binding proteins (PBPs) in the cell wall) or in porins, which allow the beta-lactam antibiotics to enter the cell[29].

The **folic acid metabolism inhibitor** combination of sulphamethoxazole and trimethoprim is active against many different bacterial infections[24]. Bacteria must synthesise *de novo* folates that can act as cofactors for various other biosynthetic pathways; without these cofactors their growth is inhibited[34]. Sulphonamides, such as sulphamethoxazole, and trimethoprim, a diaminopyrimidine antibiotic, inhibit enzymes involved in different stages of the folic acid biosynthetic pathway and thus this combination is considered synergistic[24]. It is a common first-line treatment for infections including urinary tract infections (UTIs), traveller's diarrhoea, methicillin-resistant *Staphylococcus aureus* (MRSA) skin infections, respiratory tract infections, and cholera[35]. Resistance to trimethoprim is mediated by mutations that increase the expression of trimethoprim targets so that the antibiotic is outnumbered and its effect limited[29]. Mutations that alter the targets themselves or the acquisition of genes that encode less sensitive targets are also known to result in trimethoprim resistance[29]. Sulphonamide resistance is common and mainly due to plasmid-borne genes encoding alternative enzymes that are less sensitive to the antibiotic[36]. This is a method of horizontal gene transfer (HGT): the movement of genes between bacterial cells that bypasses vertical transmission.

The **nitroimidazole**, metronidazole, inhibits DNA synthesis and causes DNA damage in anaerobic bacterial cells[35]. Strict aerobic bacteria lack electron-transport proteins with sufficient negative redox potential to activate the prodrug form once it is inside the cell, thus metronidazole has the opposite spectrum to aminoglycosides[37]. It is the first-line option for infections caused by anaerobic bacteria, such as dental abscesses (e.g. by *Prevotella* or *Streptococcus)*[38] or bacterial vaginosis caused by *Gardnerella vaginalis*[37]. Resistance is mediated by decreased uptake or altered reduction efficiency (that also leads to decreased uptake)[37]. Efflux and drug inactivation are also possible, as is the acquisition of *nim* genes from other bacteria via HGT. These *nim* genes encode alternative reductase enzymes to convert the prodrug into a non-toxic alternative[37].

The **nitrofuran**, nitrofurantoin, has multiple antibacterial properties – none of which are fully understood yet[39]. Like nitroimidazoles, it is a prodrug, and is activated inside the bacterial cell by the action of nitroreductases[39]. The intermediate metabolites produced bind to bacterial ribosomes and enzymes involved in DNA and RNA synthesis, plus other metabolic processes[39]. It is broad-spectrum with activity against both Gram-negatives and Gram-positives, though some *Klebsiella* and *Pseudomonas* species are intrinsically resistant[39], and it is a first-line treatment for uncomplicated, lower UTIs[39]. Resistance is thought to be mediated by mutations in nitroreductases[39].

Tetracyclines such as doxycycline are another type of protein synthesis inhibitor that act upon conserved sections of the 16S rRNA in the 30S subunit, preventing translation[24]. It has broad spectrum and is commonly used to treat sexually transmitted infections (STIs), Lyme disease, skin infections and MRSA[40]. Resistance is mediated by tetracycline-specific efflux pumps or target-protection proteins that dislodge tetracycline when it is bound to the ribosome[29].

1.3.2 Watch antibiotics: first or second line of defence with high resistance potential

Watch antibiotics have higher resistance potential and may be of broader spectrum[21]. These antibiotics are also featured on the Highest Priority Critically Important Antimicrobials (CIA) list[41], which is intended to ensure that these antibiotics are prioritised for stewardship strategies and used prudently.

Piperacillin with tazobactam is another penicillin/beta-lactamase inhibitor combination, as described earlier. *Pseudomonas aeruginosa* is an opportunistic, Gram-negative pathogen that can cause infections in humans, but is intrinsically resistant to penicillin antibiotics caused by the production of a beta-lactamase enzyme[42]. Piperacillin with tazobactam has **anti-pseudomonal** activity and therefore is a common option for the treatment of *P. aeruginosa* infections. This bacterium has become a major contributor to infections that are hospital-acquired and/or in immunocompromised or critically ill patients[43]. It is important then that antibiotics capable of treating these infections are only used when appropriate to limit the development of resistance and conserve their utility.

There are two other sub-classes of beta-lactams that are on the Watch list: **third generation cephalosporins** and **penems**. Third generation cephalosporins have a specific type of modification to the basic beta-lactam structure that increases their specificity to binding cell wall proteins[44]. They have a broader spectrum than previous generations, although again it varies between drugs. For example, cefotaxime and ceftizoxime have the best Gram-positive coverage of third generation cephalosporins[44], though they are typically used to treat Gram-negative infections in hospitals associated with meningitis and sepsis[44]. Penems, including carbapenems, have the broadest spectrum of activity of the beta-lactams and are relatively resistant to most types of beta-lactamases[45]; hence why they are often used to treat beta-

lactam resistant or MDR infections[45]. Carbapenem resistance can be conferred by single nucleotide polymorphisms (SNPs) that affect PBPs in the cell wall[46] or in the porin that enables carbapenem import into the cell[46], or by increased activity of efflux pumps[46]. The most clinically important cause of carbapenem resistance is carbapenemases enzymes[29].

Fluoroquinolone antibiotics, such as ciprofloxacin, are highly active against both Gram-negatives and Gram-positives[47]. Newer fluoroquinolones in particular, such as moxifloxacin, have very broad spectrums of activity, including against anaerobes[47]. They work by inhibiting DNA gyrase, an enzyme involved in DNA replication and transcription[24]. Fluoroquinolones are very commonly prescribed drugs, including for ear, gastrointestinal (GI) and respiratory infections and UTIs[47]. Resistance is commonly mediated by mutations in the target enzymes, as well as over-expression of efflux pumps and production of Qnr, a protein encoded by plasmids that competes for the fluoroquinolone binding site[29].

Glycopeptides are similar to beta-lactams in that they inhibit cell wall synthesis, however they do this by binding to the D-Ala-D-Ala molecules rather than the enzymes that process synthesis[24]. These antibiotics target Gram-positive organisms only; due to their size, they are typically unable to cross the outer membrane (OM) of Gram-negatives[48]. Teicoplanin is more potent than vancomycin, but both are crucial antibiotics used to treat infections caused by Gram-positive bacteria such as *Enterococcus* or *Clostridioides difficile*[48]. Resistance to vancomycin is mediated by the acquisition of a *van* gene cluster, which act to replace the final D-Alanine in the cell wall molecules with D-lactate or D-serine, preventing vancomycin binding[29]. The normal D-Ala-D-Ala molecules are also destroyed by the function of the van gene operon[29].

Lincosamides, such as clindamycin, and **macrolides**, such as azithromycin, have similar mechanisms of action. They inhibit translocation, an early stage in protein synthesis, by targeting the conserved sequences of the 23S rRNA in the 50S ribosomal subunit[24]. Lincosamides target Gram-positives and anaerobes, but typically not Gram-negative or strictly aerobic organisms[49]. They are often used to treat head, neck, respiratory, bone, soft tissue, abdominal, and pelvic infections[50]. Macrolides have a similar broad spectrum of activity, plus some Gram-negative aerobes or facultative anaerobes, but Enterobacteriaceae tend to be resistant due to having relatively impermeable cell walls[51]. They are also used for respiratory tract, skin and soft tissue infections[51]. Resistance to lincosamides and macrolides can be mediated by mutations in the ribosome, modifications of the ribosome (e.g. methylation), or by efflux pumps[29].

1.3.3 Reserve antibiotics: last resort

Reserve antibiotics are those that should only be used as a last resort; that is, in the case of a serious or life-threatening multi-drug resistant infection that is not responding to first or second line treatments[41].

