Chapter 6: Discussion

6.1. Key messages and future work

In this thesis, I explored the capacity of the human gut microbiome as a reservoir of antibiotic resistance. I determined that the majority of bacterial members of a normal, healthy gut microbiota possess genetic antibiotic resistance determinants using genomic studies of a unique human gut bacteria culture collection. I also generated antibiotic resistance phenotypes to link to these genotypes and identified the presence of resistances to antibiotics essential in medicine that were not predicted using a range of genome-based databases and methods. To my knowledge, this is the first time that such a large phylogenetically diverse collection of human commensal gut bacteria has been tested for resistance to multiple clinically relevant classes of antibiotics using phenotypic and/or genotypic approaches. This identified multiple instances of antibiotic resistance that could not be predicted using genomics, signifying that the full extent of antibiotic resistance in the microbiome is currently unknown. In addition, I combined deep culturing with reference genome-based metagenomics to study the impact of amoxicillin on the gut bacteria in mice with humanised microbiota, identifying the acquisition of a clinically relevant beta-lactamase by a potential opportunistic gut pathogen via HGT. This unique combination of techniques therefore enabled strain-level discrimination of antibiotic-induced genetic changes. Overall, this thesis highlights the power of a combined genomic and culture-based approach to investigate the gut resistome.

The overall widespread distribution of resistance determinants in the HBC (Chapter 3) is concerning in its implications for the potential horizontal transfer of antibiotic resistance genes among resident and transient members of the gut microbiome. With approximately 52

% of commensal gut bacteria possessing ARGs there are more opportunities for these HGT events to occur than if only a small minority of gut bacteria harboured ARGs. Thus, the majority of the human microbiome is in theory capable of contributing to the spread of antibiotic resistance. Indeed, this was in part demonstrated by the acquisition of the *cfxA* beta-lactamase gene in isolates of *Odoribacter splanchnicus* following exposure to amoxicillin (Chapter 5); this transfer event conferred high-level beta-lactam resistance in the recipient *O. splanchnicus* strain. This shows how antibiotics can lead to the movement of clinically relevant ARGs and cause increased antibiotic resistance in gut bacteria. If this occurs in pathogens or opportunistic pathogens, then this could result in antibiotic-resistant infections. Though *cfxA* has not currently been detected in bacteria belonging to other phyla, possibly due to phyla HGT barriers, *O. splanchnicus* has been described as an opportunistic pathogen and thus this event has potentially direct clinical impacts. Moreover, if *cfxA* were to become located within a more promiscuous mobile element or plasmid, it could hypothetically spread to more distantly related taxa such as Enterobacteriaceae and cause highly beta-lactam resistant infections.

However, antibiotic resistance in commensal gut bacteria can also be a benefit to the gut microbiome: for example, beta-lactamase-producing resident bacteria may be protected against the effects of beta-lactam therapy. The action of the beta-lactamase to degrade the antibiotic may also protect other resident commensal bacteria. Moreover, antibiotic resistant commensals can protect against colonisation by antibiotic-resistant pathogens[294]. As antibiotic resistance in commensals can be considered both an issue and a benefit, it is important in the future to define which antibiotic resistance genes are most likely to spread to opportunistic or obligate pathogens, plus which commensal gut bacteria are most likely to cause opportunistic infections.

That isolates representing novel taxa were also found to contain known antibiotic resistance genes highlights that we cannot fully understand which bacteria carry antibiotic resistance genes until we have identified all bacterial species that inhabit the gastrointestinal tract. One approach for this would be continuing to predict the presence of antibiotic resistance genes and mutations in genomes of newly cultured human gut bacteria as they become publicly available. For example, PATRIC[194] provides a genome annotation services that includes CARD[163,164] for annotating antibiotic resistance genes; NCBI has its own AMRFinderPlus[295] tool for identifying antimicrobial resistance genes in whole genome sequences. These tools could be programmed to run automatically when new bacterial genomes are uploaded, allowing antibiotic resistance genotype to be readily available and curated. An alternative approach for determining which bacteria carry antibiotic resistance genes or mutations would be to search for known genetic antibiotic resistance determinants in metagenome-assembled genomes (MAGs); this may help to increase the phylogenetic diversity being studied. However, MAGs are limited due to the absence of isolates for phenotypic analysis plus can result from mixed-assemblies of closely related strains[296], potentially introducing spurious results. Unless this can be resolved, culturing and studying whole genome sequences remains the most accurate way of surveying the distribution of genetic determinants of antibiotic resistance.

