The effects of antibiotic exposure on selection of antimicrobial resistance in the human gut microbiome

Leon William Peto

St Anne's College, Oxford

Nuffield Department of Medicine

A thesis submitted for the degree of Doctor of Philosophy

Michaelmas Term 2019

The effects of antibiotic exposure on selection of antimicrobial resistance in the human gut microbiome

Leon William Peto, St Anne's College, Oxford

The gut is a major reservoir of human pathogens, and increasing antimicrobial resistance (AMR) in these organisms is an important problem. Selection of AMR by antimicrobials occurs against a background of disruption in a rich bacterial ecosystem, the gut microbiome. Our understanding of this has recently been transformed by new techniques, such as metagenomic sequencing, that can describe the whole community and directly detect AMR genes. Efforts to control AMR would be greatly helped by reliable measures of the impacts of different antimicrobials on the gut microbiome, but these are generally not available. The aim of this thesis is to address this problem.

A significant limitation of using metagenomic sequencing to study AMR is its inability to detect important resistance genes present at low abundance, and in initial work I explore methods to improve the detection of these using plasmid DNA extraction and selective culture-enrichment. These approaches improve the limit of detection of specific resistance mechanisms, but prove unsuitable for use in a quantitative assay.

I then analyse data from an observational study that I conducted, which recorded antimicrobial exposures and collected stool samples from hospital patients and healthy volunteers. Using multiple regression models with data from metagenomic sequencing, I estimate the effects of specific antimicrobial exposures on global microbiome diversity, the abundance of specific taxa, and the abundance of specific classes of AMR genes. The study utilised both cross-sectional and longitudinal sampling frames, which I analyse separately, providing independent estimates that are supportive of one another. These data allow the direct, quantitative, comparison of many different antimicrobials used in the study. The work also develops approaches to the analysis of these types of observational data that could be used in future studies.

Acknowledgements

I am grateful most of all to my supervisors, Sarah Walker and Martin Llewelyn, for their insight, guidance, and patience. Also to Tim Peto and Derrick Crook for strategic advice. Particular thanks go to Nicola Fawcett, who originally set up the ARMORD study that was taken over by me on when I started the DPhil. Thanks also to Claire Scarborough, Lorrayne Jefferies and Musa Kamfose, who at various times helped with recruitment to the study. I owe thanks to many people in the Modernising Medical Microbiology group, but especially to Ali Vaughan, Louise Pankhurst and Dai Griffiths for helping to set me up in the lab, Phuong Quan and Fawad Jamshed for data extraction, and Nicole Stoesser for frequently discussing shared problems. Also to Chris Butler, who provided samples from the POETIC study that are analysed in Chapter 6. Finally, to Lisa Peto for putting up with me whilst this was written.

Funding and Ethical Approval

The research was funded by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at the University of Oxford in partnership with Public Health England (PHE) [HPRU-2012-10041] and the NIHR Oxford Biomedical Research Centre. The ARMORD study was approved by Leicester Research Ethics Committee (reference 15/EM/0270). All participants provided informed consent.

Declaration and Attributions

The work contained in this thesis is my own, but could not have been done without the assistance and previous work of several others, outlined below. All laboratory work, bioinformatic and statistical analysis was done by myself, with advice from my supervisors.

Chapter 3

My experiments built on previous work by others in the Modernising Medical Microbiology group, as described in section 3.1.1. Bacterial strains were provided by Nicole Stoesser. Plasmid copy number analysis was performed by Anna Sheppard. I conceived and wrote the manuscript published in PLOS ONE that describes these results, with some comments from co-authors.

Chapters 4, 5, and 6

The majority of data in these chapters comes from the ARMORD study. This was started by the Modernising Medical Microbiology group before I was involved, with Nicola Fawcett primarily responsible for the initial design and ethical approval. I took over the study in August 2016, and ran it until its close in March 2019, during which time the majority of patients were recruited (76%) and samples collected (91%), as detailed in sections 4.1.1 and 5.3.1. In order to improve recruitment, I altered the focus of the study, making a substantial ethical amendment in June 2017. After taking over, I performed the majority of patient recruitment myself, but had substantial assistance from Claire Scarborough (Clinical Research Fellow), and Musa Kamfose and Lorrayne Jefferies (Research Nurses), who were funded by the NIHR Clinical Research Network.

In Chapter 6 I also analyse samples from fourteen women taken during a previous study (POETIC) run by Chris Butler from the Nuffield Department of Primary Care Health Sciences.

Papers arising

Peto L, Fawcett NJ, Crook DW, Peto TEA, Llewelyn MJ, Walker AS. Selective culture enrichment and sequencing of feces to enhance detection of antimicrobial resistance genes in third-generation cephalosporin resistant Enterobacteriaceae. PLoS One. 2019 Nov 8;14(11)

A manuscript based on the work in Chapters 5 and 6 is currently being prepared.

Table of Contents

List of Figures

List of Tables

Abbreviations

A&E	Accident and Emergency
AIC	Akaike Information Criterion
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
ARMORD	Antibiotic resistance in the microbiome Oxford study
bp	Base pairs
CDI	*Clostridioides difficile* infection
CFU	Colony forming units
CPE	Carbapenemase producing *Enterobacteriaceae*
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
EPR	Electronic patient records
ESBL	Extended spectrum beta-lactamase
ESPAUR	English Surveillance Programme for Antimicrobial Utilisation and Resistance
GPC	Gram positive cocci
GvHD	Graft versus host disease
HSCT	Haematopoietic stem cell transplant
HGT	Horizontal gene transfer
ICU	Intensive care unit
KPC	*Klebsiella pneumoniae* carbapenemase
NBG	Nutrient Broth with 10% Glycerol
NDM	New Delhi Metallo-beta-lactamase
NE	Not estimable
NEWS2	National Early Warning Score 2
OUH	Oxford University Hospitals NHS Foundation Trust
PCA	Principal component analysis
PCR	Polymerase chain reaction
pDNA	plasmid DNA
POETIC	Point of care testing for urinary tract infection in primary care trial
RDE	Relative detection efficiency
RPKM	Reads per kilobase per million
UTI	Urinary tract infection
VRE	Vancomycin resistant enterococci
WCC	White cell count

1 Introduction

1.1 The antimicrobial resistance problem

The development of antimicrobials in the mid 20th century, along with effective vaccination and sanitation, has led to the near disappearance of premature death from primary infectious causes in high-income countries [1]. What had been the main cause of death throughout most of human history is now largely avoidable. With increasing development, a similar shift has been occurring throughout most of the world, although progress is uneven and far from complete [2]. As well as their transformative effect in curing primary infections, antimicrobials now also underpin many other medical advances, allowing patients to survive surgery or therapeutic immunosuppression that compromises their normal defences and leaves them vulnerable to secondary infection.

However, at the same time that this transition has been happening, our power to contain infection is being eroded by the increase in antimicrobial resistance (AMR) [3]. The development of resistance to antimicrobials is an almost inevitable consequence of the evolutionary selective pressure they place on microorganisms. Resistance has developed to antimicrobials in all major human pathogens, and there is not truly one resistance problem, but many, with different considerations applying to different organisms. All of these AMR problems share common themes, and some of them are intimately interconnected.

Drug resistance is a major problem in three of the most important human infections: HIV, tuberculosis, and malaria [4-6]. However, each is usually treated with its own specific antimicrobials that are rarely used for other infections, so AMR in each can be largely dealt

with in isolation. The aim of treating these infections is eradication or permanent suppression of the infecting organism, and fear that inadequate treatment may allow resistance to develop motivates the use of combination antimicrobial therapy.

Resistance in human commensal bacteria provides a very different problem. AMR in these organisms, which are responsible for hundreds of thousands of deaths per year [2], is a major concern, and is the focus of this thesis. In contrast to the pathogens mentioned above, resistance in each commensal cannot be treated in isolation. The human microbiome consists of thousands of bacterial species that live on, and in, us [7], but only a handful of these account for the large majority of bacterial infections, in particular *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae, Neisseria meningitidis, Escherichia coli*, *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. All of these pathogens contain virulence mechanisms allowing them to cause infection, particularly when a host's normal defences are compromised, but in each case the vast majority of carriers do not develop infection and causing disease is presumably incidental to the pathogen's evolutionary fitness.

The antibiotics used to treat these pathogens have overlapping activity against other commensal bacteria, so their use typically exerts selective pressure on many different organisms simultaneously, nearly all of which are not involved in infection. The aim of treatment is usually just to cure the invasive infection with little regard for eradicating colonisation with the infecting organism, which is often not feasible and in any case might be short lived. In this context, efforts to control resistance include minimising the total

amount of antibiotic exposure across the population and using antibiotics with the lowest impact on non-infecting bacteria, with little requirement for combination therapy [8].

Horizontal transfer of DNA between commensal bacteria exacerbates the resistance problem as resistance genes from one can be acquired by another, potentially allowing the accumulation of resistance mechanisms in more virulent or transmissible organisms. Many high consequence resistance mechanisms have entered pathogens by horizontal transfer from non-pathogens, including penicillin resistance in *S. pneumoniae* [9], methicillin resistance in *S. aureus* [10], and third generation cephalosporin resistance in *Enterobacteriaceae* [11].

This thesis focusses entirely on the flora of the large intestine. This is the most diverse commensal ecosystem and contains the vast majority of all human commensal organisms, which are often heavily exposed to antibiotics when these are used to treat infection at distant sites. It is also the principal ecological niche of some commensal pathogens of outstanding clinical importance, in particular the *Enterobacteriaceae* and, to a lesser extent, *Enterococcus* species, along with many other gut organisms that are occasionally involved in infection.

1.2 AMR in gut commensals

Of *Enterobacteriaceae* colonising the gut, *E. coli* is typically the most abundant and is practically ubiquitous in humans, although species from several other genera are often present as well, chiefly *Klebsiella*, *Enterobacter* and *Citrobacter* [12,13]. These are among the commonest causes of bacteraemia worldwide, and are principal causes of urinary tract

infections (UTIs), intrabdominal infections, neonatal sepsis, and most nosocomial infections [14]. They are also one of the most concerning species in terms of AMR, with horizontal transfer of resistance genes occurring frequently, and many strains now resistant to multiple antibiotics [15]. Resistance has developed to most commonly used antibiotics, and the recent global spread of carbapenem resistant *Enterobacteriaceae* (CPE) represents a major clinical problem, as these are often resistant to all first line antibiotics used for empirical therapy [15,16]. Resistance is now spreading even to antibiotics of last resort [17].

Enterococcus species, although an uncommon cause of infection in healthy people, are an important cause of healthcare associated infection [14], and AMR in these organisms is also widespread. In particular, *Enterococcus faecium* is usually resistant to beta-lactams, and is commonly resistant to glycopeptides. As with the *Enterobacteriaceae*, horizontal transfer of resistance genes occurs frequently, allowing the accumulation of multiple AMR mechanisms in the same strain [18].

1.3 The impact of AMR

The current global burden of disease caused by AMR in commensal bacteria is hard to estimate, partly because in much of the world the facilities to diagnose resistant infection are lacking, but also because determining how AMR contributed to a particular death or illness is usually difficult [19,20].

It is clear that in some places, such as India, AMR in *Enterobacteriaceae* is already at critical levels and is leading to many deaths through treatment failure [20]. For example, a recent study of neonatal sepsis in Delhi found that *Enterobacteriaceae* were the commonest cause

of infection, and 25% (89/350) of isolates were carbapenem resistant [21]. In another nationwide Indian study of over 18,000 bacteraemias, *Enterobacteriaceae* were the commonest pathogens and carbapenem resistance was found in 12% of *E. coli* and 57% of *K. pneumoniae* isolates [22].

AMR prevalence in Europe is heterogeneous, but in several Eastern European countries the majority of invasive *K. pneumoniae* isolates have combined resistance to third generation cephalosporins, aminoglycosides and fluoroquinolones, and in Greece 65% of invasive *K. pneumoniae* are carbapenem resistant [23]. Resistance rates in Northern Europe remain at relatively low levels, and in the UK the proportion of bloodstream infections with resistance to key antibiotics has remained stable over the past few years, although in 2017 there were still over 16,000 bloodstream isolates resistant to one or more key antibiotics [24]. However, there are some alarming trends in the UK. Even though CPE account for <1% of invasive *Enterobacteriaceae* isolates, there has been a sharp rise in recent years (**Figure** 1.1) and a prolonged outbreak of CPE in the North West of England has demonstrated the difficulty in controlling such resistance when it becomes endemic [24,25].

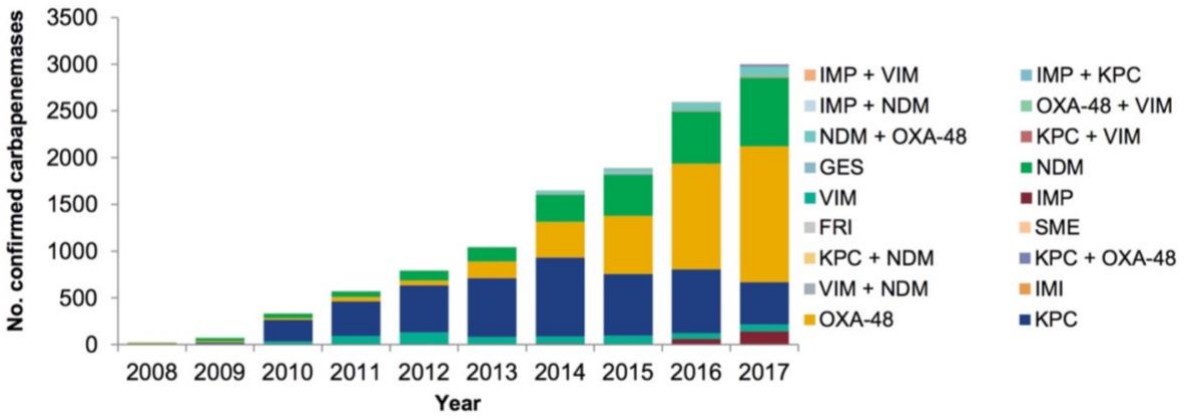

Figure 1.1 Confirmed CPE isolates referred to Public Health England

Bars coloured by type of carbapenemase enzyme. Reproduced from the 2018 English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) report [24]

In keeping with the variation in AMR in clinical isolates between countries, studies in healthy volunteers from around the world have also shown large differences in the average abundance of AMR genes in the gut flora depending on country of origin [26]. These differences correlate with country-level antibiotic usage in both humans and animals [26-28]. Average antibiotic usage per person varies several-fold between countries, even between European countries at similar levels of economic development [26], suggesting that changes in national policy could reduce antibiotic use and the prevalence of AMR.

1.4 Efforts to control AMR

The rising threat of AMR has prompted global action to improve surveillance, limit agricultural use of antibiotics, and improve the regulation of human use [29]. In the UK there has been increasing attention on AMR, reflected in the 2011 report by the Chief Medical Officer and the 2016 Review on Antimicrobial Resistance [3,30]. Recent declines in the incidence of methicillin resistant *S. aureus* and *Clostridioides difficile* infection demonstrate that epidemics of AMR organisms can be reversed [31]. Efforts to reduce antibiotic use are starting to succeed, and from 2013–2017 antibiotic use decreased by 9% in humans and by 40% in animals in the UK [32], which may partly be responsible for the recent stabilisation in resistance in clinical isolates.

An essential part of AMR control is antimicrobial stewardship, whereby unnecessary antimicrobial use is avoided and drugs are chosen to minimise the selection of resistance [8]. The human gut is a major reservoir of resistant organisms, and minimising the selection of resistance in the gut is a central concern of stewardship [33]. However, there is a significant gap in evidence to inform these efforts, as stewardship decisions tend not to be

informed by direct measurements of ecological disruption or resistance selection within the gut, but are instead largely based on the clinical spectrum of an antimicrobial or its effect on *C. difficile* associated diarrhoea [34]. Considering clinical spectrum alone could miss significant differences in the effects of antibiotics on the gut microbiome. For example, unlike other carbapenems, imipenem has negligible biliary excretion and some studies suggest minimal effect on gut flora, but the data are limited and this observation has not led to it being generally preferred for human use [35]. The risk of *C. difficile* diarrhoea associated with different antibiotics provides a useful measure of gut microbiome disruption that might be expected to be associated with AMR selection, for example suggesting that fluoroquinolones, clindamycin and certain cephalosporins produce strong selective pressure in the gut. However, this is an indirect measure, and depends among other things on the activity of the antimicrobial against *C. difficile* itself.

Many studies over several decades have measured the effect of antimicrobials on gut flora, but these have not led to widely used metrics to compare antibiotics and inform prescribing decisions. Most such studies have been small, examining a single agents, and until recently relied on the culture of only a few groups of organisms. As the spread of AMR has highlighted the need for reliable data on the effects of antimicrobials on resistance selection, new tools have become available that can complement previous techniques and improve our understanding. Metagenomic DNA sequencing, in which DNA extracted directly from a sample is sequenced, has transformed the study of microbial ecosystems by allowing culture-independent measurement of all the organisms in a community. Constituents of the gut microbiome are known to provide defence against the selection of resistant pathogens [36], but until recently could only be studied indirectly. The selection of AMR in the gut

occurs in the context of the disruption of a rich ecosystem, and metagenomic sequencing gives a much fuller picture of this, both of the microbiome and also the 'resistome' – the complete complement of resistance genes themselves. This may produce insights that can help us to counter the rise in AMR.

1.5 Thesis overview

In this thesis I use faecal metagenomic sequencing to better understand the effects of antimicrobials on the gut microbiome and selection of AMR in the gut.

Chapter 2 provides a background review of relevant literature, firstly, of the human gut microbiome, including techniques used for its study and some practical considerations in metagenomics, and secondly, measurement of AMR in the gut, in particular the metagenomic detection of resistance genes. Finally, I discuss previous studies looking at the effect of antimicrobials on the gut flora, including an overview of culture-based studies and a review of recent studies performed using culture independent methods

A significant limitation of using metagenomics to study AMR is its inability to detect resistance genes present at low abundance. In **Chapter 3** I describe experiments in which I aim to develop a method to improve the limit of detection of important resistance genes whilst retaining the benefits of direct sequencing. I trial plasmid DNA extraction and a short period of culture-enrichment prior to sequencing, both of which improve detection of specific resistance genes but are unable to retain quantification, so are not taken forward in later work.

The Antibiotic Resistance in the Microbiome Oxford study (ARMORD) is a clinical study that I ran at the Oxford University Hospitals NHS Foundation Trust (OUH), which collected stool samples from patients and healthy volunteers and provides the basis of the remaining results. In **Chapter 4** I describe the methods used in the study and for the analysis performed in the remaining results chapters. I also briefly discuss methodological considerations regarding the choice of laboratory and bioinformatic methods used in ARMORD, and present some of my own data to justify the choices made.

In **Chapter 5** I present the results of the cross-sectional analysis of data from the ARMORD study, in which previous exposures are related to the baseline gut microbiome and resistome of participants to assess the effects of different antimicrobials. I discuss important issues in relation to modelling the cross-sectional data, in particular, how to define a measure of antibiotic exposure that can be used in multiple regression models, and how to adjust for confounding. My primary aim in this analysis is to provide reliable, quantitative data to directly compare the effects of different antimicrobials on the gut microbiome, including overall measures of diversity, and the abundance of specific taxa and AMR genes.

A subset of the ARMORD participants, who were undergoing haematopoietic stem cell transplant, had serial stool samples collected during admission, and most of these patients received antimicrobials. In **Chapter 6** I discuss issues relating to analysis of longitudinal data, then analyse these data to obtain independent estimates of the effects of different antimicrobials, comparing them to estimates from the cross-sectional study. I also analyse data from a small number of samples taken before and after antibiotics that were available from a previous trial, the POETIC study. The POETIC results, which were actually available

before the main ARMORD results and informed their analysis, highlight the difficulty of analysing a dataset in which limited sample size and data on possible confounders makes modelling impossible.

Finally, in **Chapter 7** I discuss my work in the context of other studies, highlighting the limitations of the data, and considering possible future directions of the field. In particular, I outline how a future, much larger, observational study could provide the level of accuracy needed to optimally inform antimicrobial stewardship by distinguishing between the disruptive effects of broad-spectrum antibiotics.

2 Background and Literature Review

2.1 The human gut microbiome

The human microbiome consists of many different communities of microorganisms occupying different ecological niches in the body, in particular the skin, mouth, upper respiratory tract, gut and vagina [7]. The occupants are extremely diverse, including bacteria, viruses, archaea, fungi and protozoa (**Figure** 2.1) [37]. The large bowel contains the vast majority of human commensals, with approximately 10^{11} per gram of faeces, or 10^{14} in total, roughly equal to the number of cells in the host [38]. Excluding viruses, bacteria are by far the most abundant constituents of the gut microbiome, usually making up the vast majority of organisms [39].

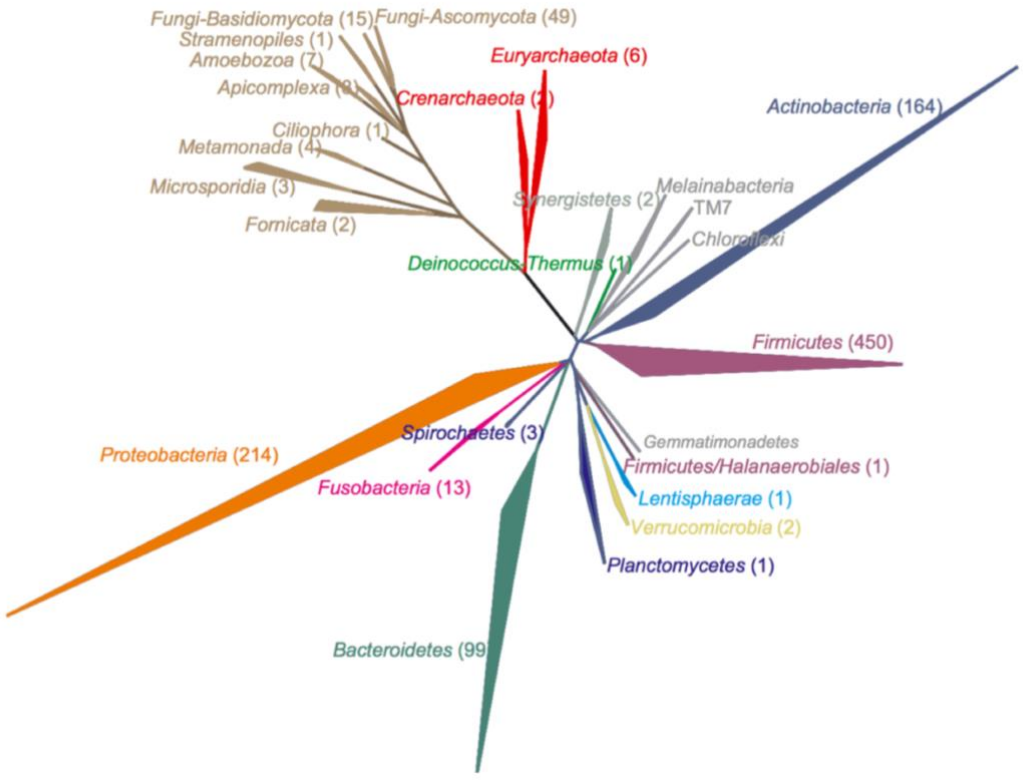

Figure 2.1 Diversity of the normal constituents of the human gut microbiome

Phylogenetic tree of species isolated from human gastrointestinal tract, including Bacteria, Archaea and Eukaryotes. Number of isolated species from each taxon are shown in parentheses (reproduced from Rajilic-Stojanovic 2014 **[39]**)

Over the past 20 years culture-independent methods have revealed the extraordinary diversity of the human microbiome [40], and this discovery is still in progress. Two recent analyses combined metagenomic data from previous microbiome studies and were able to assemble the genomes of thousands of bacterial species, most of which were previously unknown, including some only distantly to related to known species [37,41]. The gut is the most diverse human ecological niche [42], and the total repertoire of species identified from gut microbiome studies is now over 2,000 [41,43]. Another surprising feature of the gut microbiome is the marked heterogeneity between individuals, often with almost no overlap in the predominant species present. Compared to this large inter-individual variability, the composition of an individual's gut microbiome is relatively stable over time [44,45].

The predominant constituents are anaerobic bacteria, mostly from the phyla *Bacteroidetes* and *Firmicutes*, but with members of the phyla *Actinobacteria*, *Verrucomicrobia*, *Proteobacteria* and *Fusobacteria* also being common [7,46]. Several factors are known to affect the composition of the gut microbiome, including diet, age, genetics, and a range of drugs, in particular antimicrobials [47-49]. However, these factors only account for some of the inter-individual variation observed, most of which is still unexplained [49].

For hundreds of millions of years multicellular organisms have evolved alongside the microbes that live on them. This has led to commensalism, where both sides cause little harm to another, and even mutualism, where the host and its residents actively benefit each other [50]. In the case of humans, the overwhelming majority of the gut flora are remarkably non-pathogenic, and many gut bacteria benefit the host, for example by

producing short-chain fatty acids that increase the energy available from food, and by synthesising vitamin B12 [51,52]. Normal development of the immune system relies on the presence of commensal bacteria [53,54]; there is speculation that other aspects of human physiology are also dependent on a healthy microbiome [55], although it is worth noting that despite this humans can live healthily after surgical removal of the entire colon.

Differences between individuals' gut flora have been claimed to play a causal role in a range of chronic diseases, from auto-immune conditions and cancer to cardiovascular disease [56,57]. None of these have yet been definitively proven by the development of therapeutic interventions, but the potential modifiability of the gut microbiome makes such hypotheses appealing.

2.2 Methods of studying the microbiome

2.2.1 Microscopy

Study of the gut microbiome began with the development of microscopy, and van Leeuwenhoek's observation of 'animalcules' in his own stool in 1681 [58]. Although microscopy is unable to characterise faecal organisms, it has provided evidence of the large number present in faeces, and can still play an important role in their enumeration in modern studies [38,59].