There are three types of beta-lactam included on the reserve list: **fourth and fifth generation cephalosporins** and **monobactams**. Fourth and fifth generation cephalosporins have a broader spectrum of, and higher, activity[52] and are more resistant to extended-spectrum beta-lactamases[53] than previous generation cephalosporins. The fifth generation cephalosporin ceftaroline also has activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) and is now a vital drug in the treatment of MRSA infections[54]. In contrast, monobactams such as aztreonam have a narrow spectrum and are only active against Gram-negatives capable of

aerobic respiration, including *P. aeruginosa*[55]. These synthetic antibiotics are highly resistant to beta-lactamases and are used to treat multi-drug resistant Gram-negative infections[55]. However, there are certain beta-lactamases, such as TEM-3, that are capable of degrading aztreonam[29].

Daptomycin is a synthetic **cyclic lipopeptide** antibiotic first reported in 2002[56] with a unique mechanism of action. It inserts into the bacterial cytoplasmic membrane, where it disrupts the membrane functional integrity to trigger the release of intracellular ions and cause rapid cell death[57]. It has antibiotic activity against Gram-positive bacteria, such as vancomycin-resistant enterococci (VRE), MRSA and penicillin-resistant streptococci – for which there are few alternative treatments[57]. Daptomycin resistance is complicated but thought to be mediated by mutations in genes that alter the target or the entry of daptomycin into the cytoplasmic membrane[58]. Daptomycin-degrading enzymes exist in environmental bacteria but are not currently considered clinically relevant[59].

Fosfomycin (a simple form of phosphonic acid) was discovered from *Streptomyces* in 1969 and is unique in its mechanism of inhibiting the first step in bacterial cell wall synthesis[60]. It is active against both Gram-negative and Gram-positive bacteria and though it was uncommonly used for several decades, in recent years it has been revived to treat antibiotic-resistant UTIs[60]. UTIs account for a significant burden of hospital admissions and are increasingly resistant to first or second line antibiotics[61], and so reserving fosfomycin for use only when absolutely necessary is vital to continue treating these cases. Fosfomycin resistance is rare as there appears to be an associated fitness cost, but mutations in *murA*, the gene encoding the protein that initiates cell wall synthesis, can occur[62]. In addition, mutations that alter membrane transporters can prevent fosfomycin entering the cell, and bacteria can acquire plasmid-encoded genes that inactivate the drug[62].

Tigecycline is the first of the synthetic class of **glycylcycline** antibiotics, developed in the 2000s[63]. Glycylcyclines feature a modified tetracycline structure; tetracycline resistance is now rife since this antibiotic is relatively old and very widely-used[64]. The glycylcycline structure binds to the bacterial 30S ribosome, blocking tRNA entry and preventing protein synthesis[63]. Tigecycline is broad spectrum, including activity against drug-resistant Gram-positive infections and even some resistant to tetracycline, despite any presence of tetracycline-specific resistance mechanisms[65]. However, efflux pumps involved in MDR and point mutations can sometimes cause tigecycline resistance[29].

The **oxazolidinone** linezolid is another synthetic antibiotic, from the late 1980s, which interferes with protein synthesis in two separate stages: by binding the 23S rRNA in the 50S ribosomal subunit and by interacting with tRNAs to prevent protein synthesis initiation[66]. Like daptomycin, it is used to treat antibiotic-resistant Gram-positive infections such as VRE and MRSA but also has activity against Gram-negative anaerobes[67]. Gram-negative aerobic or facultative anaerobic bacteria can contain efflux pumps that are able to pump out linezolid and resist its action[67]. Resistance is also mediated by ribosomal mutations[29].

Polymyxins are non-ribosomal cyclic lipopeptides, originally discovered in 1947 during "the golden age of antibiotic discovery" before falling out of use due to nephrotoxicity. They have been revisited more recently due to the increasing cases of MDR infections[68]. Polymyxins bind to the lipopolysaccharide in the OM of Gram-negative bacteria, causing outer and subsequently inner membrane destabilisation[68]. They are also reported to induce osmotic imbalance and oxidative stress, resulting in cell lysis[68]. Polymyxins are not susceptible to the activity of efflux pumps[24], hence why they are such good options to selectively treat serious Gram-negative aerobic or facultative anaerobic infections that are resistant to other

antibiotics[69]. Colistin resistance is mediated by chromosomal mutations or acquisition of genes that alter the lipopolysaccharide[27].

From this information discussed so far, we can make two important conclusions: firstly, beta-lactam antibiotics feature in all three categories of antibiotics, encompass a large number of drugs, are among the oldest clinically-used antibiotics and are still the most prescribed drugs in the world. This underlines their critical importance in modern human medicine. Secondly, that resistance to all these clinically relevant antibiotics among bacterial pathogens has emerged[5], including to last resort antibiotics. This means that cases are arising where an infection cannot be treated with any available antibiotics – sometimes resulting in the patient's death[70]. For example, a woman died in the United States in 2017 following a carbapenem-resistant Enterobacteriaceae infection: the strain of *K. pneumoniae* she was infected with was resistant to 26 antibiotics available in the country at the time[70]. This highlights the increasing severity of antibiotic resistance.

1.4 Bacterial genetics and antibiotic resistance

There are three main ways in which bacteria can be resistant to antibiotics that are explained by their genetics: intrinsic resistance, where the bacteria are naturally resistant; DNA mutations, where mutations in the genome lead to increased resistance; and antibiotic resistance genes, where entire genes can explain increased resistance. These will now each be discussed in more detail.

1.4.1 Intrinsic resistance

There are a number of biological differences between bacteria that mean not all antibiotics are effective against all bacteria; this natural resistance is also described as intrinsic resistance. For example, Gram-negative bacteria have an OM, which Gram-positives do not possess[71]. The OM prevents access of glycopeptide antibiotics such as vancomycin to Gram-negative bacteria such as Proteobacteria[71]. Moreover, metabolic differences (e.g. aerobic versus anaerobic) can explain intrinsic resistance. For example, efflux pumps, that can pump out antibiotics from bacterial cells, are common in aerobic and facultatively anaerobic bacteria such as Proteobacteria[29]. As they require oxygen and active transport to function, they are uncommon in anaerobic bacteria. The decision of which antibiotic to prescribe must take these differences into consideration, along with a number of other factors relating to the disease-causing bacterium, the antibiotic and the patient. However, these intrinsic resistances are not involved in the development or spread of antibiotic resistance as they occur naturally; instead, these processes are caused by changes in the bacterial genome[72].

1.4.2 DNA mutations

One key way in which antibiotic resistance can develop is by changes in the DNA sequence, or mutations. Mutations occur naturally: when cells replicate and divide, DNA must also be replicated, but the process is not error-free[73]. If incorrect nucleotides are incorporated into the new DNA strand being synthesised, this could have an impact on the function of that particular DNA sequence. For example, a bacterial cell with a mutation in a gene that encodes the target of an antibiotic may have a reduced affinity for how the antibiotic binds to said target[72]. In the presence of that antibiotic, that cell is less likely to be killed by the antibiotic

and can divide to produce more cells featuring the same DNA mutation. In this way, a more resistant strain can replace and take over the previous population of bacterial cells; thus, antibiotics select for bacteria that are more tolerant of or resistant to the antibiotic being used. The problem is magnified when we consider the short generation times of bacteria: *E. coli* can divide in approximately 20 minutes, leading to millions of cells in a matter of hours[74]. This allows antibiotic-resistant bacteria to be selected for in a short time period and also for mutations to accumulate and potentially become fixed in the population[73]. Mutations can impact antibiotic resistance in several ways: they can alter the target of the antibiotic so that the two can no longer interact; they can alter an efflux pump to have increased activity and reduce the concentration of antibiotic in the cell; they can increase the amount of the target protein in the bacterial cell so the impact of the antibiotic is reduced; or increase the expression and production of antibiotic-degrading enzymes. Using sufficiently high antibiotic dosing regimens can prevent a sub-population of cells with antibiotic resistance-conferring mutations from surviving[75,76].