Continuing to culture gut bacteria from stool samples remains imperative for identifying novel isolates and studying the presence of antibiotic resistance genes and mutations. In addition, this will help improve taxonomic classification of metagenomic sequencing. In Chapter 5, despite isolating approximately 11,000 individual isolates and generating study-specific reference genomes there were still metagenomic sequences identified in metascrapes derived from faecal samples that could not be assigned taxa. Isolating additional colonies could be beneficial, though I predicted that around three times as many colonies picked in this

study would need to be isolated to recover all predicted OTUs. Presently, this would require huge manual effort, as culturing anaerobic bacteria is difficult to automate due to the space restrictions inside an anaerobic cabinet. The culturomics approach[297] could be implemented, which uses multiple types of media to select for different bacteria and mass-spectrometry to rapidly identify those bacteria. Targeted culturing such as ethanol selection for spore-formers may help reduce the number of additional colonies that need to be picked; developing more advanced selective culturing may assist further. As technology improves, it will become easier to isolate all the possible bacteria residing in the gut. There is a particular need for high-throughput, broad-scale culturing of gut bacteria from diverse peoples across the globe to recover maximum diversity.

In addition to isolating and studying novel genomes, novel antibiotic resistance genes are also important to discover for improving the annotation of antibiotic resistance in bacterial genomes and metagenomes belonging to diverse microbiomes. As shown in this thesis, rule-based methods are not accurate for predicting phenotypic resistance in gut bacteria, which as discussed could have direct clinical implications. Though other studies have shown higher rates of accuracy for rule-based genomic prediction of antibiotic resistance in pathogens[240,242,248], a recent bioRxiv preprint showed that accuracy could vary between laboratories studying the same WGS datasets of carbapenem-resistant clinical isolates[240,298]. Identifying known point mutations is especially difficult as they could be masked by sequencing errors or consensus sequences[240]. In addition, it can be hard to identify mutations causing antibiotic resistance phenotypes in the first place[299], and so databases of point mutations should be considered particularly incomplete[299].

In Chapter 4, it was shown difficult to identify or validate candidate novel antibiotic resistance genes from individual gut bacteria using functional metagenomics or cloning of a specific

candidate gene. In the future, qPCR or RNASeq of candidate resistance genes or mutations may provide insights into their expression that could explain differing antibiotic resistance phenotypes been closely related bacteria. In addition, integration sites of cloning vectors (such as of the GeneArt synthesised Group2384-carrying plasmid) could be investigated to see if this affects the expression of the insert candidate gene. Alternative recipient strains instead of *E. coli* could be used; however, classic genetics techniques are more limited for gut bacteria than for pathogens and so presently few alternatives exist. Moreover, successful shotgun cloning from pure cultures of isolated bacteria is crucial for better annotating gut bacteria and should continue to be optimised. Developing genetic techniques for gut bacteria will take time but is essential if we are to better understand our gut microbiota. In particular, improving annotation of antibiotic resistance would be useful to study antibiotic resistance on a global scale: it is possible that one day commercial microbiome sequencing services could be combined with surveillance of antibiotic resistance genes to help achieve this.