2.2.2 Culture

From the late 19th century until the close of the 20th century, characterisation of the microbiome relied entirely on culture, and our knowledge of its constituents was determined by what could be selectively cultivated. Fortunately, many gut organisms are

readily isolated by culture, including *Enterobacteriaceae* and *Enterococcus* as well as many anaerobes. When studying specific organisms that can be selected using certain growth conditions or antimicrobials, culture remains an excellent tool, and is the only way of isolating bacteria for detailed characterisation, for example to determine phenotype or full genotype.

The development of culture-independent techniques of microbial analysis in the 1990s revealed that up to 80% of species were not being identified by culture, discrediting it as a tool for studying the microbiome [60,61]. However, this view has recently been overturned by several innovative studies using high-throughput workflows that combine multiple parallel culture conditions with modern identification methods [62-65]. With these techniques, dubbed 'culturomics', around 95% of gut organisms now appear to be cultivatable [66], including many not previous identified using any method. This approach complements culture-independent methods, and should aid the translation of microbiome research into applications such as probiotics or discovery of novel antimicrobials [67].

However, such methods are not practical to routinely characterise the microbiome, and it is laborious to use culture to quantify even the few major groups that can be readily isolated in a routine laboratory. For this reason, most microbiome studies use one of two culture-independent techniques; marker gene sequencing or metagenomic ('shotgun') sequencing, both of which analyse DNA extracted directly from a mixed microbial community.

2.2.3 Marker gene sequencing

In marker gene sequencing, a conserved region of DNA that allows discrimination between taxa is amplified and then sequenced. In practice, the marker is almost always the bacterial 16S ribosomal RNA gene, as it is universally present in bacteria, has a strongly conserved sequence, and is rarely horizontally transferred [68]. These sequences are compared with a reference database to provide a quantitative description of the microbes in the sample [69]. The development of this technique, initially used to study environmental samples, has transformed understanding of microbial communities by providing almost complete descriptions for the first time. Classification to the species level is possible using long read sequencing of the 16S rRNA gene, but more often short read sequencing is used and this typically allows classification to the genus level.

2.2.4 Metagenomic sequencing

Decreasing sequencing costs have recently allowed an alternative approach to marker sequencing, in which there is no target and any DNA present in the sample is sequenced indiscriminately [70]. Such metagenomic sequencing avoids the potential bias introduced by marker amplification and can detect organisms whether or not they contain the marker gene, allowing for a more complete microbiome profile including prokaryotes, eukaryotes and viruses. This can, however, potentially be a drawback when trying identify bacteria in a sample containing an overwhelming amount of eukaryotic host DNA [71]. The main disadvantage compared to marker sequencing is that it is inefficient for determining a taxonomic profile, as the large majority of DNA sequenced will be either unidentifiable or not assignable to a specific taxon [72]. For a given depth of sequencing, 16S sequencing will detect bacteria present at a much lower abundance, which may be important if very low

level carriage with specific organisms is significant, but matters less if the aim only is to describe the main taxa, say those present above an abundance of 0.1% [73].

For the more abundant taxa, metagenomic sequencing allows identification with a much higher resolution than 16S sequencing, to the species or strain level, sometimes even allowing assembly of whole genomes [74,75]. Functional analyses looking at other genes of interest are also possible with metagenomic data, for example metabolic pathways, mobile genetic elements, or antimicrobial resistance mechanisms.

2.2.5 Other methods

Several other culture-independent methods not relying on sequencing have been used for microbiome analysis, including fingerprinting of 16S amplicons and DNA hybridisation arrays, but these are rarely used now so are not discussed further [60]. Also not discussed are the many techniques for characterising the activities of a microbial community rather than its constituents, such as metatranscriptomics, metaproteomics and metabolomics.

2.3 Methodological considerations in microbiome analysis

Describing the microbiome involves both listing the organisms present in a sample and quantifying their abundances, which raises some particular methodological issues. The final taxonomic report should quantitatively represent the composition at the ecological site of interest, but this can be affected by several factors, including the method of sample collection, storage, DNA extraction, sequencing, and bioinformatic classification, as well as the potential for contamination at every stage.

2.3.1 Sample collection

Most human gut microbiome studies have relied on faecal samples, mainly because these are straightforward to collect. This represents the luminal contents of the rectum, but not necessarily the contents of the proximal colon, which have been found to differ in some studies [76,77]. The lumen also represents a different ecological niche to the mucosa, with its associated adherent organisms. Studies comparing mucosal biopsies to faeces have highlighted differences, although are these are often performed in the context of elective endoscopy or surgery after bowel preparation, so may incorporate their own biases [78,79]. Although faecal samples may not allow complete characterisation of the intestinal microbiome, they provide a useful proxy that has marked inter-individual variation, allowing exploration of associations between the microbiome and various exposures and outcomes. A problem with using faeces as a sample is that collection is unpredictable, and several studies have compared faeces to rectal swabs, which may be more reliably taken. These show high similarity to paired faecal samples, but often with challengingly low DNA output and higher abundances of skin flora and *Proteobacteria* [80-82].

2.3.2 Storage

Sample storage conditions are important, with prolonged time at room temperature affecting composition [83]. Freezing at -80˚C preserves the microbiome composition for DNA analysis and samples should ideally be frozen as soon as possible after collection, although storage at 20˚C for 24 hours [84,85], or 4˚C for 3 days [86], has been shown to have little effect on subsequent DNA analysis. Storage at room temperature for more than 2 days can significantly affect composition, leading to increased abundance of *Enterobacteriaceae* [87].

2.3.3 DNA extraction

The efficiency with which extraction methods recover DNA from different organisms varies widely, depending in particular upon their cell wall composition [88,89]. For example, extraction from Gram positive bacteria requires more aggressive, often mechanical, lysis, and extraction methods without this may significantly underrepresent DNA from Gram positives [90]. Because of the heterogeneity of organisms within a sample, and between different samples, no single extraction method can provide a completely unbiased DNA output, and comparing results that used different extraction methods requires caution.

2.3.4 Sequencing

Sequencing can also introduce bias. The efficiency of sequencing may depend on features of DNA that vary between organisms, such as GC content, secondary DNA structure or fragment length. This is particularly true if PCR amplification is involved, as with Illumina library preparation. The number of DNA reads sequenced, called the sequencing depth, determines whether scarce organisms can be detected. This is a particularly important consideration for metagenomics, as exhaustive sequencing is not possible and the trade-off between sequencing cost and detection of low abundance organisms is pragmatic [91]. Also, because there is generally far more DNA present in a sample than can be sequenced, the output usually reflects the relative, not absolute, abundance of organisms unless a standard of known concentration is added [92-94].

2.3.5 Bioinformatics

The bioinformatic method used to classify sequence data can also affect the results. Many different software packages exist, using different approaches and producing different outputs given the same data [95]. All rely on databases of microorganisms that are incomplete (unsurprisingly as new species are continually being discovered) and that are likely to over-represent culturable taxa, particularly human pathogens.

2.3.6 Contamination

DNA is ubiquitously present in the environment, including even in laboratory reagents, so non-selective sequencing of the DNA present in a sample is prone to issues of contamination [96]. Although contamination cannot be eliminated, it can be minimised with careful workflows, for which negative controls are an essential part. Contamination is most problematic in low DNA samples, and generally less so in faecal samples, which usually have high bacterial concentration.

2.4 Methods for studying AMR in the microbiome

Taxonomic classification provides a basis for microbiome analysis, but to study the functional capabilities of a microbial community, such as the determinants of AMR, additional methods are required.

2.4.1 Selective culture

Culture is a particularly valuable tool for studying AMR, as the expression of a resistant phenotype can be used to easily and cheaply select organisms even if present at low abundance. This also allows detection of unexpected or unknown resistance mechanisms,

and isolation by culture is the only practicable way to determine the full genetic context of resistance genes, using whole-genome sequencing. However, determining the AMR genes present requires further testing after culture, and reliance on phenotype may miss important resistance genes expressed at low levels [97] or in organisms that are difficult to culture [98]. It is also impractical to use culture to screen for large numbers of different organisms and resistance mechanisms in parallel.

2.4.2 PCR

For specific AMR genes, PCR allows detection and quantification at a wide range of concentrations, including in unculturable organisms, with a limit of detection similar to culture [99]. This is particularly suited to the rapid detection of a small number of high consequence AMR genes, such as the carbapenemases [100]. A major weakness of PCR, however, has been the limited number of targets that can be included in the same assay, making it impractical to detect all important AMR genes simultaneously, although recent highly multiplex PCR approaches have allowed over 200 AMR gene targets in a single workflow [101]. PCR is also unable to determine the precise sequence of an AMR gene or its genetic context.

2.4.3 Microarray

Microarray hybridisation can incorporate a much larger panel of targets than PCR, allowing thousands of different AMR gene variants to be probed in the same workflow [102]. However, because of the lack of target amplification, microarrays have a limited sensitivity for AMR genes present at low abundance. For example, in one study evaluating a microarray it was unable to detect AMR genes in a host organism whose relative abundance

was below $5x10^{-4}$ (i.e. <5 organisms per 10,000) [103]. Another serious problem with both PCR and microarrays is the need to choose a specific set of primers or hybridisation probes, for which no standard exists. This, along with the lack of a directly quantitative output in terms of relative abundance, means that both require careful validation before use, and make it difficult to compare, let alone combine, data from different studies.

2.4.4 Metagenomic sequencing

In contrast to the methods above, metagenomic sequencing does not require any prior specification of resistance mechanisms. High throughput sequencing can easily generate millions of DNA reads, which can be screened for any sequence of interest, including the thousands of currently known resistance genes. This has even allowed retrospective tracking of the emergence of the mcr-1 colistin resistance gene in samples which were sequenced before the gene was discovered [104]. Sequence data is also directly quantitative in terms of relative abundance (at least of the reads generated) and highly portable, allowing the easy aggregation and reanalysis of data from multiple studies, for example to compare differences between the gut resistome in different populations around the world [28].

Another benefit of metagenomic sequencing is that the same data can be used to define the microbiome, providing context that may help explain patterns of AMR gene carriage. However, it is not usually possible to reliably localise AMR genes to a specific organism, particularly when using short read sequencing data (typically 150-300 base pairs (bp) in length). Localising AMR genes present on plasmids is particularly challenging, as even if complete assembly of plasmid DNA reads were possible, it would be impossible to match

these to the chromosomal DNA that could identify the host, at least with standard sequencing data. One potential way around this problem involves proximity-ligation, which creates covalent bonds between adjoining DNA strands prior to cell lysis, and which has recently been used to allow the localisation of plasmids to host organisms in metagenomic data [105].

Because it does not use selective amplification, another major limitation of metagenomic sequencing is its inability to detect genes present at low abundance. For example, in a typical Illumina (short read) sequencing experiment with 5 million paired reads, one would expect only a single read to overlap an AMR gene in an organism present at a relative abundance of 10^{-4} (assuming mean genome size 4Mb and gene target size 2kb). This would correspond to an absolute abundance of around 10^7 organisms/g in typical faeces [38], but carriage of important resistance organisms is frequently below this level. In one study, 14/26 (54%) of extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* carriers on an intensive care unit (ICU) had concentrations below this, in the range 10^3 – 10^7 colony forming units (CFU)/g, and metagenomic sequencing failed to detect an ESBL gene in all nine samples with concentrations below 10^6 CFU/g [106]. In contrast, standard PCR and culture can both detect organisms present at concentrations as low as 10^2 CFU/g [99].

2.5 Resistance genes in the gut microbiome

2.5.1 Studies using culture

Studies from the 1970s using selective culture showed that multidrug resistance had already become common among *Enterobacteriaceae* in human faecal flora, including in people with no recent exposure to healthcare or antibiotics [107]. Similar increases in resistance were

also seen in culturable gut anaerobes, along with the transfer of resistance genes between diverse gut organisms, including stable plasmid-mediated transfer of resistance genes from *Bacteroides* to *E. coli* [108,109]. Recently, the availability of whole-genome sequencing has highlighted the frequency with which horizontal gene transfer (HGT) can occur, at least between closely related bacteria. In a single hospital outbreak of *Klebsiella pneumoniae* carbapenemase (KPC), over 60 horizontal transfer events were observed between different strains of *Enterobacteriaceae* over 5 years [110]. Given the apparent ease of HGT in some settings, the absence of other transfer events is also striking. For example, *Bacteroides* species and *E. coli* are nearly always present together in faecal flora, and several broad spectrum β-lactamases are commonly found in *Bacteroides* that would appear to offer a strong selective advantage if acquired by *E. coli*. Among these is ccrA, a metallo-β-lactamase that leads to carbapenem resistance in *Bacteroides fragilis* and which can be cloned into *E. coli in vitro*, producing a resistant phenotype [111,112]. Yet these β-lactamases are not found in *E. coli* in nature, possibly because of an associated fitness cost that outweighs any advantage from resistance.

2.5.2 Culture-independent methods

Over the past decade, the development of culture-independent methods for studying AMR has provided a much clearer picture of the totality of resistance gene carriage in the gut [113-115]. A surprising finding from early studies using PCR and microarray was the ubiquity of resistance genes in faecal flora, particularly those to tetracyclines and macrolides [116,117]. Metagenomic sequencing has greatly expanded the repertoire of genes identified from the human gut, with one study of 162 samples finding the number of distinct resistance genes per individual ranged from 33 to 89 [118]. Tetracycline and macrolide

resistance genes were again commonest, but also common were resistance genes to beta-lactams, aminoglycosides, amphenicols, antifolates and quinolones.

Although the prevalence of many of these resistance genes appears to have increased in response to the introduction of antimicrobials in the mid 20th century [26,27], studies of faecal DNA from pre-Colombian mummies and previously uncontacted Amerindians show that all of these resistance gene classes were present in the gut microbiome before the human discovery of antimicrobials [119,120]. While it is possible some of these genes have functions other than antimicrobial resistance, many are almost identical to modern variants that confer resistance, and may have evolved to defend against antimicrobials produced by other members of the gut microbiome.

Better understanding of the determinants of AMR gene carriage could help to control AMR. Metagenomic studies are starting to shed light on the transfer of resistance genes between continents [121], and between humans, farmed animals and the environment [122-124]. Differences in the abundance of resistance genes between countries have been associated with increased country-level antimicrobial use [26], but to clearly define the effect of antimicrobials requires data on individual level exposure. In recent years there have been an increasing number of such studies that have used culture-independent methods to shed light on the effects of antimicrobial exposure on the gut microbiome and on AMR gene carriage, which are reviewed below in section 2.6.5.

2.6 Effects of antibiotics on the gut microbiome and resistome

2.6.1 The relevance of microbiome measurements to understanding AMR selection

In addition to their activity at the site of infection, most antibiotics have some activity in the gut, causing collateral damage to gut organisms and selection pressure for AMR [125]. Selection can occur between organisms in the same ecological niche (e.g. selection of one *E. coli* strain over another) but also via the large-scale disruption of the microbiome, allowing overgrowth of a resistant organism beyond its usual ecological niche (e.g. intestinal domination by a resistant *E. coli* following eradication of the predominant anaerobic flora). In principle, selection of resistance could occur without significant microbiome disruption, but these effects generally occur together because most commonly used antibiotics affect a wide range of commensal bacteria. Increased abundance of resistant commensal pathogens following microbiome disruption is also important in its own right, as this is associated with higher risk of invasive infection [126,127] and, probably, transmission [33]. This means that, along with direct measures of AMR, measures of gut microbiome disruption are very valuable in understanding AMR selection in the gut. A major benefit of using measures of microbiome disruption as a surrogate for AMR selection is that these can be calculated from every sample, whereas direct measurement of AMR may only be informative in a minority of samples carrying specific resistance mechanisms, limiting statistical power.

2.6.2 Design of studies assessing the effect of antibiotics

Because the gut microbiome is so variable, the same antibiotic exposure can have very different impacts in different individuals [128], making it difficult to reliably estimate its average effect. Longitudinal sampling from the same individual before and after antibiotics reduces this problem but does not eliminate it, as intra-individual variation can also be

significant, particularly in the context of illness, healthcare exposure or exposure to multiple drugs. The ideal study design would combine several features that in practice must be balanced against one another:

- A choice of the antimicrobial exposure(s) of interest
- An unconfounded comparator group (receiving placebo or an alternative drug)
- Longitudinal sampling to reduce the problem of inter-individual variation
- A large study size to reliably estimate the average treatment effect
- A population representative of future patients (who may have pre-existing microbiome disruption and high prevalence of resistant organisms)
- Concomitant measurement of clinical outcomes, such as subsequent colonisation with resistant organisms, *C. difficile* infection or other nosocomial infections

Table 2.1 summarises these trade-offs for commonly used study designs. **Volunteer studies** require recruitment of healthy volunteers, who provide longitudinal samples before and after a course of antibiotics, often with a parallel untreated group to quantify natural variability. This has been the commonest approach used, and provides a very robust experimental design for comparing different exposures. However, because of the difficulty and expense of running volunteer studies, most are small (<20 participants), reducing the reliability of their results. This also means they tend not to compare many different exposures, with most studies looking at a single antibiotic, in isolation. This makes quantitative comparison between studies difficult, particularly as study methods often vary widely. Another problem is that it is not possible to use this study design in real, unwell, patients, whose gut flora may differ from healthy volunteers and who are at risk of the clinical outcomes mentioned above.

Table 2.1 Study designs for measuring the effect of antibiotics on the gut microbiome

Design	Description	Choice of exposure	Unconfounded comparator	Longitudinal sampling	Ease of recruitment	Representative population	Clinical outcomes
Volunteer study	Antibiotics given to healthy volunteers	+	+	+	–	–	–
Randomised trial	Patients requiring antibiotics randomised between drugs	+	+	+/–	–	+	+
Longitudinal observational study	Serial samples from patients requiring antibiotics	–	–	+/–	+/–	+	+
Cross-sectional observational study	Single sample from patients who have had antibiotics	–	–	–	+	+	+

Plus and minus signs represent the possibility or relative ease of each feature for the specified study design. Longitudinal sampling means the ease of collecting samples before the start of antimicrobials

Measures of the microbiome and resistome are sometimes incorporated into **randomised trials**, usually as a secondary endpoint, and this too is a very robust experimental design. Trials comparing different antibiotics for the same indication, rather than comparisons with placebo, provide the most relevant data to inform practice and have the advantage of being performed in real patients. Sampling may be cross-sectional following antibiotic exposure, or longitudinal, with the first sample ideally taken before antibiotics have had any effect on the gut flora, although in an acute setting this may only be possible in a minority of participants. However, few antibiotic trials have included microbiome measurements, maybe in part from the desire to avoid anything that could reduce recruitment.

Observational studies collect samples from patients receiving antibiotics during routine care, so there is no control over the treatment they receive. This means that antibiotic exposure may be very heterogeneous and only antibiotics in common use can be studied. Another major problem is the possibility of confounding between measures of exposure and measures of the microbiome and resistome. The main advantage is the relative ease of recruitment of participants representative of those actually requiring antibiotics. **Longitudinal** observational studies aim to collect samples from patients before, during and after antibiotic exposure, although as with trials, this is often difficult in practice. **Cross-sectional** observational studies collect a single sample per individual and relate this to previous antibiotic exposure, comparing measurements in those with differing antibiotic exposures. The disadvantage of this is the inability to eliminate inter-individual variation and a greater potential for confounding.

For over 50 years various study designs have been used to described the effects of antibiotics on the gut microflora and on the selection of resistance, and until quite recently these were dependent on culture. Although there is no profound distinction between studies using culture alone and those incorporating culture-independent methods, reviews that summarise most of the culture-based studies have been published [125,129] and I discuss their findings in the following section. In section 2.6.4 I discuss the relevance of studies estimating risk of *C. difficile* infection (CDI) following different antibiotics. In the past 10 years, several studies have used culture-independent methods to provide new insights into the effect of antibiotics on the gut microbiome and resistome. These have not been comprehensively reviewed, so in section 2.6.5 I review these. Although much has been learned from studies performed in animals and *in vitro*, this literature is extensive and difficult to synthesise, and it is also difficult to extrapolate results to humans. For this reason the following review is restricted to studies with data about antibiotic exposure in humans.

2.6.3 Results from culture-based studies

Over the past few decades culture-based studies have provided a great deal of information about the effects of antibiotics on the gut flora. They have demonstrated differing effects of different antibiotics, some of which produce profound disruption of the microbiome, including reduction in a wide range of aerobic and anaerobic bacteria. They have also demonstrated selection of important resistant organisms with antibiotic use [36,130]. **Table** 2.2 is adapted from the most recent comprehensive review of such studies, published 18 years ago in 2001 [125]. It presents the data for selected antibiotics in common use in the UK, most of which is derived from volunteer studies.

Table 2.2 Effect of selected antibiotics on the gut microflora in culture-based studies

Drug	No. studies	No. patients in total	Abundance			Emergence of resistance		
			Aerobic GPC	Enterobacteria	Anaerobic bacteria	Enterococci	Enterobacteria	Bacteroides
Amoxicillin	6	56	↑ – ↓	↑ –	↑ – ↓	–	↑ –	–
Co-amoxiclav	1	7	↑	↑	–	–	–	–
Piperacillin-tazobactam	1	20	–	↓	↓	–	↑	–
Ceftriaxone	4	41	↑	↓↓	↓ –	–	–	–
Meropenem	1	10	↑	↓	↓	–	–	–
Clindamycin	1	10	↑	↑	↓↓	↑	↑	–
Erythromycin	1	10	↑	↓↓	↓↓	–	↑	–
Vancomycin po	3	36	↑	–	↓ –	↑ –	–	–
Metronidazole po	1	10	–	–	–	–	–	–
Ciprofloxacin	4	45	↑	↓↓	–	–	↑ –	↑ –

Adapted from Sullivan, Edlund & Nord 2001 **[125]**, GPC = Gram positive cocci, ↑ = increase, – = no change, ↓ = suppression (2-4 log10 CFU/g), ↓↓ = strong suppression (>4 log10 CFU/g). Different symbols in the same box represent conflicting results from different studies. Grey shading indicates single studies.

Even for commonly used antibiotics, the small sample size is notable, for example piperacillin-tazobactam and co-amoxiclav are both represented by a single study with fewer than a dozen individuals. Because of this there is significant uncertainty in the effects observed, which are only reported qualitatively. It may also explain some unexpected results, such as the lack of apparent effect of metronidazole or co-amoxiclav on anaerobic bacteria.

Because only qualitative results are reported, the summary does not provide the quantitative comparisons that would be most useful in balancing the impact of different antibiotics when selecting treatment. In contrast, quantitative estimates of the risk of *C. difficile* infection are available, and, although indirect, these provide some of the most useful data on the comparative effect of different antibiotics on the microbiome [131-133].

2.6.4 Results from studies of C. difficile *infection*

The results from meta-analyses of the risk of CDI in hospital and community settings are summarised in **Table** 2.3. These consistently show larger odds ratios for antibiotic use in community-associated than healthcare facility-associated CDI, but are otherwise consistent with a similar ordering of antibiotics with regard to CDI risk. The greater odds ratios seen in community-associated disease are likely to be related to the much lower risk in the control group, who are less likely to have received other antibiotics and have not had healthcare exposure. Clindamycin appears to have the highest risk, with fluoroquinolones, cephalosporins and carbapenems producing intermediate risk. Macrolides seem to have lower risk and tetracyclines appear to have the lowest risk of all. However, grouping by

antibiotic class brings together some very dissimilar agents, particularly among the penicillins and cephalosporins. These may have very different effects on CDI risk [134], and to adequately inform prescribing, data on CDI or the impact on the gut microbiome and resistome should be drug-specific. Another problem in using data on CDI to infer the effect of antibiotics on the gut microbiome is that it may underestimate the disruptive effect of drugs with activity against the prevalent strains of *C. difficile* during the study.

Table 2.3 Odds ratios for CDI following exposure to different antibiotic classes

Antibiotic class	**Healthcare facility associated CDI** [131] **(95% CI)**	**Community associated CDI** [132] **(95% CI)**
Penicillins	1.2 (0.7 – 2.2)	3.3 (1.9 – 5.6)
Cephalosporins	2.0 (1.2 – 3.2)	4.6 (1.6 – 12.5)
Carbapenems	1.8 (1.3 – 2.7)	NE
Fluoroquinolones	1.7 (1.2 – 2.4)	5.7 (4.4 – 7.3)
Clindamycin	2.9 (2.0 – 4.0)	20.4 (8.5 – 49.0)
Macrolides	1.1 (0.6 – 2.2)	2.6 (1.9 – 3.4)
Aminoglycosides	1.2 (0.7 – 2.1)	NE
Tetracyclines	0.8 (0.2 – 2.7)	0.9 (0.6 – 1.5)

Meta-analyses of observational studies, adapted from [131, 132]. NE = Not estimable.

2.6.5 Results from studies using culture-independent techniques

Culture-independent methods have been used in many studies over the past ten years, and these are summarised in **Tables 2.4-2.7**. PubMed was searched for abstracts containing reference to antibiotics and the intestinal microbiome (search strategy described in **Appendix 1**). Papers that met the following criteria were reviewed, including reference searching for additional relevant papers:

1) Contains primary data about the effect of antibiotics on the gut microbiome or resistome

2) Utilises culture-independent methods of microbiome/resistome analysis (excluding microbiome methods limited to a few specific organisms)

3) Reports data from >1 human (i.e. excluding single case reports, animal studies and in vitro studies)

4) Reports data in a form that can usefully be compared with results from other studies

5) Does not only report on specific groups that are likely to have an atypical gut microbiome, such as infants, patients with diarrhoea, colectomy patients, or bowel transplant patients.

The majority of studies have been published in the past few years, with most data coming from volunteer studies or longitudinal observational studies. Only a single treatment trial was identified that reported the effects of antibiotics on the microbiome. This may partly reflect the long time from inception to publication of trials, as these techniques would have been less widely accepted when published trials were being designed. Most studies used 16S sequencing and did not use any parallel method to detect AMR. A few studies used metagenomic sequencing, and these generally also looked at both the microbiome and AMR genes.