1.4.3 Antibiotic resistance genes and horizontal gene transfer

In addition to DNA mutations, bacteria can harbour antibiotic resistance genes (ARGs): genes that encode proteins that are capable of modifying or degrading antibiotics or their targets[72]. One of the most common examples is a beta-lactamase, an enzyme capable of degrading beta-lactam antibiotics (the class that includes penicillins). There are both different sub-classes of beta-lactam antibiotics and beta-lactamase enzymes. Different beta-lactamases can have differing levels of activity against sub-classes of beta-lactam antibiotics[25]. This means that beta-lactam antibiotics belonging to a different sub-class (e.g. a cephalosporin) could be used

to treat infections that are resistant to penicillin via a penicillin-degrading beta-lactamase. Modifying and degrading enzymes exist for different types of antibiotics, as outlined in section 1.3 and Table 1.2.

The critical issue with ARGs is that they can be transferred by HGT; there are three main processes through which this can take place. Firstly, there is transformation, where bacteria that are "competent" can take in DNA from their environment that is self-replicable or is incorporated into their genome[72]. Competence can vary between species and even within a single bacterial cell depending on its replication cycle[72]. Secondly, there is transduction, which involves the packing of bacterial DNA into bacteriophages (viruses that infect bacteria)[72]. When the phage infects another bacterium it inserts the bacterial DNA inside it into the new host bacterium, where it can be incorporated into the bacterial chromosome[72]. Thirdly, there is conjugation, involving the direct cell-cell contact or bridge-like connections between bacteria, allowing for DNA to be transferred from the donor cell to the recipient cell[72].

The movement of ARGs is therefore a major contributing factor to the spread of antibiotic resistance. ARGs are not always transferred alone but can reside in mobile genetic elements (MGEs)[77]; regions of DNA that contain more than a single gene and even elements responsible for their own replication or movement. Such MGEs include:

- Integrative and Conjugative Elements (ICEs): also known as conjugative transposons – DNA sequences that are integrated into the host bacterial genome and encode their own functional conjugation systems
- Integrons: sections of DNA that easily recombine to capture new DNA
- Plasmids: self-replicating, circular extra-chromosomal DNA transferred by conjugation
- Phages: bacterial viruses that can integrate into the host bacterial genome

- Transposable elements: transposons and insertion sequences are DNA sequences that can move position within a genome

The conjugative MGEs are especially relevant to the spread of antibiotic resistance as they aid the transfer between unrelated bacteria. A single MGE can contain multiple ARGs, meaning that a bacterium can acquire resistance to multiple antibiotics at once, even in the absence of selective pressure from those antibiotics. Furthermore, not only does antibiotic use introduce a selective pressure that selects for mutations conferring antibiotic resistance, but it also promotes mutations and HGT[78]. Antibiotic exposure can induce the SOS DNA damage response in bacteria, which arrests the cell cycle and allows any DNA damage to be repaired[79]. SOS-induced DNA damage repair is especially error prone, resulting in an increased mutation rate[79]. In addition, the SOS response is linked to increased rates of conjugative transfer[79]. Thus, antibiotic therapy both promotes and selects for a variety of antibiotic-resistance conferring changes in bacterial genomes.

1.5 Antibiotic resistance is a major global issue

Antibiotic resistance is an ancient strategy used by bacteria to survive competition from other microorganisms that produce antibiotics[5]. It is, therefore, a perfectly natural biological occurrence. However, it is problematic when it occurs in clinical isolates of bacterial infections. Antibiotic resistance is a global threat to human health that is increasing in both prevalence and severity, and is considered one of the most pressing issues of the 21st Century[80]. Some estimates place the mortality rate of drug-resistant infections (DRIs) at 10 million deaths per year by 2050[80]. The highest burden of DRIs is in developing countries, where antibiotics are becoming increasingly more accessible and are often available without a prescription[81]. In

these countries, proper sanitation (e.g. clean water, basic toilets, good hygiene practises and waste disposal) and strict antibiotic guidelines are often lacking[82]. South-East Asia in particular is a global hotspot for emerging infectious diseases due to these reasons, plus small-scale animal production and high livestock densities[83]. As a result, infections are more easily transmitted and there is a strong selective pressure for the evolution of resistant bacteria[82]. For example, enteric pathogens isolated from Vietnamese children suffering from Acute Watery Diarrhoea (AWD) are often MDR[84]. The issue is also important in developed countries: in the USA, 23,000 people die of DRIs annually[85].

Of particular concern are hospital-acquired infections (HAIs), infections acquired whilst receiving other medical treatments that total over 1.7 million cases and almost 99,000 related deaths per year[86]. The leading causes of these are the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.. These contribute significantly to healthcare and economic burdens and are often reported as resistant to multiple drugs[87]. The latter four of these ("KAPE") are all members of the phylum Proteobacteria; of 538 species of bacterial pathogens[88], 43 % (236) belong to Proteobacteria – the biggest contribution of any phyla (Fig. 1.3). Antibiotic resistance in pathogenic Proteobacteria is therefore especially relevant and understanding the spread of antibiotic resistance, plus discovering alternative or novel treatments for MDR pathogenic bacteria, is of high priority[89]. Indeed, the top three microbial infections of critical priority for new treatment R&D according to the World Health Organisation (WHO) involve three of the KAPE Proteobacteria pathogens: *A. baumannii*, *P. aeruginosa*, and Extended-Spectrum Beta-lactamase (ESBL)-producing *Enterobacteriaceae* (and specifically isolates that are resistant to carbapenems)[90].

Carbapenems are typically reserved for infections that are resistant to multiple other antibiotics, including other beta-lactams. As mentioned, beta-lactam antibiotics are some of the most commonly used drugs in the world, and beta-lactamases are some of the most common and well-studied types of ARGs[91]. These often exist on MGEs, and have contributed significantly to the burden of DRIs. For example, the aminopenicillin amoxicillin is one of the