Discovering novel antibiotic resistance genes and mutations will help improve the accuracy of rule-based antibiotic resistance prediction methods. It is important for these methods to be accurate: if WGS-AST becomes a routine procedure in healthcare, we need to ensure patients are receiving antibiotics that are effective but not causing unnecessary harm to their gut microbiota. False predictions of susceptibility or resistance may result in patients being treated with antibiotics that do not work or with antibiotics that cause significant impacts to their gut microbiota when a narrower-spectrum antibiotic would have been sufficient. Fortunately, rule-based methods have been shown to be more accurate for specific species of pathogenic bacteria[299]. If this type of study is expanded with additional isolates per species or isolation source, comparable to that of studies in pathogens, we may be able to increase the accuracy of genome-based antibiotic resistance predictions in gut bacteria. Studying more

isolates would allow the approach developed in this thesis to define gut bacteria resistant or susceptible to be limited to more specific taxa (such as species rather than across all four phyla studied here), which may further improve WST-AST accuracy. Measuring MIC instead of zone of inhibition would also provide more direct information and enable antibiotic concentration breakpoints to be determined for gut bacteria. Moreover, expanded databases with genetic antibiotic resistance determinants from a wide range of bacterial sources may result in less bias towards pathogenic Proteobacteria. In the future, WGS-AST could become a gold-standard method for inferring phenotypic antibiotic resistance in commensal gut bacteria, as well as clinical isolates of bacterial pathogens.

Expanding the number of bacteria (e.g., the entire HBC) and range of antibiotics (e.g. all of those on the WHO essential list) studied in this thesis would provide further insights into the impact of antibiotic spectrum on the gut microbiome. For example, in Chapter 4 gentamicin was effective against four Firmicutes bacteria considered strict anaerobes. Aminoglycoside uptake is thought to require quinones, lipid-soluble membrane electron carriers required for aerobic respiration[300] – which are typically only present in Alpha-, Beta- and Gammaproteobacteria[300]. These findings highlight how current knowledge of antibiotic spectrum is based on a limited set of bacterial species and that some antibiotics – such as gentamicin – may have under-realised impacts on the gut microbiota. This is important to understand more fully since the use of antibiotics is known to have long term impacts on the gut microbiome with potentially serious implications for our health. This knowledge could eventually be used to establish a framework to guide antibiotic selection in a clinical setting and ultimately guide microbiota-dependent personalised medicine. Furthermore, with research into gut microbiota-based therapeutics advancing rapidly, perhaps in the future a scenario will exist where when taking an antibiotic the patient is also prescribed a specific

probiotic containing bacteria likely to be harmed by that antibiotic or that aid gut microbiome recovery. Mouse models with humanised microbiota are essential for early testing of these proposed probiotics or any gut microbiota-based therapy.

Though the mouse model system described requires optimisation, it resulted in remarkable discrimination that identified the acquisition of a beta-lactamase by a commensal strain of gut bacteria. Eight other OTUs were observed to have gained several SNPs following antibiotic therapy, but these require further investigation and validation to confirm their hypothetical role in increased amoxicillin resistance. Moreover, the model could be easily altered to study other antibiotics, such as those of last resort, or gut microbiomes with different features from alternative human donors. Off-target effects of particular antibiotic therapy (e.g. increased resistance to other antibiotics that were not used as the therapy) could also be studied. These experiments will help to understand the species- and strain-level ecological dynamics of antibiotic resistance that occur in the gut following antibiotic treatment. This information could aid personalised microbiome-based medicine.

6.2. Concluding remarks

High-throughput ‘omics’ studies have been extremely useful for gaining understanding into the gut microbiota and resistome, and microbiomes more generally. In this thesis, whole genome sequencing was used to map the distribution of genetic antibiotic resistance determinants in individual isolates of phylogenetically diverse commensal gut bacteria and identify many unpredicted resistances to antibiotics considered essential in medicine. Moreover, high-throughput culturing and whole genome sequencing identified a 1 in 11,000 horizontal gene transfer event of a clinically relevant beta-lactamase. However, a return to

classical microbiology to complement these techniques would enable better resolution of high-throughput and large-scale sequencing studies, as well as increased understanding of the biology of the individual bacteria themselves. This is critical if we are to fully understand the role of each member of the gut microbiota and how they contribute to the overall microbiome function and therefore our health. Only when this is achieved will understanding the spread of antibiotic resistance, the complete gut resistome, detailed microbiome function and personalised microbiome-based medicine become possible.