Tables 2.4-2.7 show results of studies assessing the impact of antibiotics on the microbiome/resistome using culture independent methods

Sampling times given in days after start of exposure unless otherwise specified (d = days, w = weeks, m = months, y = years).

HSCT = Haematopoietic stem cell transplant, ARG = Antimicrobial resistance gene, abs = antibiotics

Table 2.4 Effects of antibiotics on the MICROBIOME in INTERVENTIONAL studies

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Volunteer	Jernberg 2007 [135]	7d clindamycin po	0, 7, 21, 3m, 6m, 9m, 1y, 18m, 2y	4 + 4 controls	Bacteroides culture & typing	↓ diversity of *Bacteroides*	Resistome results in Table 2.6
Volunteer	Dethlefsen 2008 [136]	5d ciprofloxacin po	-2m, -6, -2, -1, 3, 5, 1m, 6m	3 (no controls)	16S sequencing	↓ diversity at 7d Almost resolved by 1m Some taxa still missing at 6m	
Volunteer	Dethlefsen 2011 [137]	5d ciprofloxacin po (repeated after 6 months)	Daily 1w pre – 2w post abs, 1m, 2m, 3m, 4m, 5m, 6m	3 (no controls)	16S sequencing	↓ diversity within 3d Often nearly normalised 7d after treatment Some changes at 3 months	
Volunteer	Vrieze 2014 [138]	a) 7d amoxicillin po b) 7d vancomycin po	0, 10	a) 10 b) 10 (no controls)	16S array (HITChip)	a) ↔ diversity or major taxa b) ↓ diversity & *Firmicutes* ↑ *Enterobacteriaceae*	Obese participants

Table 2.4 **continued overleaf**

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Volunteer	Raymond 2015 [128,139]	7d cefprozil po	0, 7, 3m	18 + 6 controls	Metagenomic sequencing (75M reads)	Small ↓ diversity ↓ *Bifidobacterium/Coprococcus/Dorea/ Eubacterium/Roseburia/Veillonella* ↑ *Enterobacter cloacae/Clostridium bolteae/ Parabacteroides/Flavonifractor* Largely resolved at 3m	Resistome results in Table 2.6
Volunteer	Zaura 2015 [140,141]	a) 10d amoxicillin po b) 10d ciprofloxacin po c) 10d clindamycin po d) 10d minocycline po	0, 7, 1m, 2m, 4m, 1y	a) 14 b) 10 c) 9 d) 10 + 23 controls	16S sequencing	a) ↔ diversity b) large ↓ diversity, largely resolved by 2m ↓ *Alistipes/Faecalibacterium/Ruminococcus* ↑ *Bacteroides* c) large ↓ diversity, largely resolved by 2m ↓ *Coprococcus/Dorea/Roseburia/Ruminococcus* d) moderate ↓ diversity, resolved at 1m	Effect of abs on gut microbiome greater than on oral microbiome. Resistome results in Table 2.6
Volunteer	Abeles 2016 [142]	a) 3d amoxicillin po b) 3d azithromycin po	0, 3, 7, 2m, 6m	a) 12 b) 12 + 32 controls	16S sequencing	a) ↓ diversity ↓ *Bacteroides/Lachnospiraceae/Veillonella* ↑ *Fusobacterium* b) ↓ diversity ↓ *Bacteroides/Lachnospiraceae/Veillonella* ↑ *Alcaligenes*	

Table 2.4 **continued overleaf**

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Volunteer	Isaac 2016 [143]	14d vancomycin po	0, 14, 6w, 3m, 5m	9 + 12 controls	16S sequencing	Large ↓ diversity, ↓ *Bacteroides*/*Clostridiales* ↑ *Proteobacteria*/*Fusobacterium*/*Veillonella* Some taxa still missing at 140d	Outpatients with rheumatoid arthritis
Volunteer	Reijnders 2016 [144]	a) 7d amoxicillin po b) 7d vancomycin po	0, 7, 2m	a) 18 b) 19 + 19 controls	16S array (HITChip)	a) ↔ diversity or major taxa b) ↓ diversity & *Clostridiales* ↑ *Enterobacteriaceae*/*Veillonella*/*Lactobacillus* Mostly resolved by 2m but some residual effect	Obese participants
Volunteer	De Gunzburg 2017 [145]	5d moxifloxacin po	0, 3, 6, 9, 16, 37	14 + 30 controls	Metagenomic sequencing (70M reads)	↓ diversity ↓ *Alistipes*/*Enterobacteriaceae*/*Coprococcus*/ *Faecalibacterium*/*Roseburia*	
Volunteer	Doan 2017 [146]	1d azithromycin po	0, 5	40 +40 controls	16S sequencing	↓ diversity ↓ *Fusobacterium*/*Lactobacillus* ↑ *Blautia*/*Roseburia*	Rectal swabs Included some pts <1 year
Volunteer	Kabbani 2017 [147]	7d co-amoxiclav po	0, 3, 7, 10, 13, 21	24 + 25 controls	16S Sequencing	↓ diversity ↓ *Roseburia* ↑ *Enterobacteriaceae*/*Parabacteroides*	½ had *Saccharomyces* probiotic
Volunteer	Haak 2018 [148]	7d ciprofloxacin po & metronidazole po & vancomycin po	0, 9, 49, 8-31m	9 + 3 controls	16S sequencing	↓ diversity just after abs ↓ *Bacteroides*/*Faecalibacterium* ↑ Staphylococci/Streptococci/Lactobacilli after abs Nearly resolved at 8-31m but some residual effect	

Table 2.4 **continued overleaf**

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Volunteer	MacPherson 2018 [149]	7d co-amoxiclav po	0, 7, 14, 21	70 (no controls)	Metagenomic & 16S sequencing	↓ diversity ↓ *Lachnospiraceae/Coriobacteriaceae/Clostridiales* ↑ *Enterobacteriaceae/Bacteroidaceae/ Porphyromonadaceae*	½ had *Lactobacillus* probiotic. Resistome results in Table 2.6
Volunteer	Palleja 2018 [150]	4d meropenem po & gentamicin po & vancomycin po	0, 4, 8, 6w, 6m	12 (no controls)	Metagenomic sequencing (35M reads)	↓ diversity ↓ *Bifidobacterium/Coprococcus/Eubacterium/ Faecalibacterium/Methanobrevibacter/Roseburia* ↑ *Enterobacteriaceae/Enterococcus/Fusobacterium/ Clostridium bolteae/Veillonella* (~24h post abs) Nearly resolved at 6w, but *Bifidobacterium/ Coprococcus/Methanobrevibacter* still missing at 6m	Note all drugs given orally. Resistome results in Table 2.6
Volunteer	Oldenburg 2018 [151,152]	a) 5d amoxicillin po b) 5d azithromycin po c) 5d co-trimoxazole po	0, 10	a) 29 b) 31 c) 30 + 30 controls	16S sequencing	a) Non-significant ↓ diversity at 10d b) Significant ↓ diversity at 10d c) Non-significant ↓ diversity at 10d	Rectal swabs. Resistome results in Table 2.6
Volunteer	Burdet 2019 [153]	a) 3d cefotaxime iv b) 3d ceftriaxone iv	-15, 0, 4, 7, 10, 1m, 6m	a) 11 b) 11 (no controls)	16S sequencing	Both similar ↓ diversity & *Firmicutes/Actinobacteria*	
Randomised trial	Wei 2018 [154]	3d azithromycin po	14, 13-31m	30 + 29 controls	16S Sequencing	At 14d ↓ diversity & *Bifidobacterium* Normalised at 13-31 months	Azithro vs placebo for asthma age 1-3

Table 2.5 Effects of antibiotics on the MICROBIOME in OBSERVATIONAL studies

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Longitudinal	Jakobsson 2010 [155]	7d clarithromycin po & metronidazole po & omeprazole po	0, ~10, 1y, 4y	3 + 3 controls	16S sequencing & 16S RFLP	↓ diversity & *Actinobacteria* at 7d Some residual effect at 4y	Peptic ulcer vs non-ulcer dyspepsia. Resistome results in table Table 2.7
Longitudinal	Taur 2012 [126]	a) metronidazole b) fluoroquinolones	Various	94 pts total a) 30 b) 38	16S sequencing	a) ↑ *Enterococcus* b) ↓ *Proteobacteria*	HSCT patients Groups not mutually exclusive
Longitudinal	Panda 2014 [156]	a) 7d co-amoxiclav po b) 7d levofloxacin po	0, 7	a) 7 b) 8 (no controls)	16S sequencing	Both ↓ diversity & *Firmicutes* ↑ *Bacteroidetes*	Inpatients on antibiotics for any infection
Longitudinal	Stewardson 2015 [157]	a) 10d ciprofloxacin po b) 5d nitrofurantoin po	0, ~7, ~35	a) 10 b) 10 (20 controls)	16S sequencing	a) ↓ similarity to baseline, mostly normalised at 28d ↓ *Alistipes*/*Bifidobacterium*/*Faecalibacterium*/ *Ruminococcus* ↑ *Bacteroides*/*Blautia*/*Eubacterium*/*Roseburia* b) Little effect on similarity to baseline ↓ *Clostridium*, ↑ *Faecalibacterium*	Outpatient UTI (cipro: upper UTI nitro: lower UTI) vs uninfected outpatients

Table 2.5 **continued overleaf**

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Longitudinal	Vervoort 2015 [158]	3-15d nitrofurantoin po	0, ~10, ~35	5 + 4 controls	16S sequencing	↔ diversity, ↑ *Bifidobacterium*	Outpatient UTI vs non-infected outpatients
Longitudinal	Shono 2016 [159]	piperacillin-tazobactam iv	Various	106 (no controls)	16S sequencing	↓ *Clostridia*/*Bacteroidetes*/*Actinobacteria*	HSCT patients (same centre as Taur 2012)
Longitudinal	Grall 2017 [106]	3-20d imipenem iv	0, 3, 15, 30	10 (no controls)	Metagenomic sequencing (280K reads)	No consistent differences noted	Non-ICU inpatients (many multiple prior abs). Resistome results in table Table 2.7
Longitudinal	Pettigrew 2018 [160]	a) piperacillin-tazobactam iv b) vancomycin iv	Various	109 pts total a) 35 b) 38	16S sequencing	a) ↓ *Blautia*/*Faecalibacterium*/*Lactobacillus* ↑ *Enterococcus* b) ↓ *Bifidobacterium*	ICU patients, a&b not mutually exclusive
Longitudinal	Martin-Nunez 2019 [161]	10d amoxicillin po & clarithromycin po & omeprazole po	0, 2m	40 + 20 controls	16S sequencing	a) ↓ diversity ↓ *Bifidobacterium*/*Streptococcus*	H. pylori infected vs healthy controls

Table 2.5 **continued overleaf**

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Microbiome analysis method	Effect on microbiome	Comments
Longitudinal	Morjaria 2019 [162]	a) piperacillin-tazobactam iv b) meropenem iv c) ciprofloxacin iv/po d) vancomycin iv e) co-trimoxazole po	most days during ~4w admission	18 pts total a) 12 b) 3 c) 17 d) 15 e) 6	16S sequencing	a & b) Large ↓ anaerobes c, d & e) ↔ anaerobes	HSCT patients Groups not mutually exclusive (same centre as Taur 2012)
Cross-sectional	O'Sullivan 2012 [125]	Various	NA	42 + 143 controls	16S sequencing	↓ diversity with antibiotics	Outpatients >65 years with/without recent abs
Cross-sectional	Korpela 2015 [163]	a) macrolides (clarithro- & azithromycin) b) penicillins (amoxicillin, co-amoxiclav & pen V)	NA	a) 63 b) 140 + 54 controls	16S sequencing	a) ↓ diversity with use in past 2 years ↓ *Bifidobacterium* ↑ *Bacteroides*/*Enterobacteriaceae*/streptococci b) ↓ diversity with use in past year	Children 2-7 years in day care with/without recent abs. Resistome results in table Table 2.7
Cross-sectional	Wipperman 2017 [164]	HRZE (isoniazid po & rifampicin po & pyrazinamide po & ethambutol po)	NA	19 + 101 controls	16S sequencing	↓ diversity ↓ *Blautia*/*Coprococcus*/*Eubacterium*/ *Lactobacillus*/*Ruminococcus* ↑ *Erysipelatoclostridium*/*Fusobacterium*/*Prevotella*	TB patients on abs (median 3m exposure) vs latent TB not on abs

Table 2.6 Effects of antibiotics on the RESISTOME in INTERVENTIONAL studies

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Resistome analysis method	Effect on resistome	Comments
Volunteer	Jernberg 2007 [135]	7d clindamycin po	0, 7, 21, 3m, 6m, 9m, 1y, 18m, 2y	4 + 4 controls	ermB/F/G qPCR (direct from sample)	Increase in ermB/F/G in all subjects by 7d Still apparent at 2 years in 3/4	
Volunteer	Raymond 2015 [128,139]	7d cefprozil po	0, 7, 3m	18 + 6 controls	Metagenomic sequencing (75M reads)	↑ *Enterobacteriaceae* associated beta-lactamases in some individuals, including TEM, OXA & ampC genes	
Volunteer	Zaura 2015 [140,141]	a) 10d amoxicillin po b) 10d ciprofloxacin po c) 10d clindamycin po d) 10d minocycline po	0, 7, 1m, 2m, 4m, 1y	a) 14 b) 10 c) 9 d) 10 + 23 controls	Metagenomic sequencing	↑ in some ARGs (erm, tet) inconsistent between participants	Effect of abs on gut microbiome greater than on oral microbiome
Volunteer	MacPherson 2018 [149]	7d co-amoxiclav po	0, 7, 14, 21	70 (no controls)	Metagenomic seq & array	↑ ARGs, including CTX-M/OXA/TEM/CMY	½ pts *Lactobacillus* probiotic
Volunteer	Palleja 2018 [150]	4d meropenem po & gentamicin po & vancomycin po	0, 4, 8, 6w, 6m	12 (no controls)	Metagenomic sequencing (35M reads)	↔ beta-lactamase & aminoglycoside ARG ↓ Vancomycin ARGs	Note all drugs given orally
Volunteer	Oldenburg 2018 [151,152]	a) 5d azithromycin po b) 5d co-trimoxazole po c) 5d amoxicillin po	0, 10	a) 29 b) 31 c) 30 + 30 controls	Metagenomic sequencing (?depth)	a) ↑ macrolide & sulphonamide ARGs b) ↑ trimethoprim & sulphonamide ARGs c) ↑ sulphonamide ARGs	Rectal swabs
Volunteer	Burdet 2019 [153]	a) 3d cefotaxime iv b) 3d ceftriaxone iv	-15, 0, 4, 7, 10, 1m, 6m	a) 11 b) 11 (no controls)	Culture	No clear effects on resistant Gram negative bacilli	

Table 2.7 Effects of antibiotics on the RESISTOME in OBSERVATIONAL studies

Design	Study	Antibiotic exposure	Sampling times	No exposed + unexposed controls	Resistome analysis method	Effect on resistome	Comments
Longitudinal	Jakobsson 2010 [155]	7d clarithromycin po & metronidazole po & omeprazole po	0, ~10, 1y, 4y	3 + 3 controls	ermB qPCR	↑ *erm* B Still apparent at 4 years	Peptic ulcer vs non-ulcer dyspepsia
Longitudinal	Grall 2017 [106]	3-20d imipenem iv	0, 3, 15, 30	10 (no controls)	MG sequencing (280K reads)	No consistent differences noted	Non-ICU inpatients (many multiple prior abs)
Longitudinal	Meletiadis 2017 [165]	Various	Various	111 + 11 controls	CTX-M qPCR	↑ CTX-M	Inpatients with/ without recent abs
Cross-sectional	Korpela 2015 [163]	a) macrolides (clarithro- & azithromycin) b) penicillins (amoxicillin, co-amoxiclav & pen V)	NA	a) 63 b) 140 + 54 controls	ermB/F qPCR & selective culture	↑ *erm* genes & macrolide resistant anaerobes with macrolide use within 6 months	Children 2-7 years in day care with/without recent abs

2.6.5.1 Dynamics of disruption

The profound microbiome disruption caused by some antibiotic exposures is clearly demonstrated in several studies [136,137,140,143,148,150]. This disruption is described either by listing the taxa with increased/decreased abundance or by using a global metric for each sample, usually of diversity or similarity to baseline. Disruption of the microbiome nearly always consists of a reduction in diversity, with many taxa becoming scarce or absent and fewer taxa predominating, although it should be noted that measures of abundance are usually relative so this cannot necessarily be interpreted as overgrowth of the newly predominant taxa. Large effects can be seen within 3 days of antibiotic exposure [136,137], in keeping with previous culture studies [166]. When antibiotics are stopped, there is recovery of diversity over the following weeks, with numbers generally normalising within 2-3 months [136,137,145,153]. However, some taxa remain absent 6 months after exposure, with little longer term data on whether they eventually return [135,136,148,150]. This may be because they are completely eradicated from the host and re-exposure to them is rare in adult life, which could particularly affect non-spore forming anaerobic bacteria as these are known to survive poorly outside the host [64]. In contrast, aerobic *E. coli* survives well outside the host, and data from travellers shows that we are continually exposed to new strains [167].

2.6.5.2 Effects on specific taxa

The available data are insufficient to describe how most species are affected by individual antibiotics, but some major themes emerge. *Firmicutes* of the order *Clostridiales* are anaerobes that are a predominant component of the normal faecal flora, including genera such as *Coprococcus*, *Faecalibacterium*, *Eubacterium*, *Lachnospira*, *Ruminococcus* and

Subdoligranulum. The abundance of these groups is very often reduced with antibiotic use [128,140,142-145,147-150,157,159,160,164], and rarely increased [146]. In contrast, there is more variability within members of the *Bacteroidetes*, the other predominant anaerobic component of faecal flora, including genera such as *Alistipes*, *Bacteroides*, *Parabacteroides* and *Prevotella*. The relative abundance of these is often reduced after antibiotic use [140,142,143,145,148,157,159], but is also frequently found to be increased [128,140,147,149,156,157,163,164].

Actinobacteria, particularly *Bifidobacterium*, are often a significant component of bowel flora, and like the *Clostridiales* their abundance is often significantly reduced by antibiotics [128,150,153-155,157,159-161,163], and rarely increased [158].

Some groups of aerobic organisms often increase in abundance during or immediately following antibiotic exposure, particularly *Enterobacteriaceae* [128,138,144,147,149,150,163] and *Enterococcus* [126,150,160], although their abundance is sometimes reduced, for example with *Enterobacteriaceae* following fluoroquinolone use [126,145].

2.6.5.3 Effects of specific agents

Comparing results from different studies is difficult, and only a minority of studies have directly compared different antibiotic exposures. It is clear, however, that some agents have a much greater impact than others. Oral vancomycin [138,143,144], fluoroquinolones [136,137,140,145,156,157] and clindamycin [135,140] have all been found to have a greater impact than comparator antibiotics. Oral co-amoxiclav caused significant disruption in two

studies without comparators [147,149], and appeared similar to levofloxacin in one small observational study [156]. Only observational data are available for piperacillin-tazobactam, showing a large reduction in a range of anaerobes [159,160,162]. There are almost no data for meropenem. Interestingly, a single observational study looked at imipenem exposure and found minimal impact on the gut microbiome, in keeping with its very low biliary excretion [106].

Oral amoxicillin does cause gut microbiome disruption, but much less so than oral vancomycin, ciprofloxacin or clindamycin [138,140,144], and appears to have similar impact to azithromycin, co-trimoxazole and minocycline [140,142,151]. In two observational studies, nitrofurantoin appeared to have minimal effect on the gut flora [157,158].

2.6.5.4 Effects on the resistome

Few studies used culture-independent methods to study the effect of antibiotics on the resistome. Three studies using PCR showed an increase in the abundance of *erm* macrolide resistance genes following exposure to macrolides or clindamycin [135,155,163], in keeping with a previous culture-based study [168]. This effect appeared to persist for several years after exposure. Another PCR study found an increase in CTX-M beta-lactamase genes following exposure to antibiotics [165].

Several studies using metagenomic sequencing have suggested that exposure to an antibiotic class increased the abundance of corresponding resistance genes, including increases in beta-lactamases following exposure to an oral cephalosporin [128] or co-

amoxiclav [149], and increases in antifolate resistance genes in response to co-trimoxazole [152].

2.6.5.5 Problems with the existing literature

Despite the insights gained from these studies, the data are insufficient to properly inform prescribing decisions. One problem is the small size of most studies, with half having twenty or fewer individuals receiving antibiotics. This makes results less reliable and increases the risk of false positives given the large amount of data produced from each individual. Combining results from the studies is almost impossible, as results are reported using several different metrics for diversity or similarity, or report data only for specific taxa. Variation in sampling timepoints also makes direct comparisons difficult.

The most useful data to inform prescribing would compare drugs that might be used for the same indication, but this was rarely done in interventional studies. In one case the aim was explicitly to inform prescribing choice by comparing ceftriaxone and cefotaxime, although this study was small (22 patients in total) and did not demonstrate any difference between the drugs, perhaps unsurprisingly given the low numbers [153]. Another useful comparison would be of different durations of the same drug, especially given the increasing focus on using short courses [169] and early de-escalation or cessation [170], but no studies looked at this.

The data that are available are inadequate given the urgent need to conserve antibiotics by using them as cautiously as possible. There could be large differences between some antibiotics with similar spectrum that are unappreciated, and even moderate differences

would be important given the scale of antibiotic use. Large interventional studies or trials with hundreds of participants would provide the clearest answers, but are difficult. Larger observational studies should be easily practicable, and might generate useful hypotheses.

3 Investigating methods to improve the detection of important AMR genes in a gut resistome assay

3.1 Introduction

All current methods of measuring the faecal resistome have major limitations. Quantitative culture requires further genotyping to define resistance elements and can only practically be used for a small number of species present in faeces [40], and targeted molecular approaches, such as PCR, cannot simultaneously detect the dozens of AMR genes and mutations that may be present in a sample from among thousands of known types [113,171]. Because of these shortcomings, metagenomic sequencing performed directly from extracted faecal DNA is increasingly being used to characterise the gut resistome, as it produces millions of short DNA reads that can be matched to a catalogue of thousands of AMR genes, theoretically producing a representative, quantitative description of AMR genes in a sample [113,114,172].

However, a major limitation of direct sequencing is the inability to detect clinically important resistance genes present at low abundance [173]. In particular, the *Enterobacteriaceae*, which include major drug resistant human pathogens such as *Escherichia coli* and *Klebsiella* species, may be present in faeces at levels too low for AMR gene detection by direct sequencing. In one recent study, metagenomic sequencing detected an ESBL in just 12 of 26 (46%) culture positive stool samples, missing all 9 samples with an abundance of $<10^6$ CFU/g [106]. Increasing the depth of sequencing to detect scarce organisms seems appealing but is not currently feasible. For example, it would not be unusual for a resistant *E. coli* to be present at an abundance of 10^3 cells/g in faeces with a

total organism count of 10^{11} cells/g [38,106], so using short read sequencing to detect a 1kb AMR gene in this organism would require hundreds of billions of reads, costing many hundreds of thousands of dollars.

Laboratory techniques to enrich the AMR genes present in a sample could allow an effective sequencing-based resistome assay that would provide a powerful tool to study AMR selection, particularly if used alongside conventional assessment of the microbiome. Reliable quantification is an important feature of a resistome assay, as although simply classifying individuals as 'colonised' or 'not colonised' is useful in many settings, often the selection of resistance consists of increasing the abundance of organisms already present [36,130]. In addition, the abundance of antimicrobial resistant organisms in the gut has been associated with risk of invasive infection and onward transmission [126,130]. Because of this, improving the limit of detection of a sequencing based resistome assay should not be at the expense of quantification.

3.1.1 Previous work

My work follows previous experiments done by others in the Modernising Medical Microbiology group exploring conditions that might be suitable for an culture-enrichment sequencing assay. Selective enrichment of faecal samples in 20ml Mueller Hinton broth containing vancomycin and metronidazole demonstrated that *Enterobacteriaceae* were in log-phase growth up to approximately 8 hours of incubation (**Figure** 3.1). Six hours was selected as an optimal incubation period for a sequencing assay as this provides approximately 10^4-fold increase in *Enterobacteriaceae* without proceeding past log-phase growth, which would prevent estimation of the starting concentration.

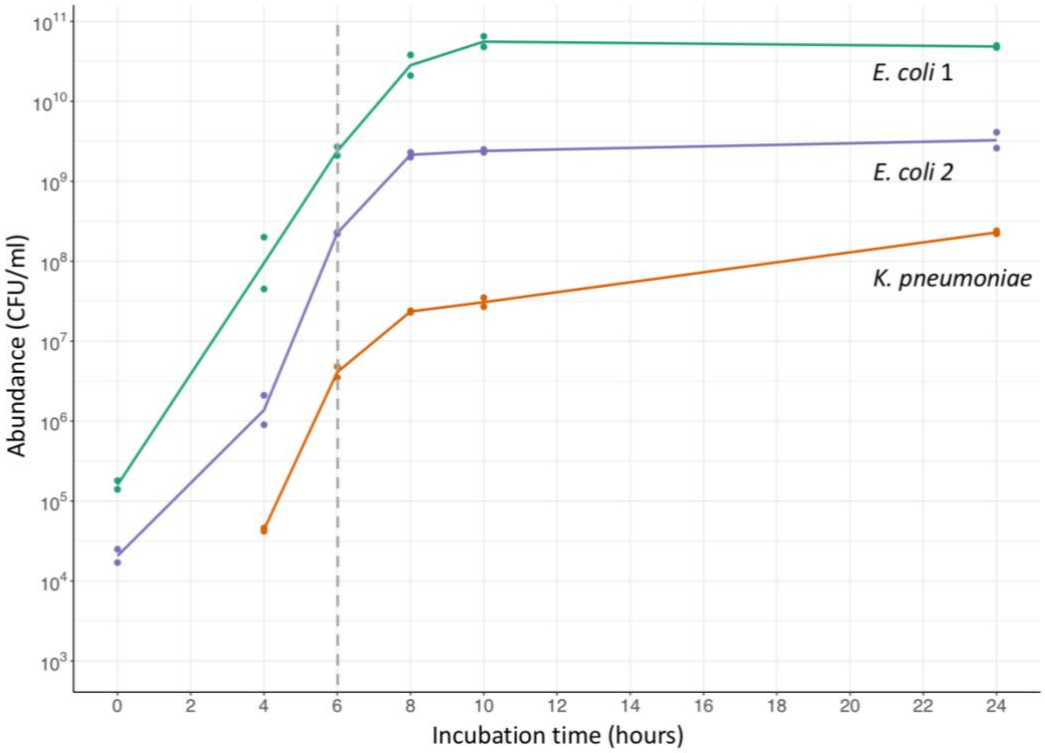

Figure 3.1 Growth of *Enterobacteriaceae* during enrichment with vancomycin and metronidazole
Two morphotypes of E. coli were present. No *K. pneumoniae* were isolated at 0 hours, implying presence below the limit of detection of 10^3 CFU/g. Dashed line indicates 6 hour incubation period selected for subsequent enrichment experiments. Lines join means of 2 replicates using the same stool sample. Data from N Fawcett.