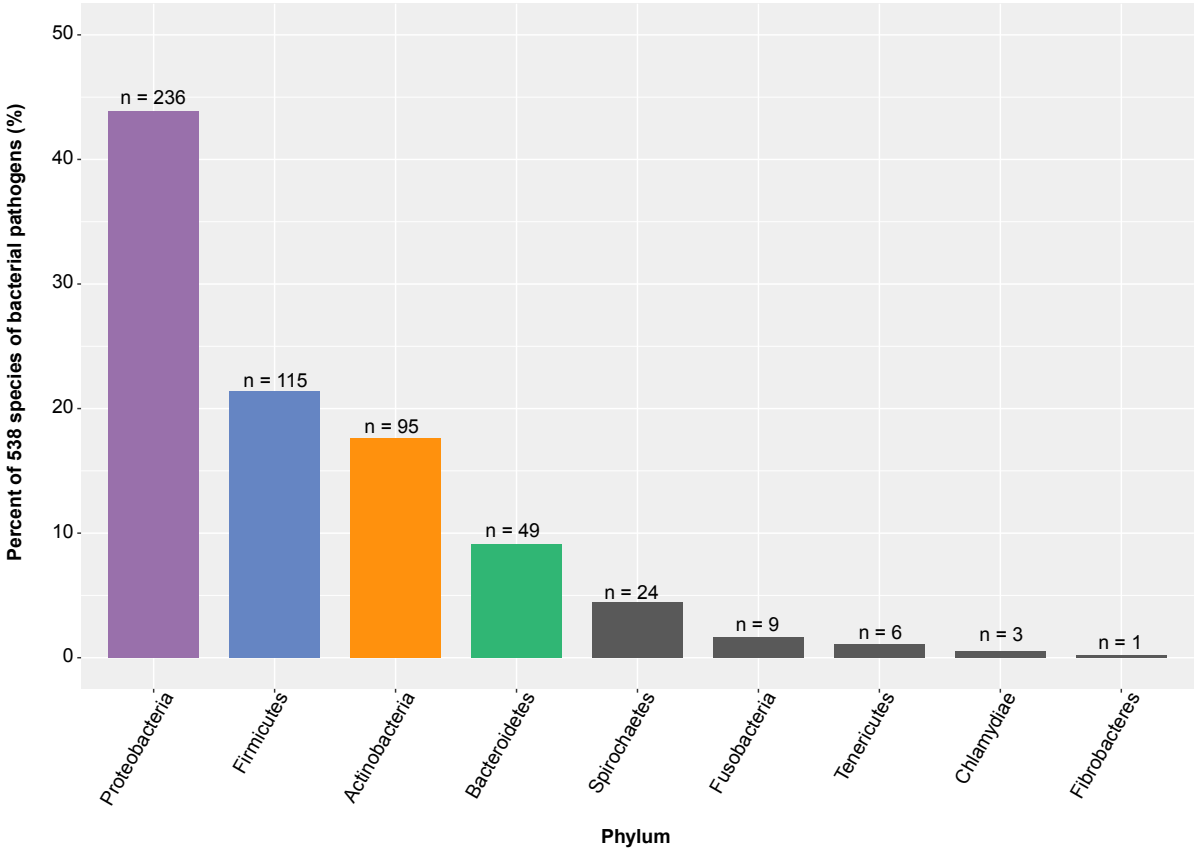

Figure 1.3. Proportions of 538 species of bacterial pathogens belonging to particular phyla. The list of bacteria species was taken from Taylor 2001[88] and the phyla they belong to identified with the online NCBI taxon identifier tool (https://www.ncbi.nlm.nih.gov/Taxonomy/TaxIdentifier/tax_identifier.cgi). The bacterial pathogens belong to nine different phyla, with over 40 % belonging to Proteobacteria and 20 % to Firmicutes. The numbers above the phyla names refer to the number of species observed in each phyla to be bacterial pathogens according to Taylor 2001[88]. The four coloured phyla highlight the four key phyla of the human gut microbiota.

most prescribed antibiotics globally due to its role as a first-line drug in the treatment of otitis media ear infections[33]. These infections are the most frequent reason doctors in the USA prescribe antibiotics[33] and are often caused by bacteria such as the Gram-negative,

facultatively anaerobic, Proteobacteria *E. coli*[92]. Nowadays, up to 95 % of *E. coli* isolates in national collections are resistant to amoxicillin (Fig. 1.4). Although there are a wide range of enzymes that confer extended-spectrum beta-lactam resistance[93], the highly mobilisable CTX-M genes are the most common and important types of ESBL due to their ease of dissemination and prevalence in human, animal and environmental isolates[94]. ESBLs were originally defined as enzymes that could hydrolyse the beta-lactam ring in most beta-lactam sub-classes except carbapenems[93]. However, some ESBLs, such as OXA-23, OXA-40 and OXA-48, have been reported as conferring resistance to carbapenems[95].

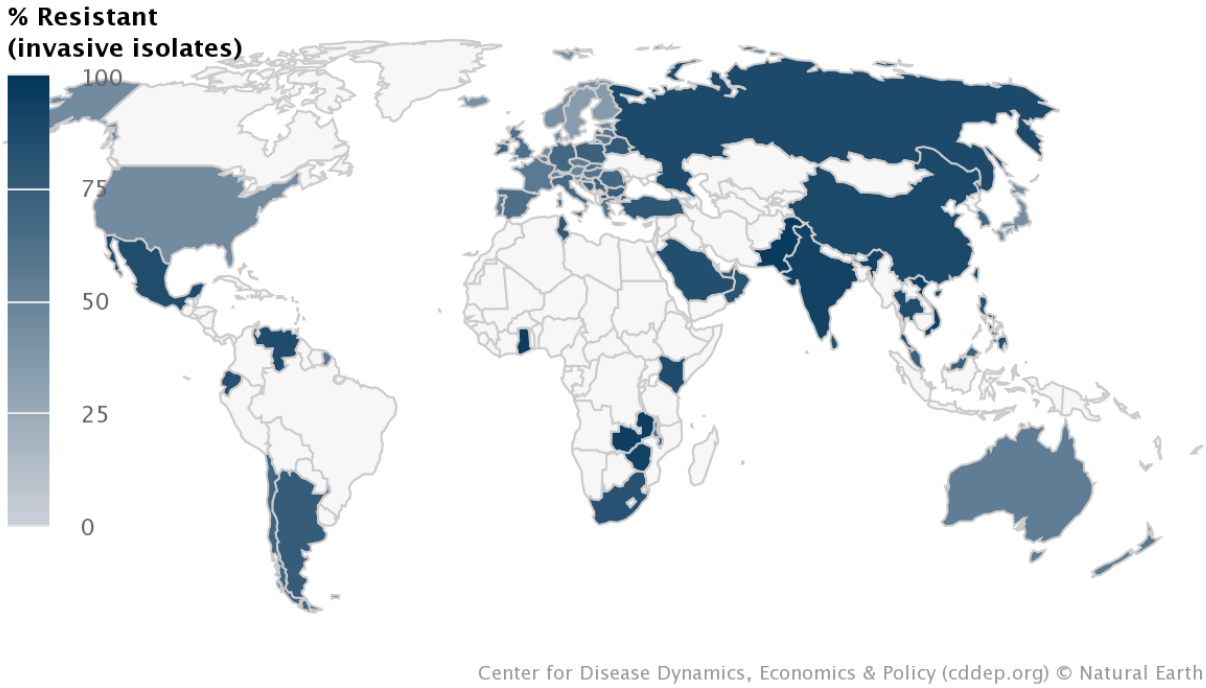

Figure 1.4. Global aminopenicillin (including amoxicillin) resistance in *Escherichia coli*. Map created at https://resistancemap.cddep.org/ on 02/03/2018. Data curated by the Center for Disease Dynamics, Economics and Policy and includes aggregated resistance rates for isolates (includes intermediate resistance) from blood and cerebrospinal fluid (i.e., invasive) from inpatients of all ages. Because of differences in scope of collections and testing methods, caution should be exercised in comparing across countries. Full data available online: The Center for Disease Dynamics Economics & Policy. ResistanceMap: Antibiotic Resistance. 2018. https://resistancemap.cddep.org/AntibioticResistance.php. Data accessed: March 02, 2018.

Carbapenem-resistant Enterobacteriaceae (CRE) infections have increased by 3 % in the USA between 2001 and 2010[90] and have high mortality rates of up to 48 %[96] due to the limited

treatment options. In addition, carbapenem-resistance conferred by mobile carbapenemases is especially important clinically due to their specific ability to hydrolyse the beta-lactam ring in carbapenems and propensity to spread[97]. One treatment option in these cases is colistin, but even resistance to this drug is rapidly spreading. It was previously thought that colistin resistance only involved chromosomal DNA mutations[98]. However, the first plasmid-mediated colistin resistance gene *mcr-1* was identified in 2015[99]. MCR-1 is an enzyme that modifies the colistin target and thus reduces the drug-target interaction, first discovered in *E. coli* isolates from China[99]. Eight *mcr* variants have now been identified[100], at least two of which are globally distributed[101]. Therefore, treatment options are severely limited and emerging colistin resistance in carbapenem-resistant infections is one of the most pressing concerns for global health. The emergence and global spread of mobile carbapenemases and *mcr* genes in clinical isolates of pathogenic bacteria highlights the extent and severity of horizontal antibiotic resistance gene transfer (HGT).

1.6 Antibiotic misuse and overuse: a One Health problem

Many antibiotic treatments are unnecessary, unregulated, or do not correctly follow guidelines[102], leading to increased opportunities for bacteria to develop or spread antibiotic resistance. Underdosing is a particular problem, where bacteria are exposed to a concentration of antibiotics too low to kill them. This can occur if the concentration required to kill the infectious bacterial cells is underestimated, or if the person prescribing, selling or purchasing antibiotics is doing so without consulting official guidance. Antibiotics are now available over-the-counter, without prescription, in countries across the world, meaning someone can choose to self-medicate with antibiotics who may not understand or receive instructions on the correct dosage to take. In addition, some people stop taking antibiotics

once they start to feel better, instead of finishing the advised course of therapy, which may lead to sub-optimal doses of antibiotic that could select for bacterial cells more tolerant to or better able to resist the antibiotic[75]. This 'underdosing' phenomenon was even warned about by Fleming in his Nobel Prize acceptance speech in 1945[103]:

'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. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.' – Alexander Fleming, 1945.

However, there is uncertainty over the guidelines for prescription doses and length of treatments and how important it is to adhere to these rules[75]. Indeed, there is evidence that taking antibiotics for longer is more likely to result in increased resistance than shorter treatment durations[75,104]. Clinical trials investigating end points such as fever resolution[105] as a guide for when to stop antibiotic treatment are recommended to better understand and develop antibiotic prescribing guidelines that reduce the risk of bacteria developing resistance.

The overuse of antibiotics is also problematic – for example in the USA, up to 50 % of antibiotic use is reportedly unnecessary[106]. Reasons for overuse include: prescribing antibiotic therapy despite lack of evidence of bacterial infection or suspected non-bacterial infection; lack of prescription required to obtain antibiotics; easy access to antibiotics (e.g. over-the-counter)[107]. The contribution of these issues varies from country-to-country, leading to variable rates of antibiotic use between nations. Antibiotic use can be measured in defined

daily doses (DDD), which represents assumed average maintenance dose per day for a drug used for its main indication in adults[108]. For example, the DDD for amoxicillin administered orally is 1.5 g per day[109]. DDD can be compared across a number of factors such as inpatients in a hospital, or population per country, e.g. DDD per 1000 people per day. In the UK antibiotics can only legally be obtained with a prescription from a licensed medical practitioner, and there are national campaigns to reduce the unnecessary or inappropriate use of antibiotics. In 2015, the UK had a total 8696 DDDs per 1000 people per day across all antibiotics measured (Fig. 1.5) – this is equivalent to 8.696 DDDs of antibiotics per person per day.

In contrast, Turkey is one of the heaviest users of antibiotics in the world[110], and had 18,095 DDDs per 1000 people per day (18.095 DDDs of antibiotics per person per day). The reasons for such high usage are much the same as already described: poor medical education regarding antibiotics, pharmaceutical industry pressure and promotions, and lack of antibiotic policy or guidelines[111]. Thankfully Turkey, and most other countries, have brought in measures to reduce the overuse and misuse of antibiotics[112]. These measures, such as national campaigns to increase awareness and understanding of antibiotic resistance or more strictly regulate antibiotic use, are known as antibiotic stewardship[113]. This stewardship is designed to limit the development and spread of antibiotic resistance[113] and therefore conserve our antibiotics and prolong their lifespan as much as possible. Despite this, antibiotic use has generally increased over the last fifteen years (Fig. 1.5) and broad-spectrum penicillins such as amoxicillin continue to be the most used type of antibiotic.

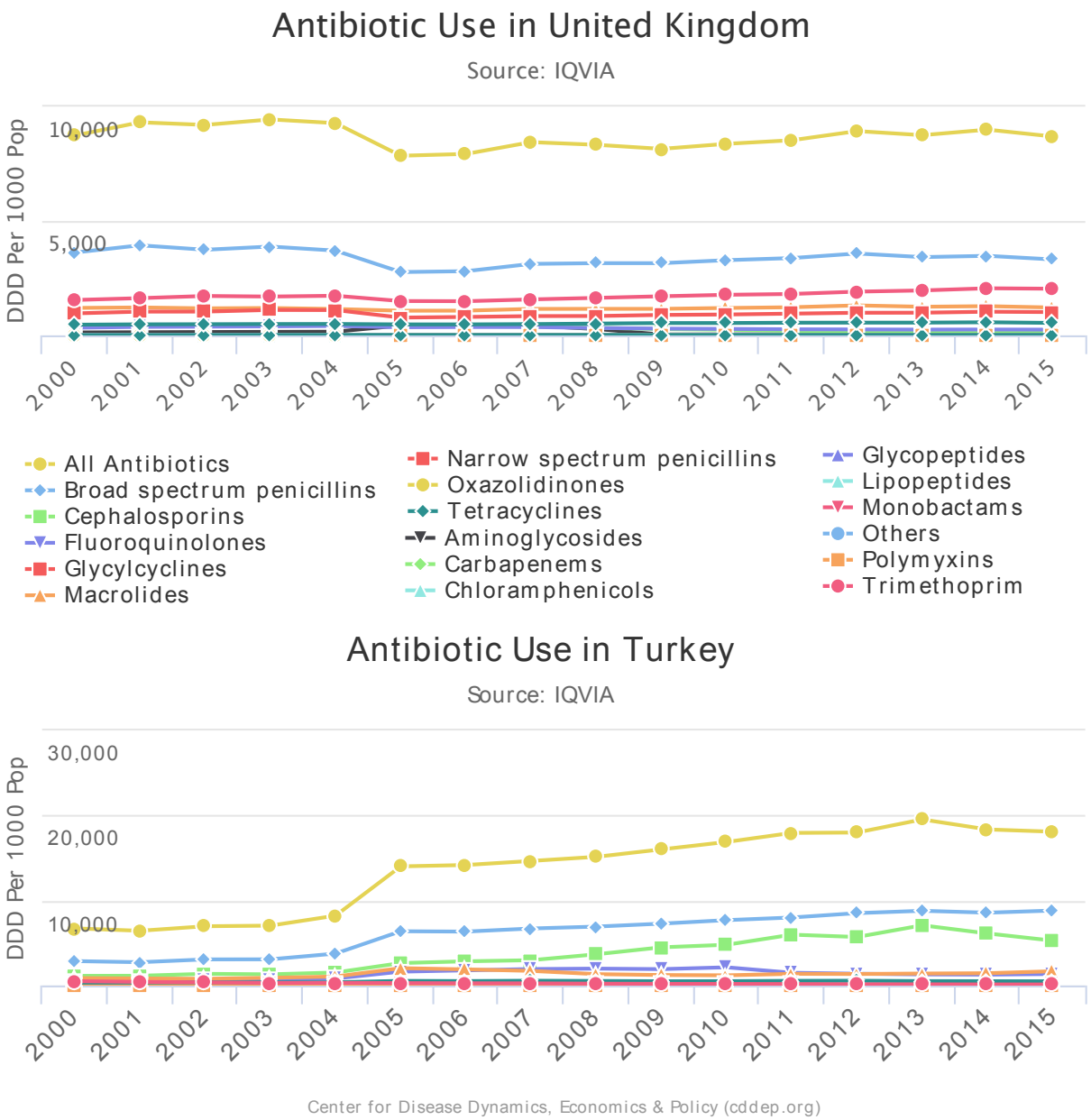

Figure 1.5. Usage of antibiotics in the United Kingdom and Turkey. The UK is the location of the current study and Turkey has the highest use of antibiotics of any country. Antibiotic use data are shown in defined daily doses (DDD) per 1,000 individuals (population, pop) per day. The Center for Disease Dynamics Economics & Policy. ResistanceMap: Antibiotic Use 2018. https://resistancemap.cddep.org/AntibioticUse.php. Date accessed: 01/03/2018.

However, it is not just antibiotic use in people that contributes to the problem of antibiotic resistance. It is a One Health problem, linking human health to the health of animals and the environment, where ensuring optimal health for all these components requires a collaborative effort across scientific disciplines and geography (e.g. locally, nationally, internationally and globally)[114]. This means we need to consider health – and specifically the use of antibiotics

and the presence of antibiotic resistance – in animals and the environment, alongside humans. For example, antibiotic use in agricultural animals is widespread[18,113,115]. Whilst often for medical purposes, antibiotics are also commonly misused in agriculture as growth promoters[116] – the exact mechanism is not well understood, but antibiotics are thought to reduce the amount of bacteria in the gut microbiome of livestock animals, reducing competition for energy derived from the animal's feed[116]. The doses used for these "medically unnecessary" purposes is typically not controlled[115] and so animals can receive sub-inhibitory concentrations[117], creating conditions that select for antibiotic-resistant bacteria.