To test these conditions in a sequencing assay, aliquots of a single stool sample were spiked with *E. coli* carrying a New Delhi Metallo-beta-lactamase (NDM) at concentrations varying from 0 – $5x10^5$ CFU/g with enrichment for 6 hours as above. Metagenomic sequencing was performed on the spiked samples before enrichment and on the pelleted broths after enrichment. In all cases the spike organism was barely detectable above the endogenous *E. coli* present in the sample. The relative abundance of *E. coli* was in the range 2.9–4.4% before enrichment and in the range 97.2–98.6% after enrichment, with no discernible effect of increasing spike concentration (**Figure** 3.2). The NDM gene was not detected in any samples before enrichment and in only 3 samples after enrichment; 2 of 3 spiked at $5x10^5$

CFU/g, and 1 of 3 spiked at $5x10^4$ CFU/g. In each case NDM was detected in just a single DNA read, corresponding to an abundance of <1 read/million.

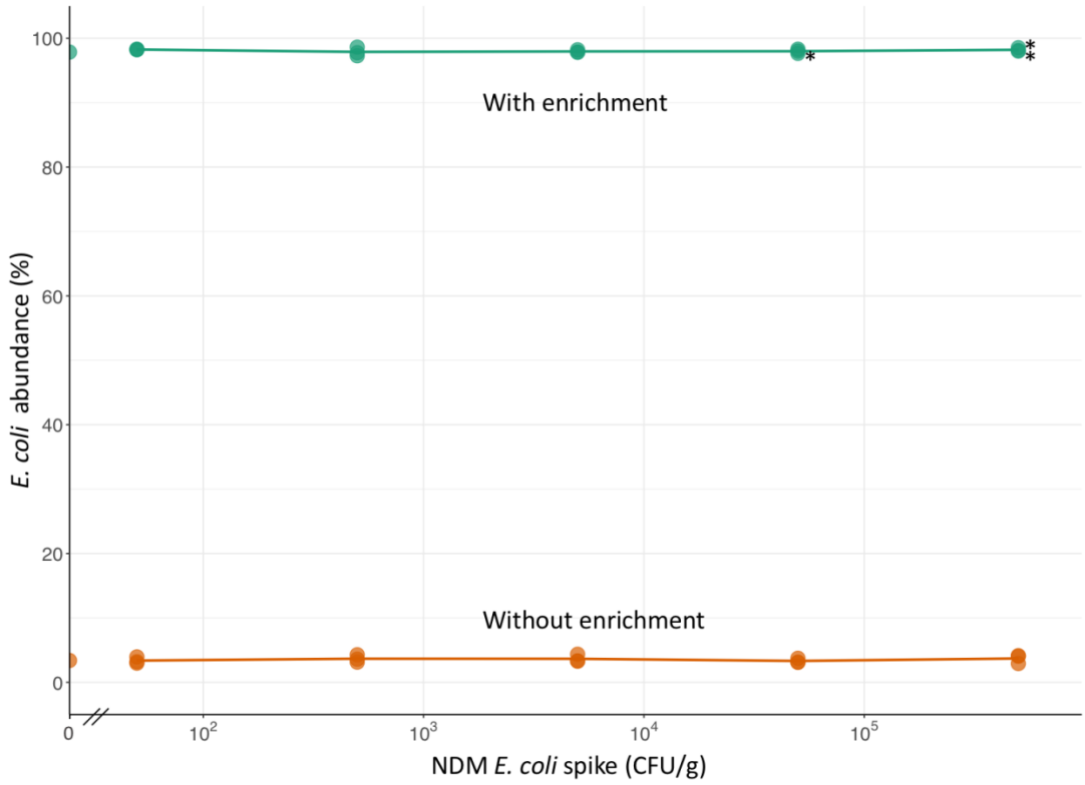

Figure 3.2 Effect of enrichment with vancomycin and metronidazole on *E. coli* abundance
Stars indicate samples in which a single DNA read mapped to NDM. Lines join mean 3 replicates using the same stool sample. Unspiked samples are shown on the y-axis. Data from N Fawcett.

3.1.2 Plasmid DNA extraction

Because of the difficulty demonstrated in these previous experiments in using selective enrichment to detect a drug-resistant subpopulation of *Enterobacteriaceae*, I decided to investigate an alternative approach. AMR genes often exist on plasmids, particularly in *Proteobacteria* [174], and many methods of plasmid extraction have been developed for use in DNA cloning [175], which exploit differences in the physical properties between small, covalently closed circular plasmid DNA (pDNA) and long fragments of chromosomal DNA [176]. The classical method, alkaline lysis, relies on the capacity of pDNA to renature and so

remain soluble following denaturation, as opposed to chromosomal DNA, which forms large tangled precipitates that can be separated by centrifugation [177]. Alternative methods exploit chromatographic differences to purify pDNA [178,179]. Although selective plasmid purification has not been compared to standard metagenomic extractions in human samples, one study that applied them to environmental metagenomic samples found that they could increase detection of some AMR genes up to 37-fold [180].

The two methods to increase detection of AMR genes that I explore in this chapter are:
i) Selective plasmid DNA purification to improve the detection of plasmid-associated AMR genes.
ii) Selective culture-enrichment of *Enterobacteriaceae* with vancomycin, metronidazole and cefpodoxime prior to sequencing.

I show that the methods tested can lower the limit of detection of selected resistance mechanisms, but that they are unable to provide reliable quantification, highlighting the difficulties of using these, or other similar approaches.

3.2 Materials and methods

3.2.1 Experiment 1 - Comparison of three plasmid DNA extraction methods

To assess the utility of plasmid purification, I compared a standard metagenomic DNA extraction method with three commercial plasmid purification kits, chosen to have different methods of action. The standard DNA extraction method is based on the QIAamp Fast DNA Stool MiniKit, modified to include mechanical lysis with bead beating to increase yield from Gram positive bacteria [181]. The plasmid purification kits were:

i) QIAGEN Plasmid Mini kit ('QIAGEN'), which uses modified alkaline lysis with DNA binding to an anion-exchange resin under high-salt conditions.

ii) QIAprep Miniprep ('QIAprep'), which uses modified alkaline lysis with DNA binding to a silica matrix under low-salt conditions.

iii) QuickLyse Miniprep ('QuickLyse'), which uses enzymatic and osmotic lysis with pDNA binding to a proprietary spin column.

Details of the extraction protocols are outlined in section 3.2.9 below.

Aliquots of 100mg of a single sample of volunteer faeces were spiked with one of two different *Enterobacteriaceae* containing plasmid-associated NDM-1 beta-lactamase (*E. coli* or *K. pneumoniae*). Spike organisms were added at a concentration of 10^9 CFU/g. DNA was extracted using each of the four methods, and sequencing performed on Illumina MiSeq. Comparison was made between the number of reads/million mapping to the plasmid-associated NDM-1 gene.

3.2.2 Experiment 2 - QuickLyse plasmid extraction versus standard DNA extraction

The best performing plasmid purification method, QuickLyse Miniprep, was again compared with the standard extraction method. Aliquots of 100mg from two different volunteer faecal sample were spiked with one of two *Enterobacteriaceae* carrying plasmid-associated beta-lactamases (*K. pneumoniae* with NDM-1 and CTX-M-15, or *E. cloacae* with KPC-2 and TEM-1). Spike organisms were added at a lower concentration of 10^8 CFU/g and negative controls with no spike were run in parallel. Plasmid extractions were performed in duplicate.

Following DNA extraction using the two methods, sequencing was performed on Illumina

MiSeq. Comparison was made between the number of reads/million mapping to the plasmid-associated beta-lactamase genes.

3.2.3 Experiment 3 - Enrichment with vancomycin, metronidazole and cefpodoxime

A modified culture-enrichment protocol was tested, based on the conditions described in section 3.1.1 with the addition of cefpodoxime to suppress growth of *Proteobacteria* susceptible to third-generation cephalosporins. DNA extraction was with the QuickLyse Miniprep Kit. To establish the performance of this protocol, another spiking experiment was performed using faecal samples from two volunteers. Aliquots of 100mg from the samples were spiked with one of two *Enterobacteriaceae* carrying plasmid-associated beta-lactamases (*K. pneumoniae* with NDM-1 and CTX-M-15, or *E. cloacae* with KPC-2 and TEM-1) at spikes ranging from 0 – $5x10^7$ CFU/g. Unenriched controls were run in parallel and all conditions were repeated in duplicate. Immediately before DNA extraction, 10^7 CFU *S. aureus* was added to all samples as a standard to allow quantification of the spike CPE using sequencing data. Sequencing was performed on Illumina MiSeq.

3.2.4 Specimens

The Antibiotic Resistance in the Microbiome – Oxford (ARMORD) Study gathers faecal samples from patients and healthy volunteers to study antimicrobial resistance with approval from Leicester Research Ethics Committee (reference 15/EM/0270). Stool samples from two ARMORD participants were used for all experiments; experiment 1 used samples from participant B, experiments 2 and 3 used samples from participants A and B. Participant A was a hospital inpatient with exposure to a third-generation cephalosporin antibiotic

(ceftazidime) 48 hours previously; participant B was a healthy volunteer with no recent antibiotic exposure. Samples were frozen at -80˚C within 2 hours of collection and were confirmed to be culture-negative for ESBL producing *Enterobacteriaceae* using overnight growth in Mueller Hinton broth containing 1mg/L cefpodoxime (Sigma-Aldrich) followed by inoculation onto ESBL Brilliance agar (Oxoid) for 24 hours growth at 37˚C in aerobic conditions [182].

3.2.5 Bacterial strains

Three CPE strains were used for spiking experiments, grown on CRE Brilliance agar (Fisher Scientific) at 37˚C in aerobic conditions.

1) *E. coli* H17, containing NDM-1 beta-lactamase on an unresolved plasmid, isolated from a hospital outbreak in Nepal (SAMN02885348) [183].

2) *Klebsiella pneumoniae* PMK3, containing NDM-1 and CTX-M-15 beta-lactamases on a 305kb plasmid, present at an estimated 1.8 copies per chromosome. Isolated from a hospital outbreak Nepal (SAMN02885389) [183].

3) *Enterobacter cloacae* CAV1668, containing KPC-2 and TEM-1 beta-lactamases on a 43kb plasmid, present at an estimated 1.9 copies per chromosome. Isolated from a hospital outbreak in Virginia, USA (SAMN03733827) [110].

3.2.6 Spiking

Overnight growth of bacteria was used to make a suspension of 5 MacFarland in nutrient broth with 10% glycerol (NBG) (Oxoid), measured using a BD PhoenixSpec nephelometer. Serial 10-fold dilutions were frozen at -80˚C with culture from thawed aliquots used to define concentrations. Stool samples were vortexed with molecular water (Fisher Scientific)

on ice at a ratio of 1:2 to create a pipettable suspension, 300mg of which was used per set of experimental conditions. In experiment 3 samples were spiked with CPE to a final concentration of $5x10^2$, $5x10^3$, $5x10^4$, $5x10^5$, $5x10^6$, or $5x10^7$ CFU/g, using 5-50µl of spiking solution, or with 50µl NBG alone for unspiked controls. In addition, to allow estimation of the number of CPE organisms in post-enrichment samples using sequencing data, a standard spike of 10^7 CFU of *Staphylococcus aureus* was added to all samples except negative controls immediately prior to DNA extraction. MRSA252 (SAMEA1705935) was used, which was grown on blood agar (Fisher Scientific) at 37˚C in aerobic conditions.

3.2.7 Enrichment

Spiked samples were added to 20ml Mueller-Hinton broth (Sigma-Aldrich) containing 20mg/L vancomycin, 20mg/L metronidazole, and 1mg/L cefpodoxime (all antibiotics Sigma-Aldrich). These were incubated at 37˚C and shaken at 190rpm for 6 hours, then, immediately centrifuged at 3200g for 10 min at 4˚C to pellet bacteria. Supernatant was discarded and the pellet resuspended in 1ml molecular water.

3.2.8 Quantitative culture

Modified track plating was used to quantify *Enterobacteriaceae* in faecal samples in experiment 3 [184]. Samples were serially diluted 10-fold in peptone water with pipette mixing at each step. Culture of 10µl from dilutions $1:10^{-1}$ – $1:10^{-8}$ was performed on CHROMagar Orientation (BD) and ESBL Brilliance agar (Oxoid). Colonies were counted after incubation at 37˚C for 16 hours in aerobic conditions.

3.2.9 DNA extraction

Four extraction protocols were used. Experiment 1 compared all four methods, experiment 2 compared standard extraction with QuickLyse extraction, and experiment 3 used QuickLyse extraction alone.

3.2.9.1 Standard extraction (bead beating followed by QIAGEN Fast DNA Stool MiniKit)

Overview: Disruption of cells with silica and ceramic beads. DNA binding to silica column, removal of impurities with proteinase and ethanol wash and elution in molecular water.

Protocol: Based on an extraction protocol previously developed in the group [181]. Samples were added to 1ml Stool Transport and Recovery buffer (Roche) in a 2ml Lysing Matrix E tube (MP Biomedicals), followed by bead beating for 2 x 40s with a FastPrep-24 5G instrument (MP Biomedicals) and incubation at 95˚C for 5 minutes. Samples were then centrifuged at 1,000g for 60s. 400ul supernatant was transferred to 1ml InhibitEX buffer, after which the manufacturer's instructions were followed for bacterial DNA extraction. Briefly, this involved mixing with 30µl proteinase K and 400µl AL lysis buffer, incubation at 70˚C for 60s, then addition of 400µl 100% ethanol followed by vortexing. The mixture was transferred to a QIAamp spin column, spun at 17,000g for 60s, washed initially with 400µl AW1 buffer and spun at 17,000g for 60s, then finally washed with 400µl AW2 buffer and spun at 17,000g for 60s. The spin column was incubated with 50µl molecular water at 55˚C for 10 minutes before elution by spinning at 17,000g for 60s.

3.2.9.2 QuickLyse Miniprep Kit extraction

Overview: Enzymatic and osmotic lysis with pDNA binding to proprietary spin column. Washed with buffer containing isopropanol and eluted in low salt buffer.

Protocol: Samples were suspended in 1ml molecular water and centrifuged at 100g for 5 minutes to remove large particles. DNA was then extracted from the supernatant according to the manufacturer's instructions. Briefly, bacterial cells were pelleted by centrifugation at 17,000g for 2 minutes and resuspended in 400µl ice-cold complete lysis solution by vortexing for 30s. After 3 minutes at room temperature the lysate was transferred to a QuickLyse spin column and centrifuged at 17,000g for 60s. The spin column was washed with 400µl QuickLyse wash buffer and centrifuged at 17,000g for 60s. After discarding the flow-through, the columns were dried by centrifuging at 17,000g for 60s. DNA was eluted from the spin column in 50 µl QuickLyse elution buffer by spinning at 17,000g for 60s.

3.2.9.3 QIAGEN Plasmid Mini Kit extraction

Overview: Modified alkaline lysis with DNA binding to anion-exchange resin spin column under low-salt conditions. Washed with medium salt buffer and eluted in high salt buffer followed by isopropanol precipitation.

Protocol: Samples were suspended in 1ml molecular water and centrifuged at 100g for 5 minutes to remove particles. The supernatant was then used for DNA extraction according to the manufacturer's instructions. Briefly, this consisted of centrifugation to pellet bacterial cells with resuspension of the cells in 300µl P1 buffer, followed by the addition of 300µl P2 buffer and 300µl P3 buffer with mixing after each step. Chromosomal DNA precipitate was removed by centrifugation at 17,000g for 10 minutes. Supernatant was transferred to a Qiagen-tip 20 pre-equilibrated with 1ml QBT buffer then washed twice with 2ml QC buffer. DNA was eluted with 800µl QF buffer, then precipitated with 560µl isopropanol before centrifugation at 17,000g for 30 minutes. The supernatant was discarded before washing

the DNA pellet with 1ml 70% ethanol. After air-drying, DNA was redissolved in 50µl molecular water.

3.2.9.4 QIAprep Miniprep kit extraction

Overview: Modified alkaline lysis with DNA binding to silica spin column under high salt conditions. Washed with high-salt buffer and eluted in low-salt buffer with no alcohol precipitation.

Protocol: Samples were suspended in 1ml molecular water and centrifuged at 100g for 5 minutes to remove particles. The supernatant was then used for DNA extraction according to the manufacturer's instructions. Briefly, this consisted of centrifugation to pellet bacterial cells with resuspension of the cells in 250µl P1 buffer, followed by the addition of 250µl P2 buffer and 350µl N3 buffer with mixing after each step. Chromosomal DNA precipitate was removed by centrifugation at 17,000g for 10 minutes. 800µl supernatant was transferred to at QIAprep 2.0 spin column and centrifuged at 17,000g for 60s. The column was washed with 500µl PB buffer and spun at 17,000g for 60s, then washed with 750µl PE buffer and spun at 17,000g for 60s. DNA was eluted in 50µl molecular water.

3.2.10 DNA library preparation and sequencing

Extracted DNA was purified using Ampure XP beads (Agilent). 50µl DNA was vortexed with 90µl Ampure XP beads and washed twice with 80% ethanol before elution in 25µl molecular water. DNA was normalised to 0.2ng/µl and libraries prepared from 1µg DNA using the Nextera XT Library preparation kit (Illumina) according to the manufacturer's instructions. Normalisation and QC of libraries was performed using a Qubit 2.0 fluorometer (Thermo Fisher) and 2200 Tapestation (Agilent). Paired-end 300bp sequencing was performed on an

Illumina MiSeq, with 10–16 samples multiplexed per sequencing run. Median sequencing depth ranged from 500,000 to 1.7 million 300bp paired reads.

3.2.11 Bioinformatic and statistical analysis

Human DNA reads (representing <4% of sequences in all samples) were identified using the Kraken taxonomic classifier and removed prior to analysis [185]. Quality control metrics were assessed with FastQC [186], and BBduk was used to remove Illumina adapters and quality-trim reads using a phred-score cut-off of 10 [187]. MetaPhlAn2 was used for taxonomic classification [188], except for normalization to *S. aureus*, where Kraken was used to classify reads to the relevant species. BBMap was used to map reads to beta-lactamase genes, *gyrA* and plasmids using the relevant genes from the assembled genomes specified above [187]. Plasmid copy number was estimated by mapping reads from sequenced isolates to a closely related closed reference genome using BWA-MEM [189]. For PMK3, the PMK1 reference was used (CP008929-CP008933), and for CAV1668, the CAV1668 reference was used (CP011582-CP011584). SAMtools was used to filter out supplementary alignments and calculate depth of coverage [190]. All statistical analyses were performed in R, version 3.5.0. Means were compared using a Welch two sample t-test, which allows unequal variances in the two groups.

3.2.12 Normalisation to S. aureus

To allow quantification of CPE spike organisms after enrichment, a standard inoculum of 10^7 CFU *S. aureus* was added to sequenced samples in experiment 3, after enrichment and immediately prior to DNA extraction. Detection of DNA reads from different organisms depends on several factors such as genome size, efficiency of DNA extraction, efficiency of

sequencing and efficiency of bioinformatic classification. These can be combined into a single measure of relative detection efficiency (RDE) compared to *S. aureus*, which is established using unenriched samples in which the concentration of the spike organism and *S. aureus* are both known. For each CPE spike organism, the mean RDE was determined from the 4 unenriched samples spiked with $5x10^7$ CFU/g CPE and 10^7 CFU *S. aureus* (shown in **Figure** 3.6), using the following formula based on the relative proportions of the known CPE spike and *S. aureus* CFU, and the relative proportions or the reads assigned to the CPE spike and *S. aureus* by Kraken:

$$\frac{Spike\ organism\ reads}{S.\ aureus\ reads} = \text{RDE x } \frac{Spike\ CFU}{S.\ aureus\ CFU}$$

The calculated RDE was then used to estimate the number of CFUs present in samples after enrichment, where the true number of CPE CFU was unknown.

3.3 Results

3.3.1 Experiment 1 - Comparison of three plasmid DNA extraction methods

The DNA output from the different extraction protocols varied over 50-fold (**Table** 3.1). The output from the QIAprep was too low for reliable Illumina library preparation, so this method was not taken forward to sequencing.

Extraction method	Minimum DNA concentration (ng/µl)	Maximum DNA concentration (ng/µl)
Standard	3.6	5.0
QuickLyse	13.5	20.8
QIAGEN	1.4	1.5
QIAprep	0.2	0.3

Table 3.1 DNA output from different extraction methods

Reads mapping to the plasmid-associated NDM beta-lactamase were detected at a mean 98 reads per Kb per million (RPKM) using the QuickLyse kit, 41 RPKM using the QIAGEN kit, and 39 RPKM using the standard extraction (**Figure** 3.3). As the QuickLyse method appeared superior to QIAGEN in NDM detection, produced better DNA output and had a more straightforward laboratory workflow, this method was taken forward for further assessment.

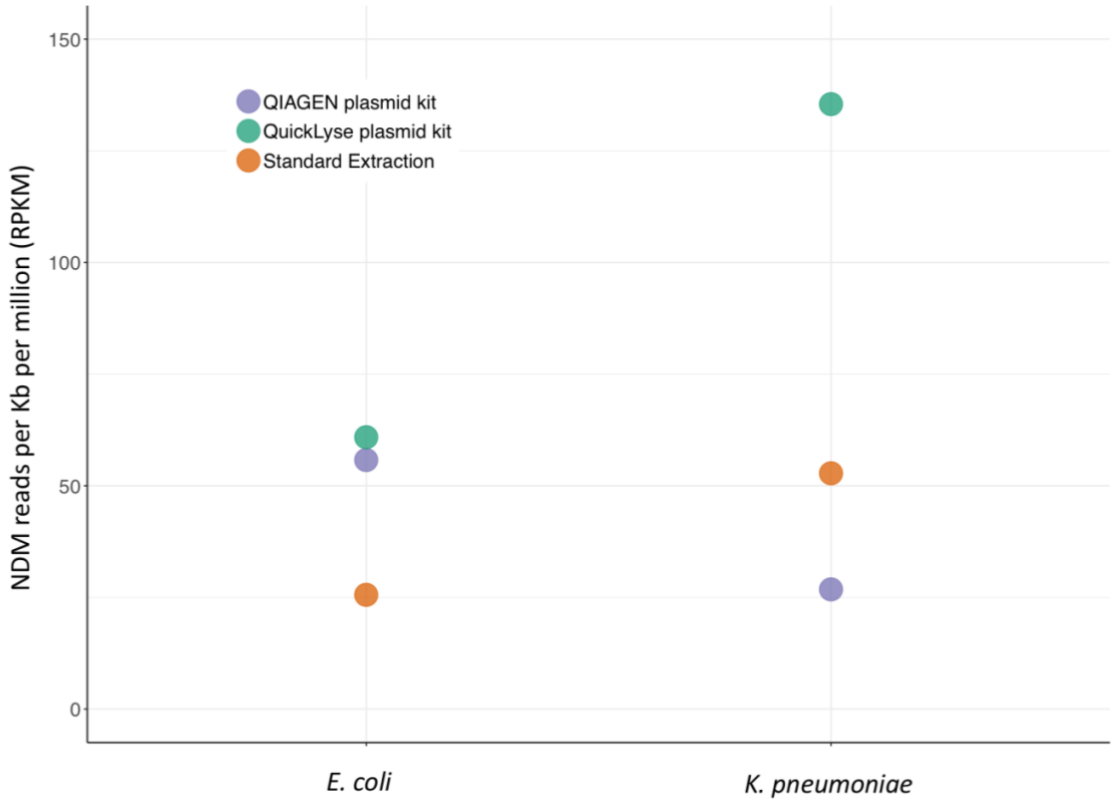

Figure 3.3 Effect of plasmid purification on detection of plasmid-associated AMR genes

3.3.2 Experiment 2 - QuickLyse plasmid extraction versus standard DNA extraction

In the second spiking experiment, the QuickLyse kit again increased the number of reads mapping to plasmid-associated beta-lactamases, to a mean of 15.7 RPKM (95% CI 6.2 – 25.1) compared to a mean of 4.4 RPKM using the standard extraction method (95% CI 2.4 – 6.4) (t-test p=0.03) (**Figure** 3.4). Although potentially useful, the 2–4 fold increase in detection of

plasmid-associated AMR genes using the QuickLyse kit was not sufficient to overcome the problems with direct sequencing outlined in the introduction, so I investigated modifications to the culture-enrichment protocol previously tested.