Clinical cases of antibiotic-resistant infections have been acquired from agricultural animals: for example, drug resistant *Salmonella* infections from poultry date back to the 1960s[118]. This "farm-to-fork" hypothesis proposes that livestock carry antibiotic-resistant bacteria that spread to humans through e.g. direct contact or contaminated animal products[99,119]. If these resistant bacteria enter a human's GI tract, they may potentially cause an infection requiring antibiotic treatment, where the first-line drug of choice may not work. In addition, several important ARGs were first identified in animal associated bacterial strains and have since spread to human-associated strains – including the *mcr-1* colistin resistance gene in colistin-resistant *E. coli* from pigs[99]. Therefore, antibiotic use in agricultural animals should also be monitored and reduced to what is only necessary for proper veterinary medicine. In some countries, such as Sweden, Denmark, the Netherlands and the UK, the use of antibiotics as growth promoters in agriculture is banned[115]. However, other countries (for example, China) continue to use antibiotics critical to human medicine – including the last-resort antibiotic colistin – for this purpose[113]. Clearly, there is still work to be done in reducing the unnecessary use of antibiotics in both humans and animals.

Since antibiotic resistance is a natural phenomenon, antibiotic-resistant bacteria are found in the environment. However, humans have increased the selective pressure on environmental bacteria to promote the development or spread of antibiotic resistance. This can be linked to agricultural use of antibiotics: faeces from livestock may contain antibiotic-resistant bacteria or antibiotic compounds themselves. Areas exposed to agricultural run-off can therefore contain concentrations of antibiotics that promote the development of antibiotic resistance in bacteria that should not have received antibiotic exposure[117]. Again, these may cause antibiotic-resistant infections – if someone ingests vegetables grown in contaminated soil[99,119], for example. Additionally, hospital waste can contaminate local water sources with antibiotics or antibiotic-resistant bacteria[120,121] – if someone went swimming in this water, they may also acquire an antibiotic-resistant infection[122]. Environments containing antibiotic-resistant bacteria and ARGs – which could be harboured by pathogenic and non-pathogenic, environmental bacteria that do not typically cause disease – are referred to as reservoirs of antibiotic resistance. In reservoirs of antibiotic resistance, it is possible that ARGs can move between diverse and multiple bacteria via HGT. Since HGT has contributed to the global dissemination of antibiotic resistance, it is important to understand its mechanisms and the environments in which it occurs. For example – where do pathogenic bacteria acquire ARGs from, and what other bacteria do they donate ARGs to?

1.7 The gut microbiome as a reservoir of antibiotic resistance

The gut microbiome, the complement of commensal microorganisms and their genes that live in the GI tract, was first proposed as a reservoir for antibiotic resistance in 2004[123]. The intestinal microbiota refers specifically to the microorganisms, including bacteria that reside within the GI tract. They make up part of the total human microbiome, along with the skin

microbiome, vaginal microbiome, and microbiomes of other organs and tissues. Humans first become colonised by microbiota during birth, for example from the vagina and faeces if born via the birth canal. Caesarean-born babies are less exposed to these sources of bacteria and are more likely to first be colonised by through skin-skin contact and contact with the environment[124]. We continue to be colonised as we age, from our diets and our environments, and our microbiome matures from its initial form into a mature, relatively stable state[124]. In the healthy adult, there is estimated to be approximately 300 species of commensals in the GI tract[125], of which the majority and most abundant belong to the Firmicutes and Bacteroidetes respectively[126]. Proteobacteria and Actinobacteria are two other common intestinal microbiota phyla, though typically less abundant and diverse in the gut microbiome than Bacteroidetes and Firmicutes[126]. These phyla vary in their respiration physiology: Actinobacteria are typically considered aerobic, with two genera described as anaerobic or facultatively anaerobic[127]; Bacteroidetes are typically considered anaerobic, though two families are thought to be strict aerobes[128]; Firmicutes includes taxa described as aerobic and others described as anaerobic; Proteobacteria are described as facultative anaerobes[129]. Since the gut contains a very low concentration of oxygen and is generally considered an anaerobic environment, most gut bacteria taxa are adapted to these conditions and are considered strictly anaerobic[130]. Facultatively anaerobic or microaerophilic bacteria are much less abundant in the gut than strict anaerobes[130].

Our intestinal microbiota are very important to our health: they help train our immune system[131], digest food and produce energy, metabolise waste products, and protect from certain diseases[132]. One particular example is providing colonisation resistance against bacterial infections such as *C. difficile*[133]; the gut microbiota can prevent this pathogen from colonising the gut and producing toxins which causes severe diarrhoea in

immunocompromised patients. Unfortunately antibiotic use, to treat or prevent bacterial infections including *C. difficile*, can also negatively affect our gut microbiota. This is because antibiotics, especially those considered broad-spectrum and delivered orally, can have a direct impact on our indigenous gut microbiota. This impact may vary at different life stages: for example, in a new-born baby to prevent infection, antibiotics can disrupt the normal colonisation process[134]. This is associated with the later development of immunological conditions such as asthma[134]. As an adult, antibiotic therapy can alter the colonisation resistance of the gut microbiota and leave the person at risk of a *C. difficile* infection[135].

We are exposed to antibiotics throughout our lives: during birth to prevent infection; during childhood to treat common infections such as ear or throat infections; more rarely in adulthood (perhaps for a wisdom tooth infection or a more severe but relatively rare infection such as bacterial meningitis); to prevent infection during labour, in immunocompromised Intensive Care Unit patients or the elderly, amongst others. Due to the use and overuse of orally-administered broad-spectrum antibiotics, our gut microbiota are also often under selective pressure to evolve antibiotic resistance[136]. There is a strong positive correlation between antibiotic consumption and proportions of antibiotic resistant bacteria and antibiotic resistance gene abundance in gut microbiomes[137]: for example, southern Europe consumes more antibiotics than Denmark and accordingly human faecal samples from Denmark have lower carriage of antibiotic resistance genes than samples from Spain or France[137]. In addition, due to the density and diversity of commensal bacteria, the potential for exposure to food-borne pathogens such as members of Enterobacteriaceae is high. It is possible transient intestinal pathogens transfer ARGs to the resident gut microbiota, rendering these ARGs theoretically accessible to any other pathogen that passes through[123], or vice versa. As just one example, conjugation events of plasmids carrying antibiotic resistance genes between

commensal *E. coli* and pathogenic *Salmonella* can occur[138]. Therefore, not only can gut microbiota acquire ARGs from pathogens, but they also harbour their own ARGs that can be transferred to other bacteria[123].

Indeed, many studies have identified the presence of ARGs in commensal gut bacteria[137,139-144] including genes with over 90% similarity to known ARG sequences from pathogenic bacteria in human stool and saliva samples[139]. Even in healthy infants under one month of age who had not been treated with antibiotics, ARGs conferring resistance to up to 14 antibiotics have been identified[141]. Tetracycline genes, such as *tetO*, *tetQ* and *tetW*, are particularly prevalent in gut microbiome samples collected from across the world[139,142]; this is thought to be due to the historical high usage of tetracycline in agriculture (now banned in the EU and USA)[137] and therefore associated with the farm-to-fork hypothesis. Macrolide and sulphonamide resistance genes (e.g. *ermB and sul2* respectively) are also reportedly common in gut microbiomes[137,141,142]; macrolides have also been used as agricultural growth promoters[137], whereas sulphonamides are very old types of antibiotics and so selective pressure for sulphonamide resistance in the gut has existed for a comparatively long period of time[137]. Clearly then, antibiotic resistance can occur in the gut even in the absence of antibiotic treatment, although antibiotic usage is associated with increase abundance or diversity of resistance genes and mutations.