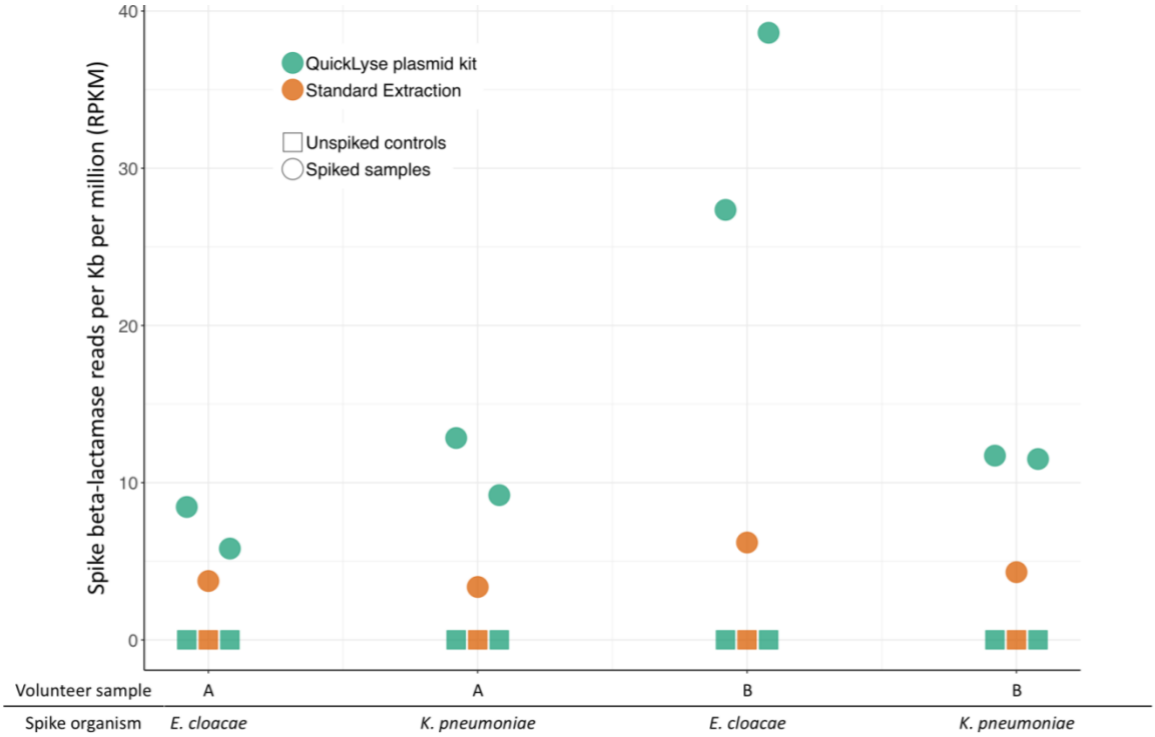

Figure 3.4 Effect of QuickLyse plasmid extraction on detection of plasmid-associated AMR genes

3.3.3 Experiment 3 - Enrichment with vancomycin, metronidazole and cefpodoxime

As enrichment with metronidazole and vancomycin had previously not allowed detection of a resistant subpopulation of *Enterobacteriaceae*, I developed a revised protocol with the addition of cefpodoxime to suppress growth of susceptible *Enterobacteriaceae* and with the QuickLyse kit used for DNA extraction.

3.3.3.1 Effect on relative abundance of Enterobacteriaceae

This enrichment protocol allowed both CPE spikes to be reliably detected even at the lowest spiking concentration of $5x10^2$ CFU/g. CPE spikes above $5x10^5$ CFU/g led to a relative

abundance of ≥90% of reads coming from the spike organism after 6 hours enrichment, as determined by the MetaPhlAn2 taxonomic classifier (**Figure** 3.5). At lower spiking concentrations, there was a marked difference between the spike organisms. At $5x10^2$ CFU/g, *K. pneumoniae* had a mean relative abundance of 58% after enrichment, compared to 3% for *E. cloacae*. In all unenriched samples the spike organism was present at a relative abundance of <10%.

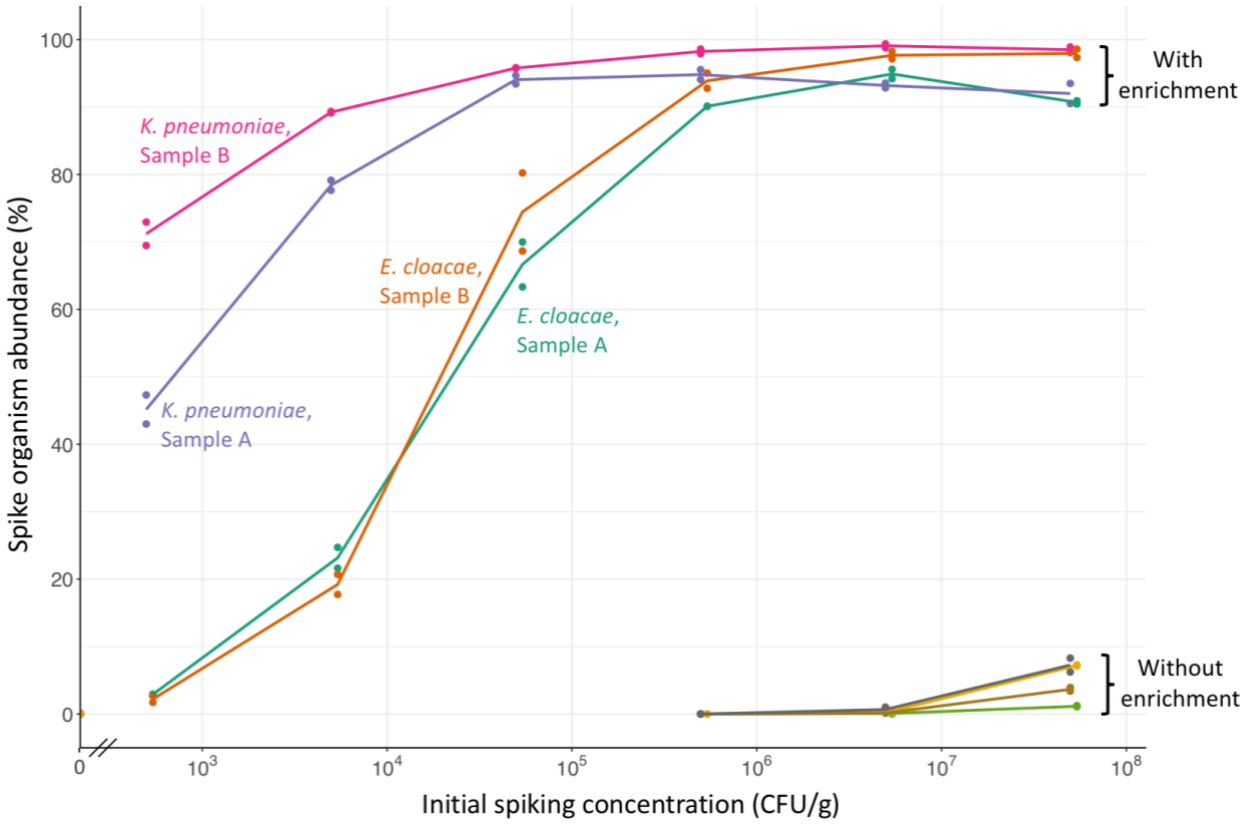

Figure 3.5 Relative abundance of CPE spike after culture-enrichment of faecal samples
Enrichment in broth containing cefpodoxime, vancomycin, and metronidazole. Lines join arithmetic means of 2 replicates. Unspiked samples are shown on the y-axis (all approximately 0%).

3.3.3.2 Effect on absolute abundance of Enterobacteriaceae

As my objective was to measure the absolute, as well as relative, abundance of organisms present in samples, I estimated the number of spike organisms after enrichment by

comparing reads assigned to the spike CPE to those assigned to the *S. aureus* standard. Abundance estimation in the enriched samples showed that the number of spike organisms increased with higher initial spiking concentration even after the relative abundance had plateaued (**Figure** 3.6), confirming the need for a standard. Comparing these sequencing-based estimates to measurement by quantitative culture showed reasonable correlation at abundances below 10^9 CFU, corresponding to initial spikes <5x10^5 CFU/g, with all estimates within 1 $\log_{10}$ of the culture value (**Figure** 3.7). However, at higher abundances, where the number of spike CFU in the sample was more than 300-fold higher than the number of *S. aureus* CFU, the number of spike organisms estimated by sequencing consistently underestimated culture values.

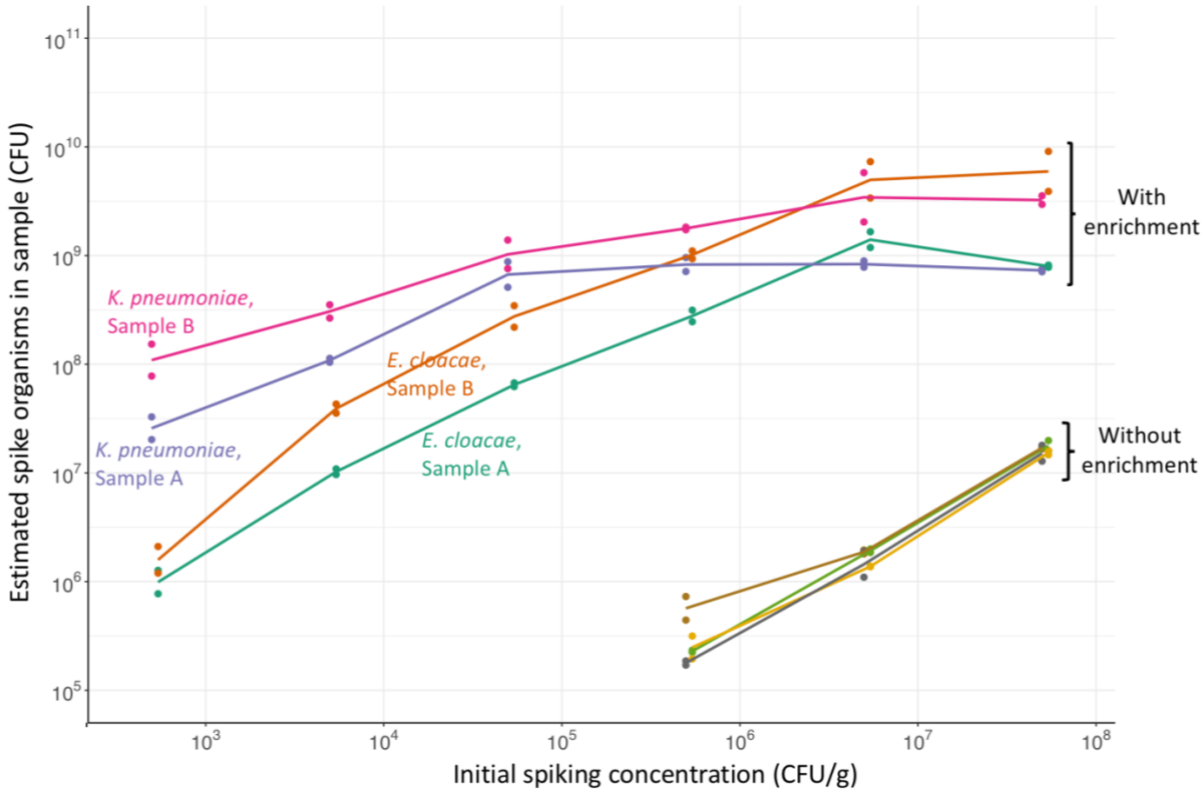

Figure 3.6 Estimated CPE organisms after culture-enrichment of faecal samples

Same experiment as Figure **3.5**. Number of CPE organisms estimated from sequence data by normalisation to *S. aureus* standard. Lines join geometric means of 2 replicates.

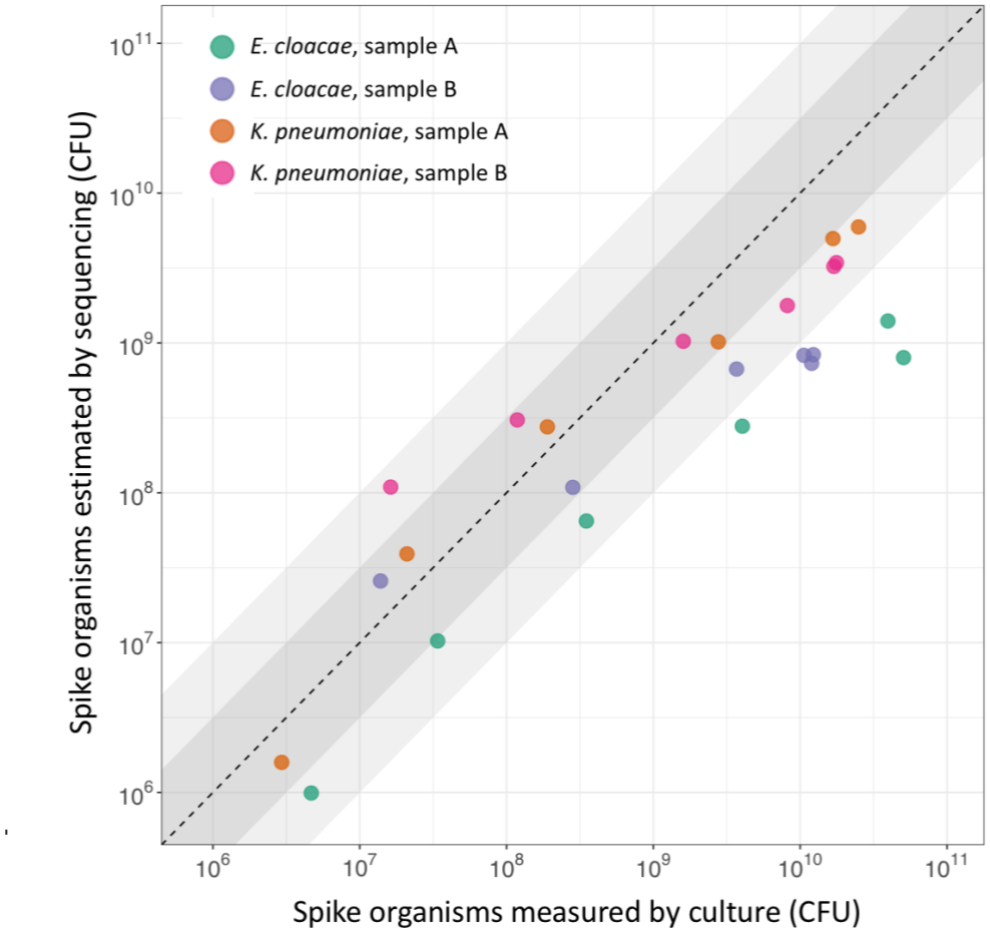

Figure 3.7 CPE organisms estimated by sequencing versus culture

Same experiment as Figure **3.5**. Points represent geometric means of 2 replicates, shaded areas are within 0.5 and 1.0 $\log_{10}$ of the line of identity.

3.3.3.3 Effects on detection of carbapenemase genes

Detection of the carbapenemase genes differed markedly between the two spike organisms (solid lines in **Figure** 3.8). The KPC-2 gene present in *E. cloacae* was detected at a mean 12 RPKM (range 5-17) at the lowest spike of $5x10^2$ CFU/g, implying a limit of detection of approximately 50 CFU/g per million reads. In contrast, the NDM-1 gene present on *K. pneumoniae* was detected at around 100 RPKM even at the lowest initial spike and hardly changed with higher spiking concentrations, in keeping with the higher abundance of *K.*

pneumoniae noted above. This prevented estimation of the limit of detection of NDM-1 in this context.

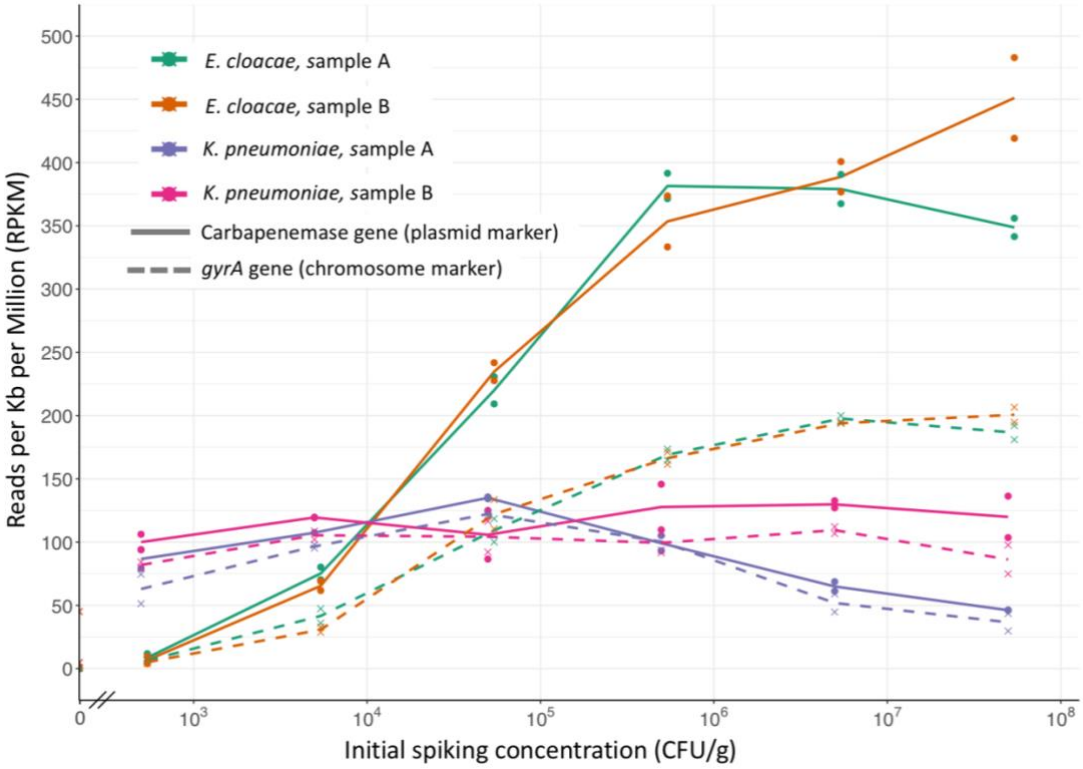

Figure 3.8 Detection of carbapenemase and gyrA genes in CPE spike

Same experiment as Figure **3.5**. Lines join arithmetic means of 2 replicates. Unspiked samples are shown on the y-axis.

3.3.3.4 Artefacts caused by plasmid extraction

At the highest spiking concentrations, the number of reads mapping to the carbapenemase gene was around 4 times higher in *E. cloacae* than *K. pneumoniae*, even though both organisms were present at >90% abundance. This difference did not appear to relate to the plasmid copy number, as in sequenced isolates the carbapenemase genes are present at similar copy numbers of 1.8 – 1.9. To explore the possibility that this difference related to efficiency of plasmid extraction, I compared the number of reads mapping to the plasmid-associated carbapenemase to those mapping to the chromosomal *gyrA* gene (dashed lines in **Figure** 3.8). In *E. cloacae*, DNA from the 43kb KPC plasmid was extracted more efficiently

than the chromosomal *gyrA* gene, leading to greater detection of KPC-2 vs gyrA. In contrast, in *K. pneumoniae* DNA from the 305kb NDM-1 plasmid and *gyrA* was extracted at similar efficiencies.

As well as the NDM-1 plasmid, the *K. pneumoniae* spike also contained DNA mapping to two small plasmids present in GenBank, pEC34A (3.8kb) and pRGRH1815 (7.1kb), although the plasmid structure(s) containing this DNA could not be resolved with available sequence data [191]. This DNA was extracted at a very high efficiency in enriched samples, and the extraction efficiency relative to chromosomal DNA increased with higher spiking concentrations (**Figure** 3.9). At the highest spikes this small plasmid DNA made up around a quarter of all reads, causing a relative decrease in other *K. pneumoniae* DNA.

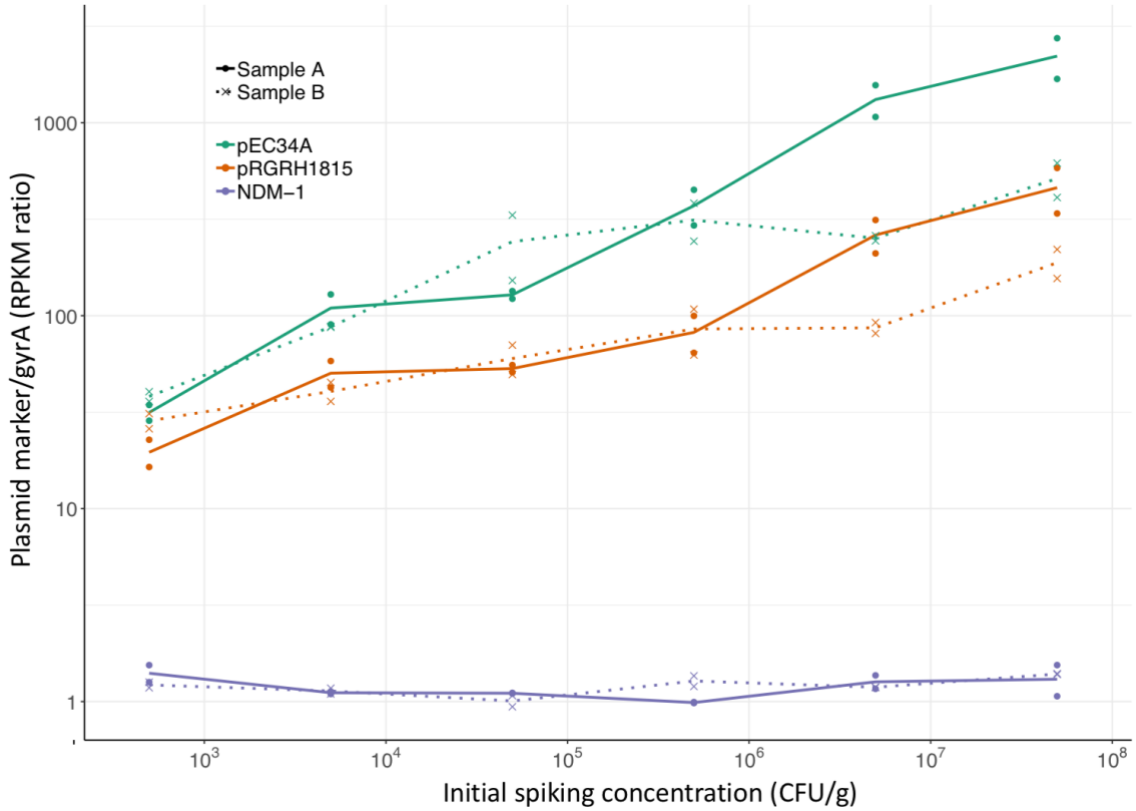

Figure 3.9 Reads mapping to plasmids markers versus chromosomal gyrA gene

Same experiment as Figure **3.5**, data from *K. pneumoniae* spike only. Lines join geometric means of 2 replicates.

3.4 Discussion

To my knowledge, this is the first study to compare pDNA purification to standard DNA extraction for resistome assessment in humans. Jitwasinkul et al [192] used pDNA extraction from stool samples followed by metagenomic sequencing to identify AMR genes carried by inpatients, but without any comparison with other methods. Plasmid extraction has been used in environmental metagenomic studies, including two analysing AMR genes [180,193,194]. One of these studies compared plasmid and standard metagenomic extraction, finding that plasmid extraction increased detection of some resistance genes 37-fold [180], but in my study the effect on the AMR genes of interest was much more modest, with increases of only 2–4 fold. This difference may relate to plasmid size, as extraction efficiency seemed strongly dependent on this. The effect was noticeable when comparing the 305kb and 43kb carbapenemase containing plasmids (**Figure** 3.8), but was much more marked in relation to reads mapping to small 4-7kb plasmids, which were extracted at extremely high efficiency compared to other DNA (**Figure** 3.9). Although some AMR plasmids in *Enterobacteriaceae* are <10kb, they are typically much larger than this, with a median size of 85kb in one recent catalogue [195]. Perhaps the main problem in this differential extraction efficiency is that it introduces large unwanted artefacts that preclude its use in a quantitative resistome assay.

Selective culture-enrichment of stool samples has been successfully used by Raymond et al to characterise low abundance constituents of the microbiome that would have been undetectable with standard metagenomic sequencing, albeit with no attempt at quantification [196]. In the current study, I aimed to create a simple, quantitative assay of resistance genes in culturable organisms using a short period of selective culture followed

by sequencing directly from the enriched sample. I chose culture conditions that would amplify a resistant subpopulation of *Enterobacteriaceae* of particular clinical importance, specifically those resistant to third generation cephalosporins, with the aim of bringing their resistance genes above the threshold of detection.

Performance of the enrichment-sequencing protocol was assessed by spiking faecal samples with known concentrations of CPE. While there were minimal differences between samples from different individuals, there were large differences between the two spike organisms. Consistency between the faecal samples, and between replicates, implies that this relates to intrinsic growth characteristics of the strains used in this experiment. The finding of such large variation in growth between different strains of *Enterobacteriaceae* precludes accurate estimation of their starting concentration unless their growth characteristics are known, undermining one of the aims of the assay. In fact, relatively small differences in growth rates could produce such differences after 6 hours. For example, if two *Enterobacteriaceae* strains had doubling times of 18 minutes and 22 minutes, then this would lead to a 12-fold difference in their abundance after 6 hours incubation.

To estimate the absolute abundance of organisms in the sample using sequence data it is necessary to use some form of standard spike. Although the use of a *S. aureus* standard allowed reasonable estimation of the number of post-enrichment spike organisms at low abundances, it correlated poorly with quantitative culture at higher abundances (**Figure** 3.7). This is likely to be related to small amounts of contaminating DNA assigned to the standard, as even in negative controls with no faecal sample a small number of reads were

assigned to *S. aureus*. This could possibly be improved by using an alternative standard with less homology to any possible contaminants or organisms present in the sample.

This study has several limitations. It was only possible to assess a limited number of conditions, and the quantitative findings of this study may have been different if using different spike organisms, culture conditions or plasmid extraction methods, although it seems unlikely that the qualitative conclusions would differ. The use of a plasmid extraction kit in the enrichment experiment means that measured bacterial abundances will be biased towards organisms that are extracted more efficiently with this method, but this effect should be consistent between conditions within the experiment. Finally, although I detected DNA mapping to known small plasmids in samples spiked with *K. pneumoniae*, it was not possible to resolve the exact plasmid structures or sizes with the short read sequencing data available.

Given the difficulties with the methods tested here, alternative ways of quantifying scarce AMR genes are needed. A promising alternative is to use target capture, in which a custom library of nucleic acid probes is used to enrich resistance genes directly from a standard metagenomic DNA extract. Using such a method, Noyes et al [197] were able to increase the proportion of sequenced reads mapping to resistance genes over 100-fold, and detected important resistance mechanisms, such as KPC, that were missed with standard metagenomic sequencing. Their workflow also incorporated unique molecular indices to improve the reliability of quantification. Using a different target capture system, Lanza et al increased the proportion of reads mapping to resistance genes 279-fold [173]. Both studies compared target capture and sequencing to standard metagenomic sequencing, but neither

were tested on samples with a known concentration of antimicrobial resistant organisms or on negative controls. My study demonstrates the need to validate such methods in well characterised conditions before they can reliably be used to make quantitative comparisons.

In this chapter I have assessed novel approaches to lowering the limit of detection of AMR genes in *Enterobacteriaceae* in a sequencing-based assay of the intestinal resistome. Assessing the differential effect of plasmid DNA extraction by plasmid size has highlighted the artefacts that make it inappropriate for use in a resistome assay requiring accurate quantification. By comparing different strains of *Enterobacteriaceae* I have demonstrated the difficulty of using culture enrichment whilst maintaining quantification. Further attempts at quantitative resistome assessment must either use different methods to amplify scarce AMR genes, or accept the likelihood of missing genes in low abundance organisms, which may include important pathogens such as *Enterobacteriaceae*.

4 Methods and Quality Control

Chapters 5 and 6 mostly describe different analyses from the same study (ARMORD). This chapter describes the clinical study, laboratory and bioinformatic methods that apply to both chapters, as well as sample quality control metrics.

4.1 Clinical studies

4.1.1 The ARMORD study

The Antibiotic Resistance in the Microbiome – Oxford (ARMORD) study was designed to investigate the effects of antimicrobials on the gut microbiome and resistome. I took over the running of the study in August 2016, after initial ethics approval had been obtained but before the majority of recruitment was carried out (260/343 (76%) of participants were recruited and 665/728 (91%) of samples were collected after August 2016). Participants were recruited from patients at the Oxford University Hospitals NHS Foundation Trust (OUH) and healthy individuals living in Oxfordshire. The study involved two sampling frames:

i) **Cross-sectional**, where participants provided a single stool sample and associations between measurements of the microbiome and resistome and previous exposures were investigated.

ii) **Longitudinal**, where participants provided serial samples and associations between changes in the microbiome and resistome and exposures that occurred between samples were investigated. This group consisted exclusively of patients admitted to the haematology ward for haematopoietic stem cell transplant (HSCT). I was responsible for the choice of this sampling frame and wrote a substantial ethics amendment allowing this. The first sample from longitudinal participants was also used for the cross-sectional sampling frame.

The study received ethical approval from East Midlands - Leicester Central Research Ethics Committee (reference 15/EM/0270).

4.1.1.1 Recruitment & eligibility

Recruits were drawn from healthy individuals responding to articles about the study in local and social media, and patients at OUH approached by a member of the study team. Recruitment at OUH was focussed on inpatient medical wards and the haematology ward.

Informed consent was obtained from all participants (consent form is shown in **Appendix 2**). Inclusion criteria were:

- Age ≥18 years
- Able to give informed consent and provide a reasonable history of recent antibiotic use
- No stoma or active inflammatory bowel disease
- For cross-sectional participants, their local hospital had to be part of OUH to improve the reliability of electronic records. This was not required for longitudinal participants as recruitment was limited by the number of patients having HSCT, who were often referred from other trusts.

4.1.1.2 Rationale for recruitment strategy

Recruitment to the cross-sectional stratum was deliberately heterogeneous, including healthy individuals, outpatients, and inpatients with varying degrees of antibiotic exposure. However, the main focus of recruitment was medical inpatients, as this group:

i. Were often resident in hospital for several days, greatly increasing the success rate of sample collection compared to healthy volunteers, outpatients or specialties with high turnover.
ii. Usually had recent antimicrobial exposure, which was often precisely recorded via electronic prescribing.
iii. Were more likely to be colonised with AMR organisms.

In contrast, recruitment to the longitudinal stratum of the study was relatively homogeneous, consisting entirely of HSCT patients. In the initial protocol, longitudinal recruitment had targeted all inpatients receiving antibiotics, but this approach was not successful. Most inpatients receiving antibiotics in hospital start them soon after admission, when it is difficult to recruit them. Also, the delay between recruitment and obtaining the first stool sample is typically 1-3 days, meaning that the first sample would often be significantly affected by antibiotics. I decided that patients receiving HSCT would provide a good population for longitudinal recruitment for several reasons:

i. They typically spend several weeks in hospital, during which time nearly all develop neutropaenic sepsis, providing a good opportunity for longitudinal sampling before and after receiving antibiotics.
ii. They have planned admissions, usually starting around one week before they become neutropaenic, during which time they are relatively well and are receiving conditioning chemotherapy. This makes identifying and recruiting patients easier.
iii. They have often have had episodes of sepsis during recent treatment for their underlying condition, usually haematological malignancy, so are often colonised with AMR organisms.

iv. The gut microbiome of particular interest in HSCT patients because enteric colonisers such as *Enterobacteriaceae* and enterococci are often responsible for neutropenic sepsis [126], and because of the possible involvement of gut organisms in triggering graft versus host disease (GvHD), a complication of allogeneic stem cell transplant [159,198].

There are, however, significant disadvantages to limiting longitudinal recruitment to HSCT patients.

i. They uniformly receive chemotherapy during admission, which is likely to affect the gut microbiome both by direct antibacterial toxicity [199], and also by causing diarrhoea via cytotoxic effects on the gut epithelium. The effect of this potential confounding needs to be taken into account in analysis.
ii. Most patients are referred for HSCT from neighbouring hospitals, where they have often received multiple broad-spectrum antibiotics during chemotherapy to induce remission. No medical records were available outside OUH, so exposure history is derived from patients and referral documents. This makes baseline samples less useful for the cross-sectional analysis than if they mostly had well known, single antibiotic exposures.

4.1.1.3 Data collection

All participants were interviewed by a member of the study team about antimicrobial exposure within the past year, travel within the past 3 years, typical diet, animal exposures, healthcare exposure and use of specific drugs, including proton pump inhibitors (PPI). The

Case Record Form is provided in **Appendix 3**. Information on recent antimicrobial use was also obtained from patients' notes.

Electronic patient records from OUH were available, which included the following data:

- Inpatient and discharge antimicrobial prescriptions
- Microbiology, haematology and biochemistry results
- Inpatient admissions, outpatient episodes and A&E attendances
- Inpatient clinical observations, which were used to calculate the National Early Warning score 2 (NEWS2), a summary score of physiological abnormality
- Discharge coding, including Charlson co-morbidity score calculated from discharge codes

4.1.1.4 Sample size

The initial recruitment target was 100 cross-sectional and 100 longitudinal participants. In August 2017 this was amended to 200 cross-sectional and 100 longitudinal participants after analysis of preliminary data suggested that this would improve the power to demonstrate modest differences between antibiotic exposures.

The sample of 200 cross-sectional participants was chosen to allow the standard deviation of AMR gene measures to be estimated within 10% with 95% confidence (2-sided alpha=0.05) [200]. The longitudinal sample of 100 participants was chosen based on an estimated standard deviation of 50 reads per million and a between-timepoint correlation of 0.6 (between measurements from the same person). 100 participants provided >80%

power to detect a difference of 50 reads per million in change from baseline in reads per million between two antibiotic groups.

Combined cross-sectional analysis of risk factors for measures of AMR gene carriage in all 300 participants was estimated to provide >80% power to detect differences of:

- 0.46 times their standard deviation between groups comprising 15% and 85% of the population
- 0.33 times their standard deviation between groups each comprising 50% of the population.

4.1.2 The POETIC study

In addition to the ARMORD study, frozen stool samples were available from a previous study, which were used for a separate analysis. The point of care testing for urinary tract infection in primary care (POETIC) trial was a randomised trial testing a point of care urine culture system for the management of uncomplicated UTI [201,202]. Women ≥18 years old presenting to their GP with symptoms of uncomplicated UTI were eligible for the study provided they had received no antibiotics for UTI in the past 4 weeks and were not taking long term antibiotics. Women kept antibiotic diaries during the study, which were used to define antibiotic exposure. Data on previous antibiotic exposure and co-morbidities was not available for this analysis.

Stool samples were collected at recruitment and after UTI treatment, allowing longitudinal assessment of the effect of antibiotics. Sample pairs were selected for sequencing to provide a range of different antibiotic exposures, with pairs excluded if the initial sample

was collected >24 hours after the start of antibiotics. The trial was approved in the UK by the Research Ethics Committee for Wales (reference 12/WA/0394), and participants provided consent for samples to be used for future research on AMR.

4.2 Laboratory methods

4.2.1 Specimen collection & storage

In the cross-sectional stratum of ARMORD, participants provided the first stool sample passed after recruitment. This was stored at ambient temperature for a maximum of 24 hours before being frozen at -80˚C.

In the longitudinal stratum of ARMORD, participants were asked to provide a stool sample every other day until discharge. These were stored at 4-8˚C for up to 72 hours before being frozen at -80˚C.

In the POETIC trial participants were asked to collect a stool sample on the day of recruitment and post it to the trial laboratory within 24 hours of collection. A second sample was collected around 14 days after the first and returned in the same manner. Samples remained at ambient temperature until received by the laboratory, where they were frozen at -80˚C.

4.2.2 DNA extraction

4.2.2.1 Extraction protocol

DNA extraction was performed using the standard metagenomic extraction technique outlined in **Chapter 3**, consisting of bead beating followed by QIAGEN Fast DNA Stool

MiniKit. This is based on a protocol previously developed in the group [181]. I made some modifications:

i. All samples, including negative controls, had a standard spike of *Thermus thermophilus* HB8 DNA (DSMZ, ref DSM 579) added prior to DNA extraction to allow estimation of absolute abundances and quantify contamination.
ii. The quantity of sample used for extraction varied depending on the consistency of the sample, as liquid samples (Bristol stool scale 6-7) were often found to have low DNA concentration.
iii. Additional precautions were taken to avoid DNA contamination (details below).

The protocol used for ARMORD and POETIC samples is given below.

Preparation

1) Prepare 2ml Lysing Matrix E tube (MP Biomedicals) by adding 1ml Stool Transport and Recovery buffer (Roche) and *T. thermophilus* DNA (DSMZ, typically 100ng, but amount varied between extractions). Label and weigh tubes.
2) Remove stool samples from -80˚C to thaw immediately before extraction.

Lysis

3) Add ~20–1000mg sample to each tube, transferring more from more liquid samples. Weigh again to establish weight of sample.
4) Bead beat tubes twice at 6m/s for 40s with a FastPrep-24 5G instrument (MPBiomedicals) with 5 minutes interval at 4˚C
5) Incubate tubes at 95˚C for 5 minutes and centrifuge at 1,000g for 1 minute
6) Transfer 900µl supernatant to 1ml InhibitEX buffer in 2ml Eppendorf, invert x 100 and centrifuge at 17,000g for 3 minutes
7) Transfer 400µl supernatant to 30µl proteinase K in 1.5ml Eppendorf
8) Add 400µl AL lysis buffer and invert x 100

9) Incubate tubes at 70˚C for 1 minute and centrifuge at 17,000g for 30s

10) Add 400µl 100% ethanol, invert x 20 and centrifuge at 17,000g for 30s

DNA binding & washing

11) Transfer 1st half of supernatant to spin column, centrifuge at 17,000g for 1 minute and transfer column to new collection tube

12) Transfer 2nd half of supernatant to same spin column, centrifuge at 17,000g for 1 minute and transfer column to new collection tube

13) Add 500µl AW1 wash buffer to column, centrifuge at 17,000g for 1 minute and transfer column to new collection tube

14) Add 500µl AW2 wash buffer to column, centrifuge at 17,000g for 1 minute and transfer column to new collection tube

15) Centrifuge at 17,000g for 1 minute to dry and transfer column to 1.5ml Eppendorf

Elution

16) Add 50µl molecular water and incubate at 55˚C for 10 minutes before centrifuging at17,000g for 1 minute to elute DNA

Following extraction, DNA concentration was measured with Picogreen and Qubit fluorometers. DNA was transferred to 96-well plates and stored at -20˚C until sequencing.

4.2.2.2 Thermus thermophilus internal standard

T. thermophilus was used as an internal standard as this extremophile bacterium has minimal homology with most human commensal bacteria and has previously been used as a standard in environmental metagenomic studies [92]. A known mass of genomic DNA purchased from a commercial supplier was used rather than intact organisms, as this was more easily scalable and avoided possible variability introduced by culturing the organisms

and relying on CFU of viable bacteria to define the spiking quantity. The absence of homologous bacteria among normal human flora is demonstrated by the virtual absence of reads assigned to *T. thermophilus* in unspiked stool samples, in contrast to *S. aureus*, which was used as a standard in the experiments in **Chapter 3** (**Table** 4.1). Also, although species from the phylum *Deinococcus-Thermus* have reportedly been identified in the human gut microbiome [203], among 478 sequenced ARMORD and POETIC samples, no species other than *T. thermophilus* made up more than 3% of reads assigned to the phylum.

Table 4.1 Reads assigned to *T. thermophilus* and *S. aureus* in 15 discarded stool samples.

Sample	Total reads	*T. thermophilus* spike	*T. thermophilus* reads/million	*S. aureus* spike	*S. aureus* reads/million
A	750,874	No	0	Yes	986
B	818,108	No	0	Yes	1500
C	330,595	No	0	Yes	638
D	2,706,497	No	0	Yes	134
E	3,137,619	No	0	Yes	20
F	3,767,887	No	0	Yes	353
G	2,768,023	No	0	Yes	102
H	1,913,003	No	0	Yes	108
I	1,738,602	No	1	Yes	78
J	1,737,707	Yes	383	No	3
K	555,252	Yes	256	No	22
L	1,791,929	Yes	510	No	4
M	860,657	Yes	286	No	2
N	1,751,931	Yes	1315	No	7
O	1,547,988	Yes	965	No	47

Discards from OUH microbiology laboratory. Sequenced on Illumina MiSeq, classified with Kraken2.

4.2.2.3 Minimising and quantifying contamination

Metagenomic sequencing is more prone to problems with contamination than whole-genome sequencing, as there is no consensus or reference to compare reads to [96,204]. A major source of contamination is other samples in the same extraction or sequencing run [205], and this is likely to be particularly true of faecal samples, where DNA output from

different samples can vary by several orders of magnitude. To minimise contamination the following principles were observed in the final extraction protocol, beyond those usually observed when performing whole genome sequencing on isolates.

1. DNA was only extracted from aliquots that had remained untouched since freezing
2. To avoid samples with high and low DNA concentration being adjacent, samples were arranged in order of consistency for extraction (as liquid samples typically have lower DNA concentration) and were transferred to 96-well plates for sequencing in order of measured DNA concentration
3. Tubes/wells from different samples were never open at the same time
4. Tubes were centrifuged briefly after mixing steps to minimise aerosol formation
5. Tubes with unbound DNA were only opened in a fume hood or laboratory cabinet to minimise persistence of aerosols
6. Pipetting was done slowly and without producing bubbles to minimise aerosol formation
7. Gloves were changed regularly and after any possible contamination

Negative controls were used to quantify contamination. For each DNA extraction and sequencing batch, two negative controls were placed amongst the samples and processed identically throughout, apart from being inoculated with a loop containing no stool sample. Addition of the same *T. thermophilus* standard to samples and negative controls allowed approximate quantification of contamination in the samples, using the formula

$$\frac{1 - TtRA_{NC}}{TtRA_{NC}} * \ TtRA_{sample}$$

where $TtRA_{NC}$ and $TtRA_{sample}$ are the relative abundance of *T. thermophilus* in the negative control and sample, respectively. For example, if 99% of reads in the negative control were assigned to *T. thermophilus* and 1% to contaminants, then in a sample from the same run with 0.1% of reads assigned to *T. thermophilus*, the expected abundance of contaminating DNA would be (1 - 0.99)/0.99 x 0.001 = 10^{-5} (or 0.001%).

4.2.3 DNA library preparation and sequencing

Samples were sequenced in batches of 56-114 at the Oxford Centre for Genomics, based at the Wellcome Trust Centre for Human Genetics. Samples had automated normalisation and library preparation using either NEBNext Ultra or NEBNext Ultra II FS kits. All samples from the same batch were pooled and sequenced across 2-8 Illumina HiSeq4000 lanes with 150bp paired-end sequencing.

4.3 Bioinformatic methods

4.3.1 Quality control, processing & subsampling

SAMtools (version 1.7) was used to reformat and merge files from the same sample [190]. BBDuk (version 37.90) was used to remove Illumina adapters and quality-trim reads using a phred-score cut-off of 10 [187]. Human DNA reads were identified using the Kraken2 taxonomic classifier and removed prior to analysis (version 2.0.6) [185]. Quality control metrics were assessed with FastQC (version 0.11.7) [186] and MultiQC (version 1.5) [206]. The number of DNA reads varied substantially between sequencing runs, so for comparative analyses all samples were subsampled to a depth of 3.5 million paired reads using BBDuk [187].

4.3.2 Taxonomic classification

Taxonomic classification was performed with MetaPhlAn2 (version 2.9.20 using database v292) [188], and with Kraken2 [185] using a standard database containing Bacteria, Archaea and viruses downloaded from RefSeq, plus the human genome (built on 23/8/2018). The reasons for these choices and some general considerations on taxonomic classification are given below.

4.3.2.1 Taxonomic classification

Many taxonomic classification packages exist for use with metagenomic data, all of which aim to define the organisms present in a sample by assigning DNA reads to particular taxa [95,207]. These vary in several important respects, in particular:

i. Speed of classification. This is often a limiting factor when analysing large datasets. Approaches that required exact matches to a reference tend to be faster than those allowing some mismatches, although sometimes at the expense of classifying fewer reads. Methods that can be efficiently run in parallel across many processing cores are much faster.

ii. Organisation of reference database. A larger reference database leads to more complete and accurate classification. The size of the database may be limited by how it is represented in computer memory, with some methods having more efficient structure than others.

iii. Use of marker genes. Some methods match reads only against selected core marker genes that are used to define species, which increases the reliability of assignments but reduces sensitivity for low abundance taxa, as most DNA reads remain unclassified. Other methods attempt to classify all reads regardless of whether they

may represent accessory DNA, potentially misclassifying DNA that is horizontally transferred and present in another species in the reference database.

Two taxonomic classifiers are used in this analysis. MetaPhlAn2 was initially developed for analysing human microbiome data and uses a set of ~1.5 million clade-specific markers across ~100,000 species, mostly bacteria but also archaea, viruses and fungi [188]. To classify species as present, at least 10% of marker genes for that species must have been detected, meaning on average several hundred reads must be present from a species for it to be classified. This makes classifications relatively reliable, but means that at a sequencing depth of 3.5 million reads, the limit of detection would be a relative abundance of around 10^{-4} (0.01%).

In contrast, Kraken2 [185] uses an efficient database structure and exact k-mer based matching to rapidly classify all reads that match Bacteria, Archaea and viruses present in the NCBI RefSeq database, currently containing ~50,000 species. The main disadvantage of this method is the potential to assign the wrong host species to horizontally transferred DNA, demonstrated by the fact that running kraken on sequence data from pure isolates invariably leads to reads being assigned to a range of closely related taxa. As individual reads are assigned to species, the theoretical limit of detection is a relative abundance of $3x10^{-7}$ at a depth of 3.5 million reads. However, the problem of misclassification means that a pragmatic cut-off is usually applied before accepting a species is present; for example, one metagenomic benchmarking tool uses a cut-off of >100 reads for classification [208], which would increase the limit of detection to around $3x10^{-5}$.

4.3.2.2 Measures of diversity

As well as examining the abundance of specific taxa, it is often useful to define global measures of diversity to simplify comparisons between samples. Several diversity metrics are commonly used in microbiome studies [43,209].

The simplest and most intuitive measure is species richness, which is the number of different species present in a sample. As the number of identified species will depend on sequencing depth, samples with differing depths can only reliably be compared if using an appropriate abundance threshold, or, even more reliably, by subsampling each sample to the same depth before classification.

The Shannon diversity index is a commonly used measure of diversity and reflects both the number of species present and their evenness. It is calculated as the sum of p*ln(p) for all species, where p is proportional abundance. For example, a sample with 100 species all present at 1% abundance would have a higher diversity (Shannon = 4.6) than if one species had 50% abundance and 99 shared the remaining 50% (Shannon = 3.0). Shannon diversity is less dependent on sampling depth than richness, as it gives most weight to the predominant species, but this also means that information on scarce species from deep sequencing has minimal impact on the index.

Several other diversity measures are less commonly used, including Simpson's index, which places more weight on species evenness than the Shannon index, and some indices that attempt to extrapolate the total richness if sequencing were exhaustive, such as Chao1 and ACE [209].

In my analyses I primarily use the Shannon index in evenly subsampled sequence data as the measure of diversity. Species richness was also used for sensitivity analyses.

4.3.3 AMR gene detection

AMR gene detection in metagenomic sequence data was performed with the ARIBA software package (version 2.11.1) [210], using the CARD database and ontology (version 3.0.2) [211]. All analysis was performed in R version 3.5.0 with the ontologyIndex package (version 2.4). The reasons for these choices and some general considerations on AMR gene classification are given below.

4.3.3.1 AMR gene databases

Detection of AMR genes requires reference to a database, of which many are available [211-215]. There are important differences between databases that determine their usefulness:

i. Scope. Databases may include any AMR determinants or be limited to particular mechanisms of resistance, for example excluding mutational resistance.
ii. Completeness. The number and variety of included AMR genes varies significantly between databases.
iii. Curation. Several databases are actively maintained and updated, but some have ceased to be updated or contain redundant entries and inconsistent annotation.
iv. Structure. Databases may incorporate a hierarchical organising structure that is useful for analysis, for example to assign a read mapping to several TEM genes to the parent category of 'TEM beta-lactamase'.

My analysis uses the Comprehensive Antibiotic Resistance Database (CARD), which is well curated, regularly updated, and covers all mechanisms of resistance. A complete ontology of AMR has been created for the database, including a hierarchy of resistance genes.

4.3.3.2 AMR gene classification

In the absence of selective enrichment, AMR genes typically account for fewer than 1% of DNA reads in faecal metagenomes [118] (the motivation behind my work in **Chapter 3**). Using these to characterise the faecal resistome is difficult for several reasons:

i. AMR genes may be closely homologous to non-AMR genes present in the sample, causing false positive assignments. This causes a particular problems in identifying mutational resistance in housekeeping genes that are present in many different bacteria, such as gyrA and ribosomal RNA.
ii. When an AMR gene has many similar alleles in the database, then DNA reads from this gene will map to multiple references and it may be difficult to distinguish which is present in the sample. Yet in some cases differences of a single nucleotide have important functional consequences, for example conferring ESBL phenotype on TEM beta-lactamases [216]. If the gene is scarce then there may only be enough reads to make an approximate assignment.
iii. There may be multiple similar AMR genes present in a sample, creating problems in reconstructing minor variants, or distinguishing minor variants from sequencing errors.

Unlike taxonomic classification, where some tools are widely accepted, no standard methods exist to classify AMR genes in metagenomic data. Several tools are available that

can assign individual metagenomic DNA reads to a catalogue of AMR genes [217-220], but the use of read-by-read classification makes clear assignment of AMR gene alleles difficult. Some tools exist to fully characterise AMR alleles in short read sequence data from isolates [221-223], but these were not designed for use with metagenomic data. Recently, some tools have been published that reconstruct complete alleles from metagenomic sequence data [215,224], but none have gained widespread acceptance.

The primary tool used in this analysis is ARIBA [210], which was designed to identify AMR gene alleles in short read sequence data from isolates but which can also be used with metagenomic sequence data. Starting with a user-selected AMR database, this converts the database into clusters of genes with significant homology. DNA reads from a sample are then assigned to clusters if they have sufficient homology. Following this, the reads assigned to each cluster are assembled into a continuous sequence, which is assigned to the closest matching allele from that cluster. A major advantage of ARIBA over approaches that classify each read independently is that the output is detection of a specific AMR gene allele, which is reported along with a corresponding read count, gene coverage, and percentage identity. This allows the removal of results with low gene coverage or identity, which may represent false assignments. Another advantage is that each allele is reported with its database accession number, which can be used for hierarchical grouping, for example to select CTX-M-15, all CTX-M genes, all class A beta-lactamases, or all beta-lactamases of any kind. However, a potential problem of using ARIBA with metagenomic data is that genes with low read counts may fail to assemble and so would not be reported.

4.3.3.3 Assessing the performance of ARIBA on metagenomic data

To assess the performance of ARIBA with metagenomic sequencing data I used the data from stool samples spiked with CPE from the experiments in **Chapter 3**, which included enriched and unenriched samples as well as negative controls. I focussed on the NDM-1, CTX-M-15 and KPC-2 genes, which were known to be present in the spike organism, but should not be present in the stool samples since these were culture negative for third-generation cephalosporin resistant *Enterobacteriaceae*. For 80 available samples, the number of reads assigned to NDM-1, CTX-M-15 and KPC-2 by ARIBA was compared to those mapping to these genes with BBMap [187] (**Figure** 4.1). Detection of the genes was concordant in 206/240 (86%) comparisons; 131/240 (55%) were negative for the gene and 75/240 (31%) positive for the gene. In 34/240 (14%) comparisons a gene was not detected by ARIBA but was detected by mapping, usually at low reads numbers. However, in 12 of these cases the detected gene should not have been present in the sample, possibly representing laboratory contamination or misclassification by mapping. The number of reads assigned by ARIBA correlated closely with mapping (Pearson's r = 0.98 in samples where either or both found a gene). ARIBA identified the correct allele in 67/68 (99%) of cases where >50 paired reads were detected by mapping, but was often unable to assemble genes with <25 paired reads, meaning that their presence was not reported. Because of the advantages of ARIBA outlined above in identifying alleles and avoiding false assignments in samples where the genes present are unknown, this method was used for subsequent analyses.