Moreover, gut bacteria have been found to contain novel ARGs that have not previously been detected in pathogenic bacteria[139,144]. For example, ten novel beta-lactamase genes with between 35 and 61 % similarity to known beta-lactamases at the time were identified from human stool and saliva[139,144]. More recently, novel beta-lactamases have been detected in sewage sludge containing human faecal matter[145], including one found on a mobile element that is considered likely to be highly mobilisable between bacteria[145]. Thus, despite the

increasing evidence that the human gut microbiome harbours diverse ARGs, it is unlikely we have discovered all ARGs or mutations that cause antibiotic resistance. In addition, with studies typically focussing on Western or industrialised communities, antibiotic resistance in other communities is less well understood. Finally, since gut microbiome studies have often depended on culture-independent techniques, rare or lowly abundant members of the gut microbiota are less well studied than more abundant bacteria. As a result, the incidence, distribution and dissemination of ARGs within the gut microbiota is still not fully understood.

1.8 Studying antibiotic resistance in bacteria

Studying antibiotic resistance involves determining the sensitivity of particular bacterial taxa (e.g. isolate, strain, species, genus, family, order, class and phylum) to an antibiotic. This can involve measuring the physical response through culture and phenotyping an isolate-antibiotic combination, or studying the presence and prevalence of antibiotic resistance-conferring mutations or genes (genetic determinants of antibiotic resistance) in bacterial genomes or metagenomes. How these methods have been used to study antibiotic resistance, particularly in gut microbiota, will now be discussed in turn.

1.8.1 Phenotyping

Microbiology has its foundations in culturing bacteria and studying their physical characteristics (i.e., phenotypes). Pathogenic bacteria have come under much scrutiny because of their propensity to cause disease. As described earlier, the majority of clinical pathogens tend to be Gram-negative, facultatively anaerobic bacteria that are amenable to

culture, allowing their antibiotic resistance phenotypes to be measured and recorded on a large scale. This has resulted in huge quantities of data being collected regarding the variation in antibiotic resistance and susceptibility to various antibiotics in common pathogenic bacteria. This data can be used to can define cut off points of susceptibility or resistance, such as those by the European Committee on Antibiotic Susceptibility Testing (EUCAST), the Clinical Laboratory Standards Institute (CLSI) or the British Society of Antimicrobial Chemotherapy (BSAC). These consider bacteria as resistant if they require over a certain concentration of antibiotic to be killed, and bacteria that are killed with lower concentrations are considered susceptible. For example, the EUCAST guidelines (version 7.1, valid from March 2017) list the amoxicillin breakpoint MIC for Enterobacteriaceae as 8 mg/L: Enterobacteriaceae isolates with an amoxicillin MIC equal to or less than 8 mg/L are considered susceptible and those with MICs over 8 mg/L are considered resistant. This type of data also allows for antibiotic resistance over time and space and between related isolates to be monitored. This is critical for prescribing practices – if there is an increasing trend in resistance to an antibiotic by a bacterial pathogen in a particular area, then the local doctors can include this in their consideration when choosing which antibiotics to prescribe.

The situation is very different for bacteria that are typically considered non-pathogenic, such as gut bacteria, which until recently have been comparatively under-studied. Due to their adaption to conditions in the gut, including the requirement for anaerobic conditions[146], they have also been hard to culture and study physically. The spectrum of antibiotics is therefore often only based upon the testing of Gram-negative, facultatively anaerobic or aerobic pathogens plus a select few Gram-positive or anaerobic species[147], such as *Enterococcus faecalis* or *Bacteroides fragilis* (both opportunistic pathogens)[147]. Therefore, antibiotic breakpoints of resistance/susceptibility in commensal gut bacteria are not defined. Moreover,

it means we do not fully understand the impact of different antibiotics across the diversity of our gut microbiota. However, since gut bacteria can sometimes act as opportunistic pathogens (like *B. fragilis,* a common cause of post-operative infections[148]) and in combination with their role as an ARG reservoir, it is important to understand their variation in antibiotic sensitivity. In particular, it would be useful to know which antibiotics might be useful for treating infections caused by opportunistic pathogenesis by our commensal gut bacteria, which antibiotics we should avoid if we wish to limit harm to out gut microbiota, and what types of antibiotic resistance these organisms might contribute to spreading. Fortunately, with recent developments in culturing of gut bacteria[146,149-151], we can now culture over 90 % of species of gut bacteria found in an individual[146]. This offers an exciting opportunity to investigate phenotypic antibiotic resistance in gut bacteria at an unprecedented scale.

1.8.2 Genome sequencing

In addition to phenotypic antibiotic resistance, antibiotic resistance genotypes can also be studied. Genomic-based predictions of resistance is typically relied upon for the surveillance of antibiotic resistance in non-pathogenic bacteria, including intestinal microbiota[152]. Databases and tools designed for this purpose (summarised in Table 1.3) have been used to study antibiotic resistance genotypes of whole genomes, either from raw sequence reads or assembled contigs. These types of methods have become popular for monitoring or tracking antibiotic resistance genes in bacterial isolates over time and/or space. This is largely used for clinical isolates to see trends in increasing or more widespread antibiotic resistance; such as the Global Pneumococcus Project studying 20,000 isolates of *Streptococcus pneumoniae* that found resistance genotypes for five antibiotics of different classes were strongly associated

with the country of isolation[153]. Relatively few studies of this type have been performed in gut microbiota, due to a proportionate lack of multiple cultured isolates and thus whole genomes for a particular species: for example, a study of the genus *Bifidobacterium*, a dominant component of the early-life microbiome, assessed ten classes of antibiotic resistance genotypes and phenotypes of 91 isolates from 54 subspecies[154].

In addition, these described tools have been used in combination with whole genome shotgun metagenomics (sequencing all the DNA in a sample) to study the resistome[155] (the collection of antibiotic resistance genes, mutations and their precursors) in diverse microbiome environments – such as soils, wastewater or sludge, smog and sediments[156]. These whole genome shotgun metagenomic studies have demonstrated that antibiotic resistance genes are widespread throughout natural and human-associated environments[156]. However, the databases are often based upon knowledge from clinically relevant pathogens – which as demonstrated earlier in Figure 1.2 are predominantly Gram-negative, facultatively anaerobic Proteobacteria. Since the majority of commensal gut bacteria are strictly anaerobic Gram-positives[126,157], they are distantly related to clinically relevant pathogens and may harbour currently unknown antibiotic resistances[139]. These have the potential to become clinically relevant either through direct opportunistic pathogenesis of commensal species[158] or through the transfer of ARGs to pathogens[152].

Table 1.3. Summary of databases and tools for predicting antibiotic resistance genotypes from sequence data. A brief summary of each database and/or tool is provided alongside an overview of the advantages and disadvantages.

Resource	Type	Advantages	Disadvantages
Antibiotic Resistance Database (ARDB)[159]	• Database of resistance gene nucleotide sequences	• Large number of sequences • Extensive metadata	• Last updated 2009 • Redundancy
Antibiotic Resistance Identification by Assembly (ARIBA)[160]	• Tool	• Tool uses sequence reads • Detailed, customisable, easily interpretable output • Removes redundancy from databases used • Can identify resistance mutations including new variants	• Relies on input databases • Not really suited for metagenomic data
ARG-ANNOT[161]	• Database	• Extensive list of resistance gene sequences combined from multiple sources • Can be used to identify mutations	• Redundancy • Last updated 2017 • Data no longer appears to be available on website
Bacterial Antimicrobial Resistance Reference Gene Database	• Database of antibiotic resistance genes	• Combines data from several sources • Regularly updated	• Redundancy • Does not include resistance mutations
Bush-Jacoby Database[162]	• Database of beta-lactamases	• Highly curated database of sequences and metadata associated with beta-lactamases	• Website no longer appears to work
Comprehensive Antibiotic Resistance Database (CARD)[163,164]	• Database of resistance genes • Tools to identify resistance genes/mutations	• Regularly updated • Includes both genes (including all ARDB sequences) and mutations • Online tool to apply methods and command-line tool available • Extensive metadata • Can account for gene or mutation conferring resistance to multiple antibiotics • Tool has built in option to look for candidate novel resistance genes	• Redundancy • Ontology complex • Tool requires assembled genomes

Resource	Type	Advantages	Disadvantages
DeepARG[165]	• Database of resistance genes • Tool using machine learning to characterise and annotate resistance genes	• Online and command-line tool • Can be applied to sequence reads (e.g. for metagenomic samples) and assembled genomes • Highly curated database • Does not just rely on best-hit	• Database last updated 2017 • Does not account for mutations
Lactamase Engineering Database[166]	• Database of beta-lactamases	• Highly curated database of sequences and metadata associated with beta-lactamases	• Only two families of beta-lactamases included • Unsure when last updated, website does not appear up to date or completely functional
MegaRes[167]	• Database of resistance gene nucleotide sequences • Tool to identify resistance genes	• Simple ontology and metadata • Good for population level analysis (e.g. count-based analyses in metagenomic samples) • Easy to interpret results • Tool uses sequence reads • Non-redundant	• Does not include resistance mutations • Cannot account for gene or mutation conferring resistance to multiple antibiotics • Last updated 2016
PointFinder[168]	• Database of chromosomal resistance mutations • Tool to identify mutations	• Good for chromosomal point mutations • Online or command-line tool, can be run together with ResFinder • Regularly updated	• Focuses on small range of species and genes • Difficult to analyse many samples at once
ResFams[169]	• Database of profile HMMs of resistance genes	• Combines resistance genes from multiple sources • Includes HMMs based on resistance genes identified in functional metagenomic screens • Can be used to identify candidate novel resistance genes	• Last updated 2015 • Mainly intended for use for functional metagenomic screens, rather than surveillance of resistance genes

Resource	Type	Advantages	Disadvantages
ResFinder[170]	• Curated database of resistance genes • Tool to identify resistance genes	• Regularly updated • Online tool, can be run together with Pointfinder • Add-on function for functional metagenomic screens	• Difficult to analyse many samples at once
Resqu https://1928diagnostics.com/resdb/	• Database of resistance genes	• Highly curated database • Non-redundant • DNA and protein sequences	• Does not account for mutations • Focuses only on genes that can be transferred horizontally • Database does not appear to be publicly available • Last updated 2013
SRST2_ARG-ANNOT[171]	• Curated database of resistance genes • Tool to identify resistance genes or mutations in genomes	• Uses sequence reads • Removes redundancy from ARGANNOT • Can identify mutations	• Relies on ARGANNOT database • Last updated 2017 • Can only identify pre-defined mutations or variants
Structured ARG database[172]	• Database of resistance genes • Tool to identify resistance genes	• Nucleotide sequences and profile HMMs • Online analysis pipeline and command-line tools • Good for metagenomic samples • Claims to frequently update	• Redundancy • Does not account for mutations • Last updated 2018 • Not designed for whole genomes

Functional metagenomic studies, such as Sommer *et al*. 2009[139], have shown that ARGs unlike those seen in pathogens exist in gut bacteria. This technology clones DNA fragments that have been extracted directly from an environmental sample, such as a stool sample, into another bacterium, often *E. coli,* and screened for antibiotic resistance phenotypes by plating on agar containing antibiotics. This bypasses the issue of culturing gut microbiota, making functional metagenomics a very powerful tool for studying a community in a relatively unbiased way. Indeed, this method has been important in understanding antibiotic resistance genes in a range of microbiomes and environments, including but not limited to: chicken guts[173]; uncontacted Amerindians[174]; faeces from domesticated animals as well as soil, water and sanitation facilities of rural villages and "shanty" towns in Peru[143]; seawater[175]; and Alaskan soil[176]. From functional metagenomic studies, we have learned that antibiotic resistance genes not currently found in pathogenic bacteria are both diverse, abundant and widely distributed. In addition, bacteria do not have to have been exposed to clinical antibiotics to harbour antibiotic resistance genes, even ones that have been found in pathogenic bacteria. However, these studies tend to rely on cloning of genes into *E. coli*[143] and may miss genetic determinants that cannot be expressed in this organism; plus, there is extensive bacterial diversity across the planet that has not been studied in depth[177]. This leaves the possibility that there are still more unknown antibiotic resistance determinants waiting to be discovered – not just in the gut, but in the Earth's total microbiome.

Genomic methods can be combined for more in-depth analyses of antibiotic resistomes. An important study of reservoirs of antibiotic resistance used 16S rRNA gene sequencing (amplification and sequencing of variable regions in the 16S rRNA gene, used as markers for bacterial species or genera), functional metagenomics and whole genome shotgun metagenomic sequencing to explore the similarity of resistomes between different

environments[143]. This included the human gut, domesticated animal gut microbiomes, soil, water, and sanitation facilities[143]. This multi-genomics approach allows antibiotic resistance genes to be quantified in terms of their relative abundance and associated with particular taxa, rather than just observing their presence. The resistomes of the different samples correlated with the phylogenetic diversity of each sample across an ecological gradient but certain antibiotic resistance genes were able to move between more diverse habitats and were linked to mobile genetic elements[143]. For example, the sulphonamide resistance gene *sul2* was found in 50 % of samples from six of seven environments studied and appeared to be localised in integrons – indicating it has the potential to transfer between bacteria[143]. Combining next-generation sequencing methods is therefore a powerful tool for understanding resistomes.

In addition to using genomics to discover or monitor ARGs, it has been used to assess the impact of antibiotics on communities of bacteria. For example, 16S rRNA gene sequencing of gut bacteria following antibiotic treatment in humans has shown that diverse gut taxa are impacted by antibiotic therapy; however, the extent varies between individuals[178]. Moreover, 16S rRNA gene-based studies have revealed the long-lasting impact of antibiotics on the gut microbiota, where often the gut microbiome does not fully return to its pre-antibiotic treatment state[179]. However, 16S rRNA gene sequencing only allows for species- or genus-level resolution, meaning detailed analysis of species or strains is not possible. Whole genome shotgun metagenomics adds more resolution, and has been used to identify that the initial state of the gut microbiome determines the impact antibiotics will have[180]. However, both 16S rRNA gene sequencing and whole genome shotgun metagenomics depend on reference 16S rRNA gene or genome sequences[181]. Therefore, the effect of antibiotics on bacteria for which reference genomes are not available cannot be readily detected. As previously discussed,

there are uncharacterised organisms without reference sequences, meaning there is still much to be learned about the development of antibiotic resistance following antibiotic therapy in humans.

1.9 Thesis aims

Clearly antibiotic resistance is a global issue and whilst the gut has been described as an antibiotic resistance reservoir, the full capacity for antibiotic resistance and which antibiotics are effective against gut bacteria are not well defined. This thesis therefore sets out to characterise the antibiotic resistance potential of human gut bacteria. To do this, I will exploit recent developments in culturing of gut bacteria and characterise the genotypic and phenotypic resistance profiles of intestinal microbiota. Moreover, I will seek direct experimental evidence of the selection of antibiotic resistance within communities of commensal human microbiota to help understand the dynamics of antibiotic resistance in the gut microbiota. The thesis can be broken down into three key parts:

- **Characterisation of genomic antibiotic resistance in commensal gut bacteria:** Determine a comprehensive overview of antibiotic resistance genes and mutations in commensal human gut bacteria representing the phylogenetic diversity of the human gut microbiome.
- **Determination of phenotypic antibiotic resistance in commensal gut bacteria and the accuracy of genotypes:** Measure antibiotic sensitivity phenotypes in commensal human gut bacteria representing the phylogenetic diversity of the human gut microbiome and compare this to antibiotic genotypes.
- **Modelling the development of antibiotic resistance *in vivo*:** Assess the impact of amoxicillin therapy on amoxicillin resistance in mice with human-derived gut microbiota at both community- and strain-level.