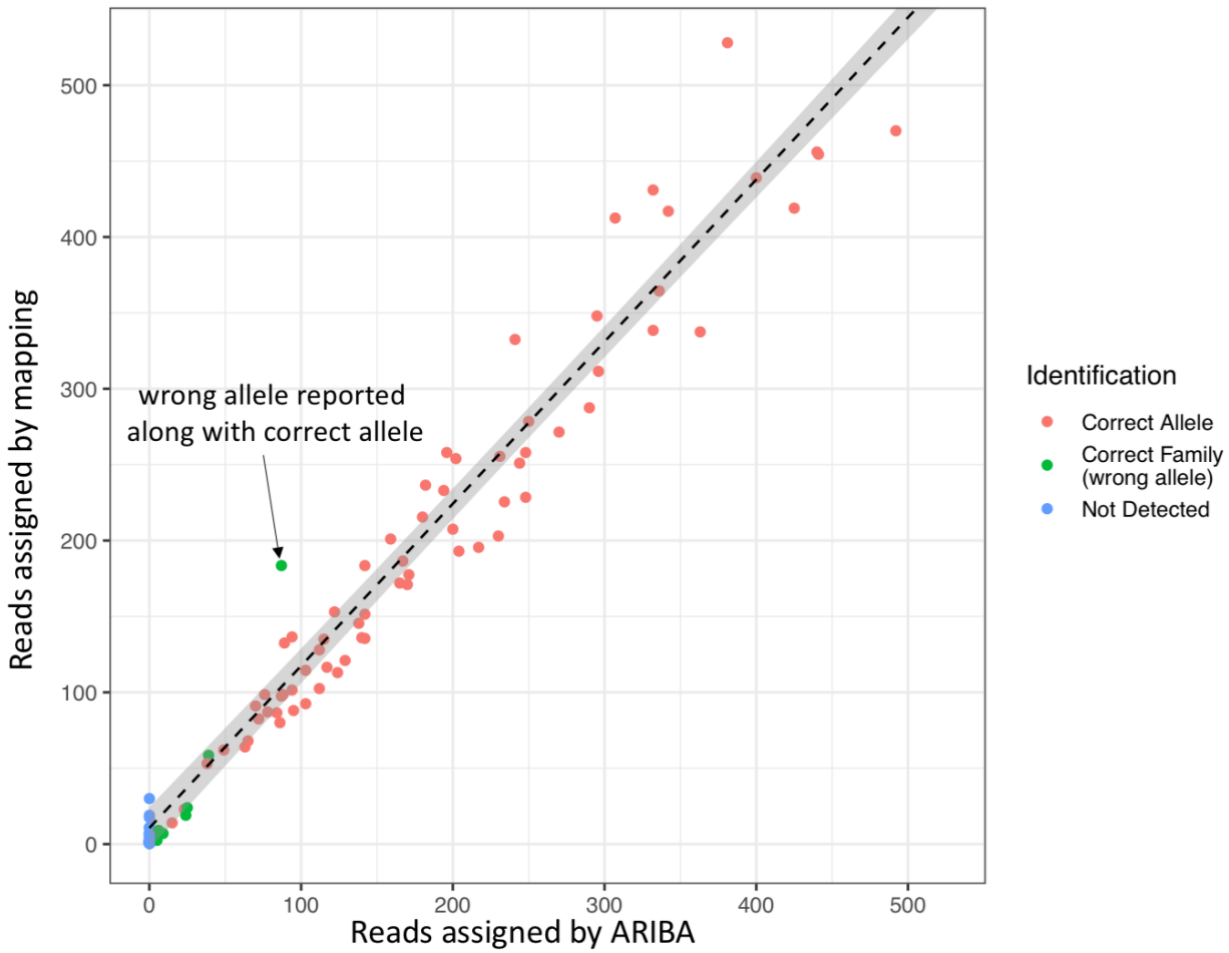

Figure 4.1 Paired reads assigned to known AMR genes by ARIBA and mapping

Composite of results for NDM-1, KPC-2 and CTX-M-15 genes. Line represents best fit linear model with 95% confidence interval.

4.4 Statistical methods

All statistical analysis was performed using R (version 3.5.0) and RStudio (version 1.1.383).

Detailed statistical methods are provided in **Chapters 5 and 6**.

4.5 Sample quality control

4.5.1 Human DNA content

Of 504 samples from ARMORD and POETIC sent for sequencing, 478 (95%) were successfully sequenced, defined as having ≥3.5 million non-human reads available for analysis after quality filtering. This was the depth chosen for even subsampling, selected after sequencing to maximise the number of samples available whilst maintaining adequate depth for analysis. The DNA output per gram of sample varied 30,000 fold, from 17ng/g to 510,000ng/g, and samples with low DNA output were more likely to fail sequencing. Variation in stool consistency explained some of this variation in DNA output, with more liquid samples tending to have lower DNA output (**Figure** 4.2).

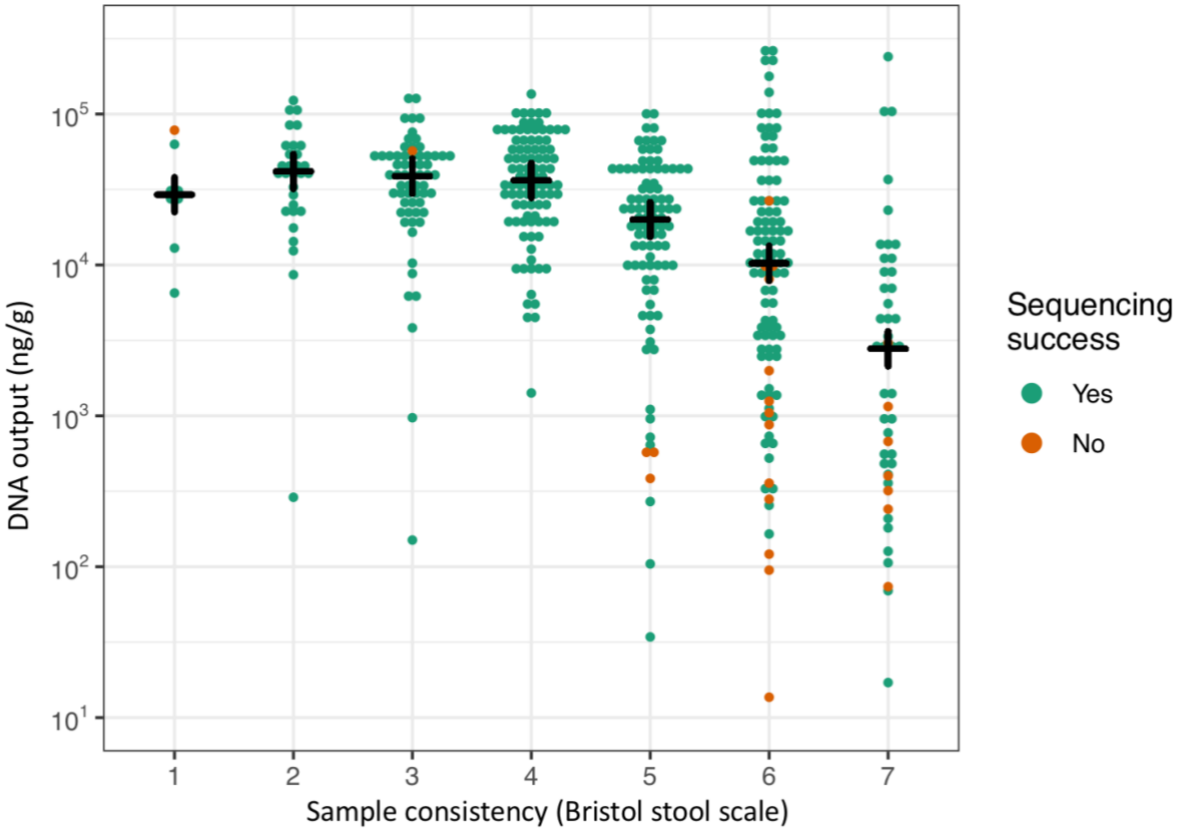

Figure 4.2 DNA output by sample consistency

Black crosses represent median output.

Failed samples also typically had high human DNA content, with a median human DNA abundance of 76% of reads. Among successfully sequenced samples, median human DNA abundance was 0.3%, and in only 17 (4%) samples was it >10% (**Figure** 4.3).

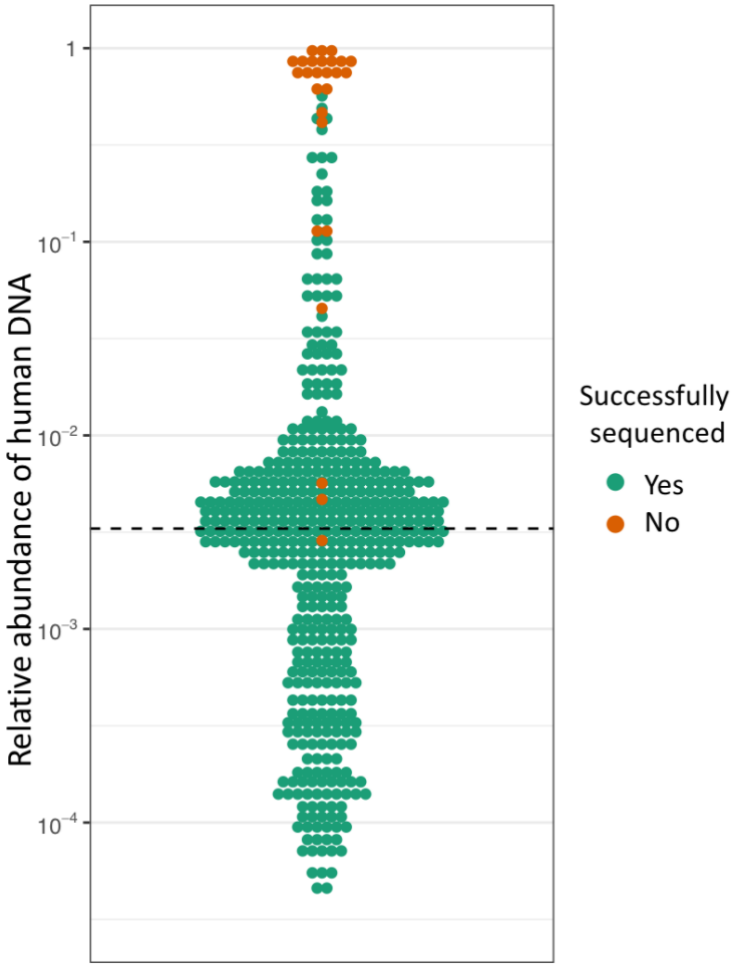

Figure 4.3 Abundance of human DNA in sequenced samples
Line shows median of successfully sequenced samples.

4.5.2 Sample contamination

Sequencing of negative controls indicated that the level of laboratory contamination was generally low, but in two cases >10% of classified reads in negative controls were assigned to taxa other than *T. thermophilus* (**Table** 4.2). In both cases the other taxa consisted of typical faecal flora, indicating probable sample-to-sample contamination. In the case of

negative control 2B, the pattern of faecal flora closely matched that from a sample in an adjacent well on the sequencing plate. This lead to the additional precaution of ordering samples by DNA concentration in 96 well plates prior to sequencing, to avoid low- and high-concentration samples being adjacent. All subsequent negative controls had minimal contamination.

Table 4.2 *T. thermophilus* abundance in negative controls.

Sequencing run	Negative control	% classified reads assigned to *T. thermophilus*
1	A (single)	**84.1**
2	A	99.2
2	B	**26.1**
3	A	99.5
3	B	96.8
4	A	99.4
4	B	97.0
5	A	99.3
5	B	99.6

Estimating the relative abundance of contamination in samples suggested that this would generally have little effect on measures of the microbiome or resistome, with a median estimated abundance of contaminants of 1.1×10^{-4} and 3.4×10^{-5} using the more- and less-contaminated controls from the same batch, respectively (**Figure** 4.4). However, one low DNA sample on plate 2 would have been dominated by contamination if it had received the same amount of contaminating DNA as negative control 2B (outlier in **Figure** 4.4). Also, the negative controls from sequencing run 2 suggest that when contamination occurs it is very variable from sample to sample, making it difficult to reliably estimate contamination without estimating this variability, which would have required many more negative controls per run.

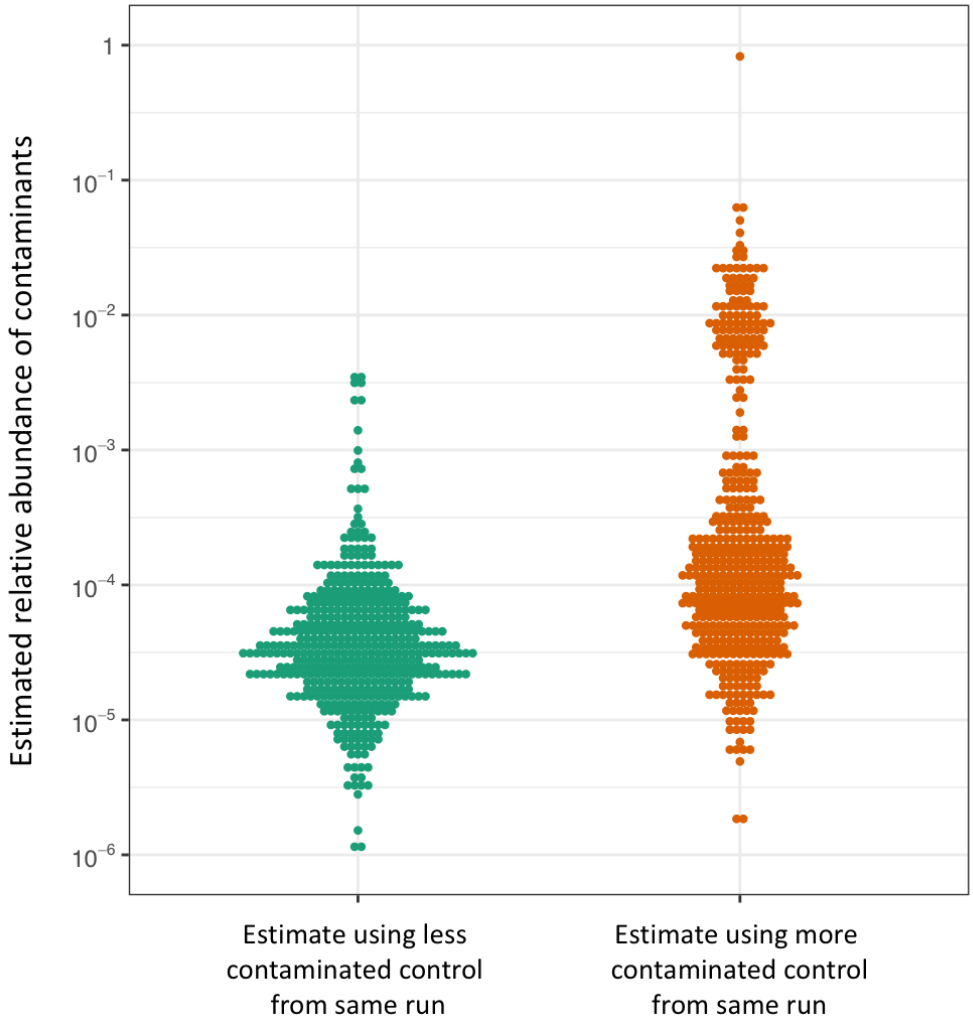

Figure 4.4 Estimated contamination in sequenced samples

4.5.3 Similarity of microbiome measurement in replicates

Technical replicates were not routinely performed, but some samples were sequenced twice because the initial attempt failed. These repeat samples allowed assessment of variability related to extraction and sequencing, although they were not typical as they tended to have a lower yield of poorer quality DNA than samples that did not require repeat sequencing, and often had a high abundance of *E. faecium*. Principal component analysis (PCA) was performed on all sample pairs where both had sufficient depth for crude taxonomic analysis, using a cut-off of >10,000 reads. Kraken2 was used to classify the log abundance of

predominant species, defined as those whose sum relative abundance was >5% across all samples. This was imputed as 10^{-4} for samples where measured abundance was ≤10^{-4}. Eight sample pairs were available for analysis, demonstrating that microbiome profiles were similar on repeat sequencing, with clear separation between different samples (**Figure 4.5**).

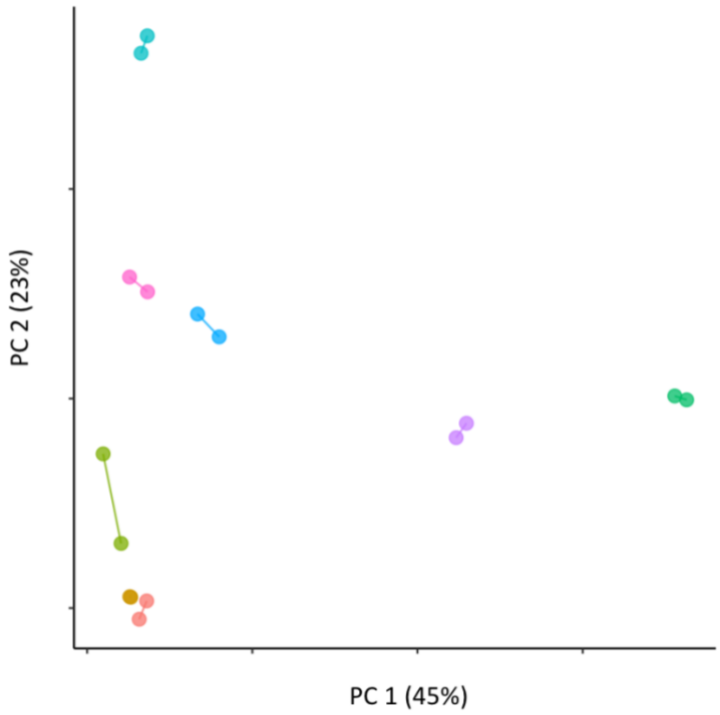

Figure 4.5 **PCA of microbiome profile with repeat sequencing of eight samples**
Coloured by sample

4.5.4 Effect of time to freezing on microbiome diversity

Among ARMORD samples, the median delay before freezing was 6 hours for those stored at ambient temperature and 24 hours for those stored at 4˚C. In POETIC samples, the median delay before freezing was 59 hours. The median number of species detected per sample was 83, and there was no evidence of an effect of time to freezing on species richness (change of -0.18 species/hour (95% CI -0.72 to +0.36) at ambient temperature in ARMORD, -0.11 species/hour (95% CI -0.29 to +0.08) at 4˚C in ARMORD, and -0.10 species/hour (95% CI -0.26 to +0.06) at ambient temperature in POETIC, all p >0.2 by univariate linear regression).

5 Cross-sectional analysis

5.1 Introduction

Most data on the effects of antimicrobials on the gut microbiome and resistome come from studies with longitudinal sampling, with only a few studies using cross-sectional sampling. Just three cross-sectional studies using culture-independent methods were identified in my review in **Chapter 2** [125,163,164], and only one of these directly compared different antimicrobial exposures [163]. However, cross-sectional sampling could potentially overcome significant limitations of other study designs. The studies that used longitudinal sampling were mostly small, having on average 45 participants, which may explain why most examined a single antimicrobial exposure. To provide reliable comparisons between several different exposures would require larger studies, but both recruiting volunteers to interventional studies and identifying patients at the correct time for longitudinal sampling is difficult on a large scale. In contrast, cross-sectional sampling is far simpler to do with larger numbers, as any patient that can provide a stool sample and has a record of antimicrobial exposure can potentially be recruited, which includes a substantial fraction of all hospital inpatients. This could provide a large, heterogeneous sampling frame, allowing comparison of the effects of many different antimicrobials simultaneously.

There are three significant obstacles to using cross-sectional data in practice. Firstly, because there is substantial variation in the microbiome between individuals, cross-sectional analyses, where exposed individuals are compared with different, unexposed, individuals, will have larger error in their effect estimates compared with longitudinal analyses of a given sample size. Whether or not this can be outweighed by the increased

ease of recruitment is difficult to estimate from published studies, and can only be answered with real-life data by comparing estimates from cross-sectional and longitudinal studies.

Secondly, observational data rely on antimicrobial use that occurs independently of the study, so there is no control over exposure. This means that only agents that are used reasonably often in normal clinical practice can be studied. More problematically, the antimicrobial use that does occur may be very difficult to classify, because of variation in treatment duration, time since exposure, and exposure to multiple different agents. A 2015 point prevalence survey of hospital antimicrobial prescribing across 53 countries found that 34% of inpatients were currently on antimicrobials, with an average of 1.4 agents per recipient [225]. Given the short duration of most prescriptions, this implies that many more patients would have had recent exposure, often to more than one agent. To allow for this in analysis, accurate data on antimicrobial exposure are essential, but may be very difficult to collate from paper records and patient interview. The increasing availability of Electronic Patient Records (EPR) makes collection of this data from large numbers of patients much more practicable and could greatly improve cross-sectional studies. Even with complete data on antimicrobial use however, classifying exposure in a way that allows robust, generalisable comparisons is a major difficulty.

Thirdly, confounding is a particular problem in cross-sectional studies, as individuals who have received a particular antimicrobial are likely to differ from those who have received different (or no) antimicrobials, in ways that could affect their microbiome and resistome. Adjustment for potential confounding aims to largely remove these biases, to the point

where they are minimal in comparison to the effect of the antimicrobials themselves. Again, the increasing availability of EPR makes doing this in large studies much more practicable, as it is easy to collect high-resolution data on potential confounding factors related to acute illness, co-morbidity and recent or concurrent use of other antimicrobials. An advantage of studying the effect of antimicrobials, as opposed to other exposures, is that their impact on the microbiome is often profound, meaning that important differences between them may be readily detectable above any residual biases in observational data.

In this chapter I analyse cross-sectional data from ARMORD participants, from whom hospital EPR data are available, to describe the impact of different antimicrobials on metagenomic measures of the gut microbiome and resistome. I give particular consideration to how antimicrobial exposure might be classified in observational studies, and how confounding might be controlled for in a multiple regression model.

5.2 Methods

5.2.1 Defining a measure of antimicrobial exposure

To analyse an observational dataset containing exposures to many different antimicrobials, each individual must have a quantified exposure to each one, both to estimate its effect and adjust for it when estimating the effects of other antimicrobials. Different routes of administration of the same drug should be treated separately, as they may have different effects on the gut flora, identification of which could be useful.

Comparison between drugs is greatly aided if exposure to each antimicrobial is defined as a single measure on a common scale, for example ranging from 0 (no exposure) to 1 (full

exposure), even though this may simplify reality. This is partly for statistical reasons, as there may be dozens of different drugs and using more than one measure for each multiplies the number of variables in the model, increasing variability in estimates and potential for false-positives (Type I error). There is also a strong practical reason for having a single exposure measure if the aim is to produce usable quantitative comparisons between exposures, as it allows a single metric per drug/route for each outcome (microbiome diversity, selection of vancomycin resistant enterococci (VRE), etc.).

However, defining such a single measure of exposure is not straightforward. There are two important factors to consider: time since most recent exposure and cumulative amount of exposure, but there are many different ways these can be measured and combined. Unfortunately, previous microbiome studies, discussed in **Chapter 2**, provide little data to inform this, as few had frequent enough sampling to define the dynamics of recovery and none compared the effects of different durations of the same exposure. Because of this, before comparing the effects of specific antimicrobials in the cross-sectional study I used the data to derive a suitable single measure of exposure.

If the full effects of an antibiotic occurred after a single dose, then the amount of exposure would be unimportant and only the time since exposure would matter; conversely, if the impact accumulated slowly and the microbiome/resistome took a long time to recover then amount of exposure would be the most important consideration. Five different exposure measures were considered, each of which requires some arbitrarily chosen window period or half-life. These are described below and illustrated with a hypothetical inpatient with typical antimicrobial exposures.

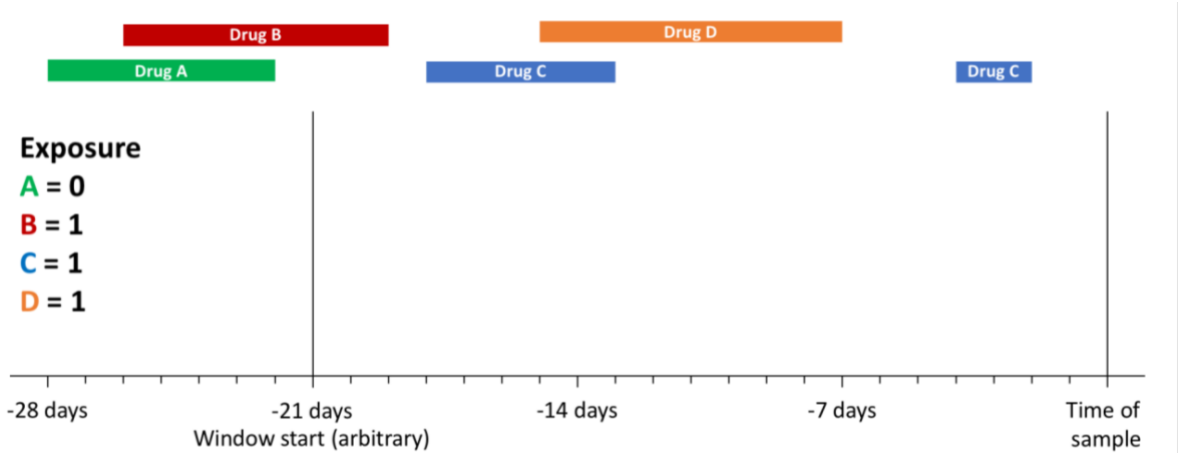

Figure 5.1 Measure 1 - Qualitative exposure in window

Figure 5.1 illustrates the simplest "qualitative" measure of antimicrobial exposure, namely exposure equals 1 if the patients is exposed to the drug in the arbitrary window at any time. This measure is simple and intuitive, but incorporates no effect of cumulative exposure and no effect of recency in the window, and is based on a sharp cut-off that is biologically implausible.

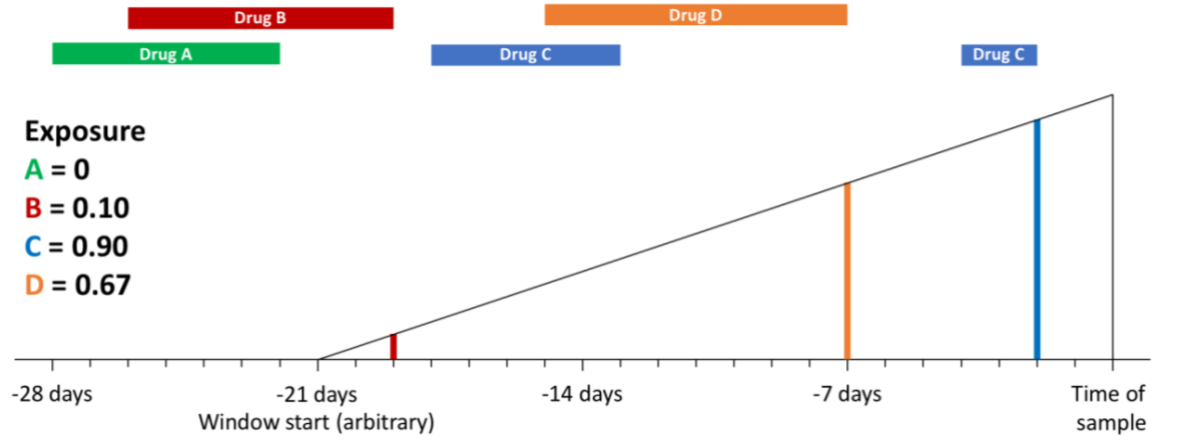

Figure 5.2 Measure 2 - Linear decay

Figure 5.2 illustrates one way that recency of exposure can be incorporated into a measure; here, exposure reduces linearly from time of sample until start of window. This measure is also fairly simple and intuitive, but incorporates no effect of cumulative exposure and makes a linear assumption about the decline in effect from the sample to the most recent previous exposure.

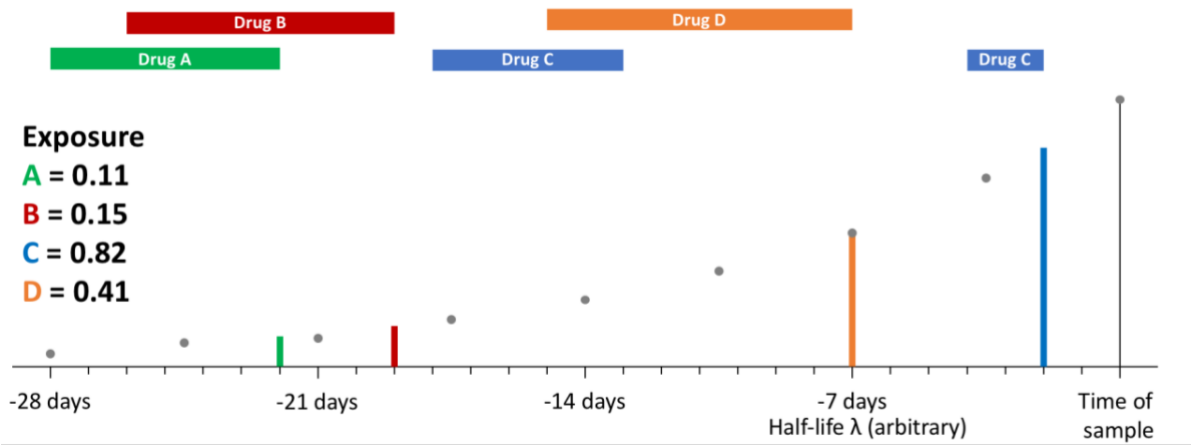

Figure 5.3 Measure 3 - Exponential decay

Figure 5.3 illustrates a similar measure, but here exposure reduces exponentially from time of sample (as $2^{-t/\lambda}$, where λ is half-life). This measure is also fairly simple and has no sharp cut-off, but does make an arbitrary assumption about λ, and again no effect of cumulative exposure is incorporated.

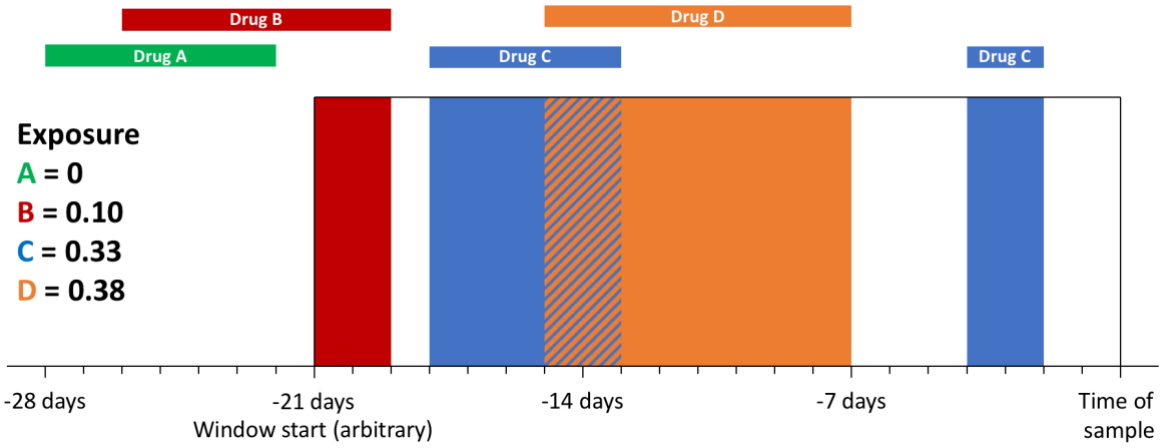

Figure 5.4 Measure 4 – Equal cumulative exposure in window

Figure 5.4 illustrates a completely different approach, focussing on the cumulative exposure but defining exposure proportional to total exposure in the window. This is also simple and incorporates cumulative exposure, but now there is no effect of recency in the window and the sharp cut-off at whatever arbitrary threshold is chosen for the window is biologically implausible.

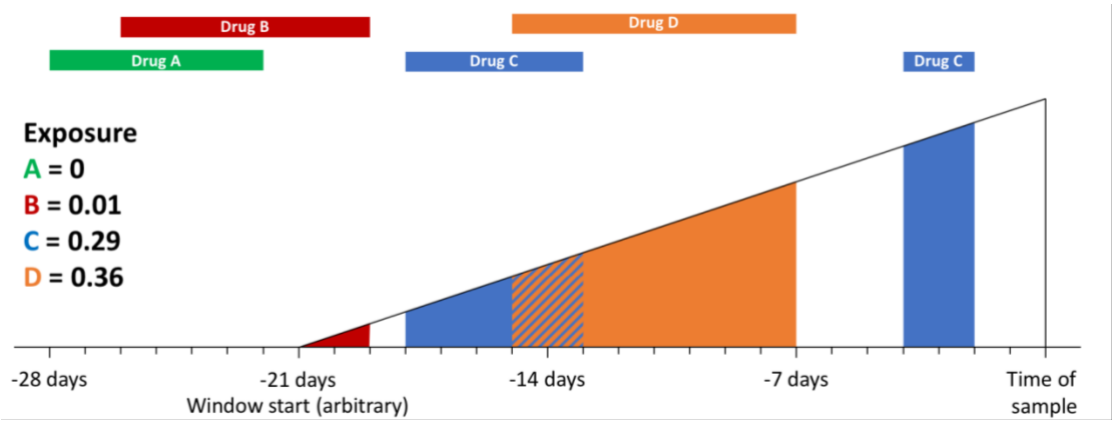

Figure 5.5 Measure 5 - Fading cumulative exposure in window

Figure 5.5 aims to incorporate all these components into one measure, by defining exposure proportional to total exposure in window with linear weighting towards recent exposure. It incorporates recency and cumulative exposure, but is less intuitive.

To assess which exposure measure to use for the main analysis, the five measures were compared in multiple regression models of the cross-sectional data using Shannon diversity as an outcome. All antimicrobials (split by route of administration) received by ≥10 participants in the year prior to sampling were included as exposure variables, with no other covariates in the model. The specific effects of different drugs were not compared during derivation of the exposure measure, only an overall metric of how well the models fitted the data. The Akaike Information Criterion (AIC) was used to compare the goodness of fit of the models [226], with lower AIC indicating better fit. Initially the five exposure measures were compared using windows/half-lives of 7, 14, 21, 28, 42, 90, 180 and 365 days. Combinations of exposure measures were subsequently compared to explore whether including long and short term metrics might produce an improved exposure measure.

5.2.2 Multiple regression

Using the best exposure measure derived as described above, multiple normal linear regression was then used to estimate the effects of specific antimicrobials on the gut microbiome and resistome. Shannon diversity was the primary outcome used for comparing exposures. Other outcomes analysed were the relative abundance of specific bacterial taxa and specific classes of AMR genes, both analysed on a log scale for approximate normality. Where a particular taxon or gene was not detected, its presence was conservatively imputed at the lower limit of detection of 10^{-6} for taxa or 10^{-5} for AMR genes. Normality of outcome variables was assessed by visual inspection of histograms and inverse-normal plots.

Covariates used in the final model to adjust for confounding are shown below. For some participants, mostly healthy volunteers, EPR data were missing, in which case values were imputed as indicated.

- Age (years)
- Sex: Male or female (reference)
- Participant category: Healthy (reference), Medical or HSCT
- Days since start of chemotherapy, truncated at 14 (0 for non-HSCT participants and HSCT participants whose first sample was taken before chemotherapy started)
- Maximum Charlson comorbidity score in the year before sampling, imputed as 0 for 85/225 (38%) participants
- Maximum NEWS2 track and trigger score in the 14 days before sampling, imputed as 0 for 52/225 (23%) participants

- Maximum C-reactive protein (CRP) in the 14 days before sampling, imputed as 0.2 mg/dL (lower limit of the reference range) for 43/225 (19%) participants
- Maximum white cell count (WCC) in the 14 days before sampling, imputed as 7.5 $x10^9$/L (middle of reference range) for 36/225 (16%) participants

Data were collected on smoking, alcohol consumption, diet, recent travel, pet ownership and use of a proton pump inhibitor, but these were not included in the model presented here as they were not significantly associated with Shannon diversity in multivariable models after correction for multiple testing, and their inclusion as covariates did not materially affect other estimates.

All antimicrobial exposures which were observed in >2 participants were included in the model to allow for confounding, but comparisons were restricted to those with >5 participants, as results from exposures with 3-5 observations were considered unreliable.

5.3 Results

5.3.1 Participants

Between July 2015 and November 2018, a total of 343 participants were recruited to the ARMORD study. However no sample was obtained from 111 and a further 7 had samples that failed sequencing, leaving 225 participants in the cross-sectional analysis (**Figure** 5.6 and **Figure** 5.7).

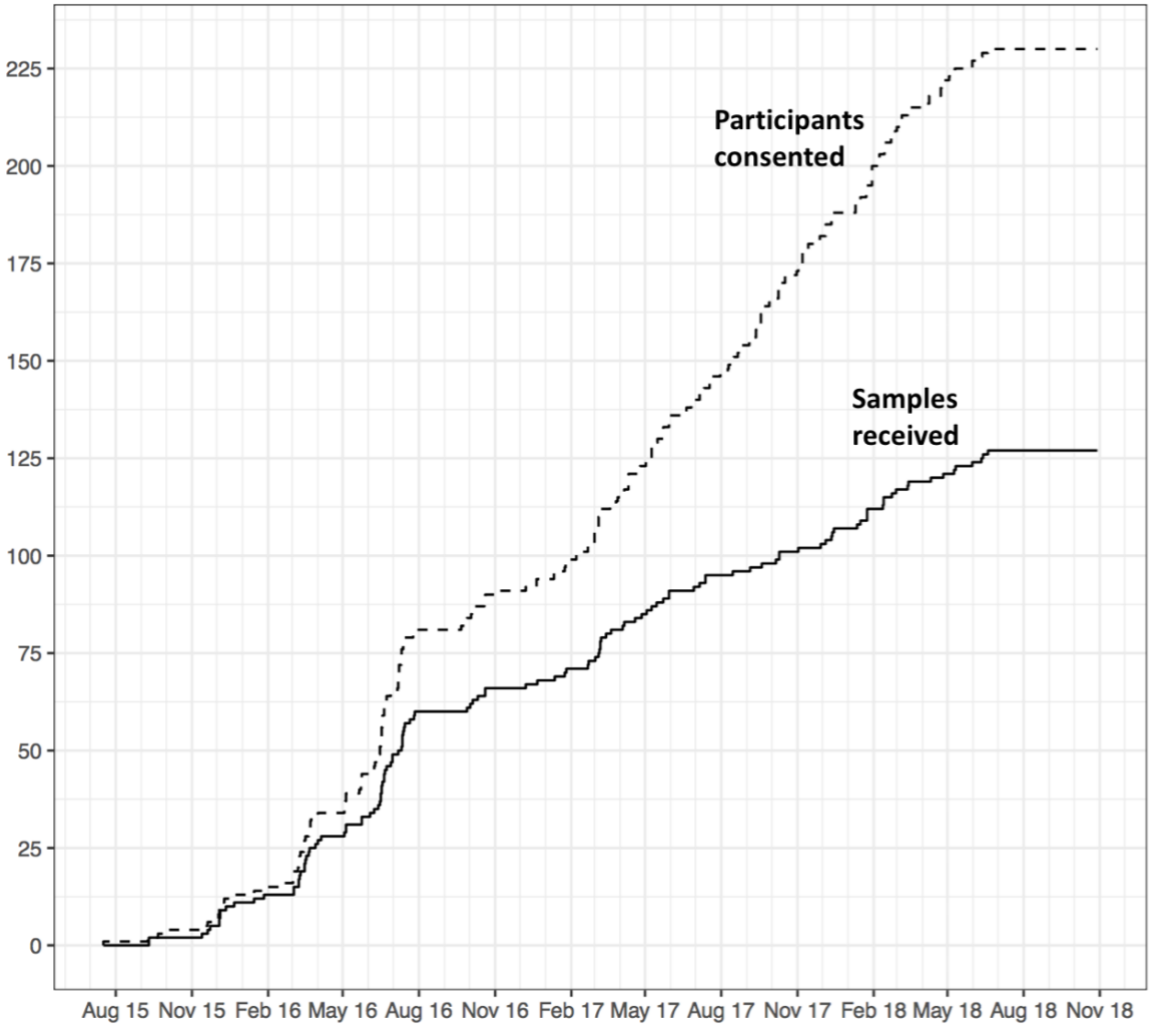

Figure 5.6 Recruitment to the cross-sectional stratum of ARMORD

Healthy volunteers and general medical patients (all provided at most one sample)

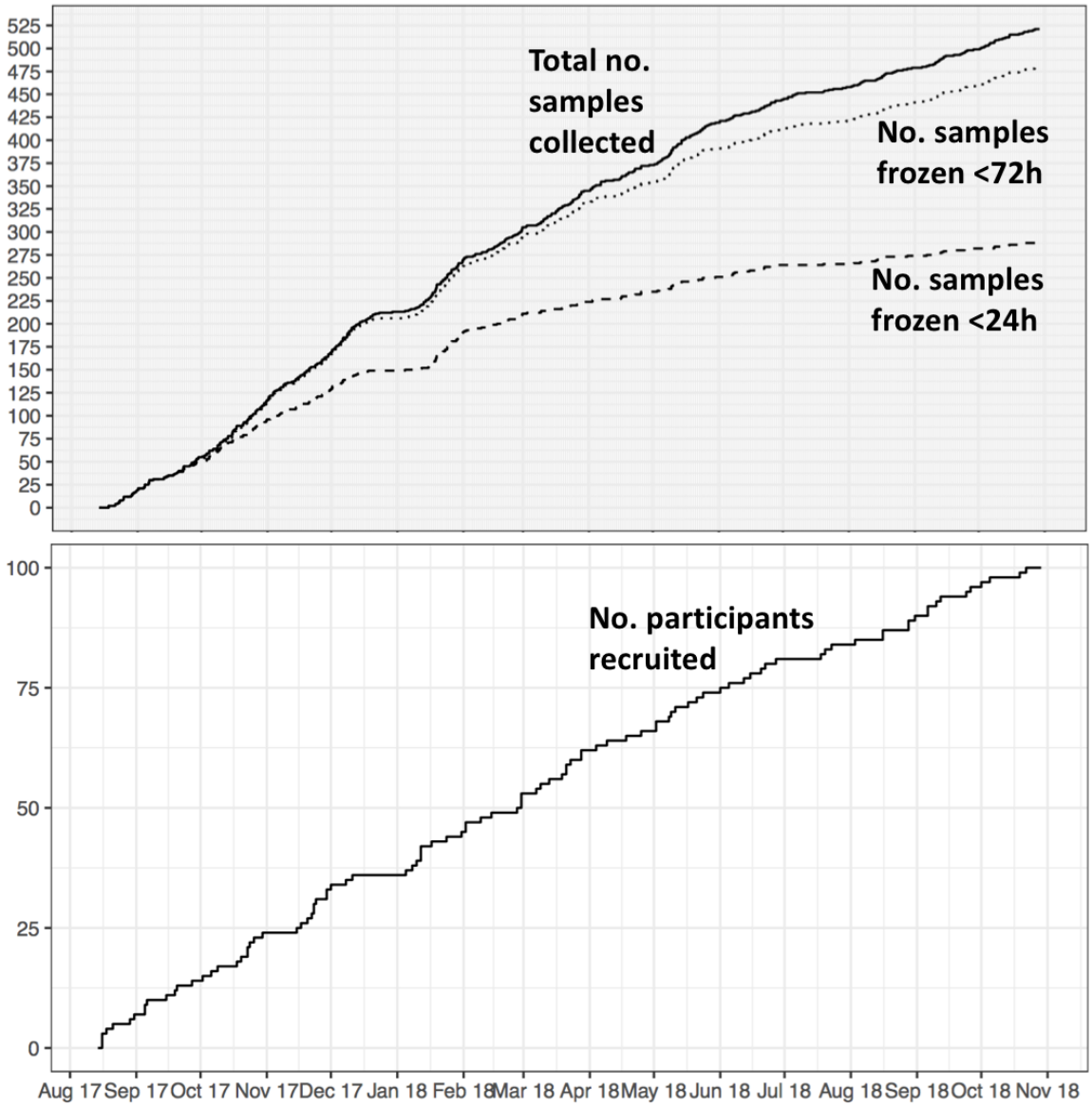

Figure 5.7 Recruitment to the longitudinal stratum of ARMORD (HSCT patients)

Details of participants included in the cross-sectional analysis are shown in **Table** 5.1. Thirty-three (15%) were healthy volunteers. One hundred and one (45%) were HSCT patients, recruited from the haematology ward at the Churchill Hospital, Oxford (of whom 80 provided longitudinal samples used for the analysis in **Chapter 6**). Ninety-one (40%) participants were patients at the John Radcliffe Hospital, Oxford, of whom 76 were recruited from medical wards, 10 from surgical wards, and 5 from the outpatient department. Participant characteristics differed by source of recruitment, with healthy

volunteers on average younger, more likely to be female, and unlikely to have recent antibiotic exposure. In contrast the large majority of hospital patents had received antibiotics in the past year, and half were receiving antibiotics at the time of sampling. Recent exposure to multiple antibiotics was common, with 99/148 (67%) of participants who had had antibiotic exposure in the past month having received >1 type of exposure.

Characteristic	Healthy volunteers	General medicine	Haematology	All
Participants N (%)	33 (15%)	91 (40%)	101 (45%)	225 (100%)
Mean age (years) (range)	41 (18–78)	73 (22–96)	58 (24–75)	61 (18–96)
Female N (%)	26 (79%)	38 (42%)	41 (41%)	105 (47%)
Antibiotics at sampling N (%)	0 (0%)	55 (60%)	42 (42%)	97 (43%)
Antibiotics in past month N (%)	3 (9%)	81 (89%)	64 (63%)	148 (66%)
Antibiotics in past year N (%)	7 (21%)	87 (96%)	97 (96%)	191 (85%)
Max NEWS2 past 14 days Median (IQR)	0 (0–0)	5 (2–8)	3 (2–4)	3 (1–5)
Max Charlson past year Median (IQR)	0 (0–0)	4 (0–13)	0 (0–8)	0 (0–8)
Max CRP past 14 days Median (IQR)	0.2 (0.2–0.2)	63 (22–163)	10 (3–69)	21 (2.4–81)
Max WCC past 14 days Median (IQR)	7.5 (7.5–7.5)	11.5 (8.4-14.4)	7.6 (5.8–10.9)	8.4 (7.4–12.4)
Conditioning day at sampling, Median (IQR)	0 (0–0)	0 (0–0)	3.0 (1.4–7.2)	0 (0–2.8)

Table 5.1 Characteristics of cross-sectional ARMORD participants

Includes imputed values, as described in section 5.2.2 (note most healthy volunteers had some values imputed)

5.3.2 Effect of any antibiotic exposure on microbiome diversity

Shannon diversity, H, in the cross-sectional samples approximated a negatively skewed normal distribution, with some samples having very low diversity (**Figure** 5.8). Although transformation as H^2 or H^3 increased the normality of this distribution, this made no qualitative difference to subsequent models, so no transformation was used for ease of interpretation of model estimates. Species richness, used for some sensitivity analyses, was more normally distributed.

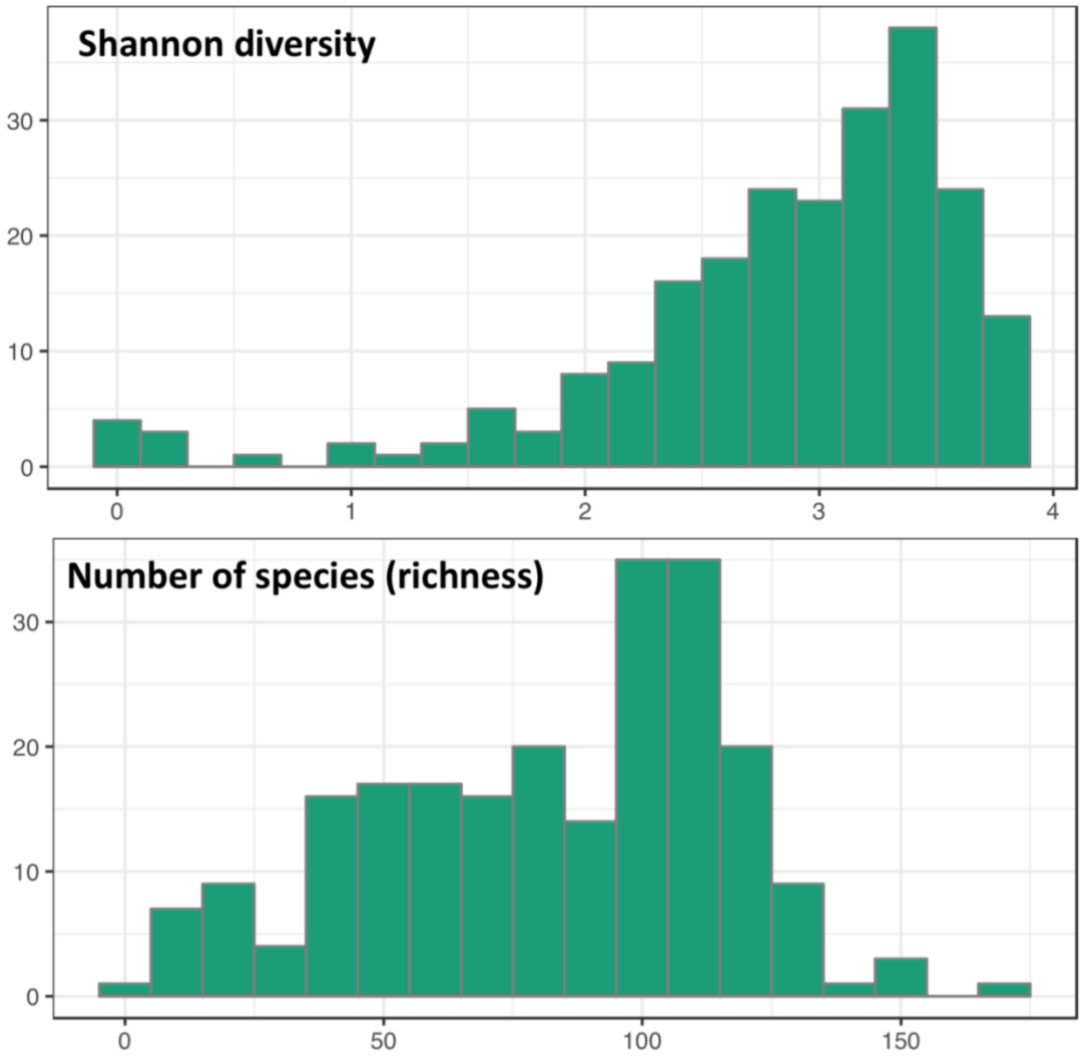

Figure 5.8 Diversity in cross-sectional samples

An initial analysis examined the effects of any antibiotic use on the gut microbiome, combining all antibiotics and routes of administration (excluding antivirals and antifungals). Current use of any antibiotic and use within the past month were significantly associated with lower diversity in univariable analysis compared with those who had not taken antibiotics in the last year (**Figure** 5.9 and **Figure** 5.10). There was no evidence of differing diversity between those with most recent use in the past 1-12 months and more than a year ago. To explore whether this reduction in diversity could be related to confounding with other participant characteristics, the same antibiotic exposure categories were used in a multivariable model that included adjustment for age, sex, and patient category (healthy volunteer (reference), general medical or HSCT). In this model, current antibiotic exposure was significantly associated with lower diversity compared to no use in the past year (-0.78, 95% CI -1.20 to -0.36, p = 0.0004), with a similar trend for use in the past month (-0.41, 95% CI -0.84 to +0.02, p = 0.06), and no evidence of an effect of use within the past 1-12 months (-0.15, 95% CI -0.58 to +0.29, p = 0.51).

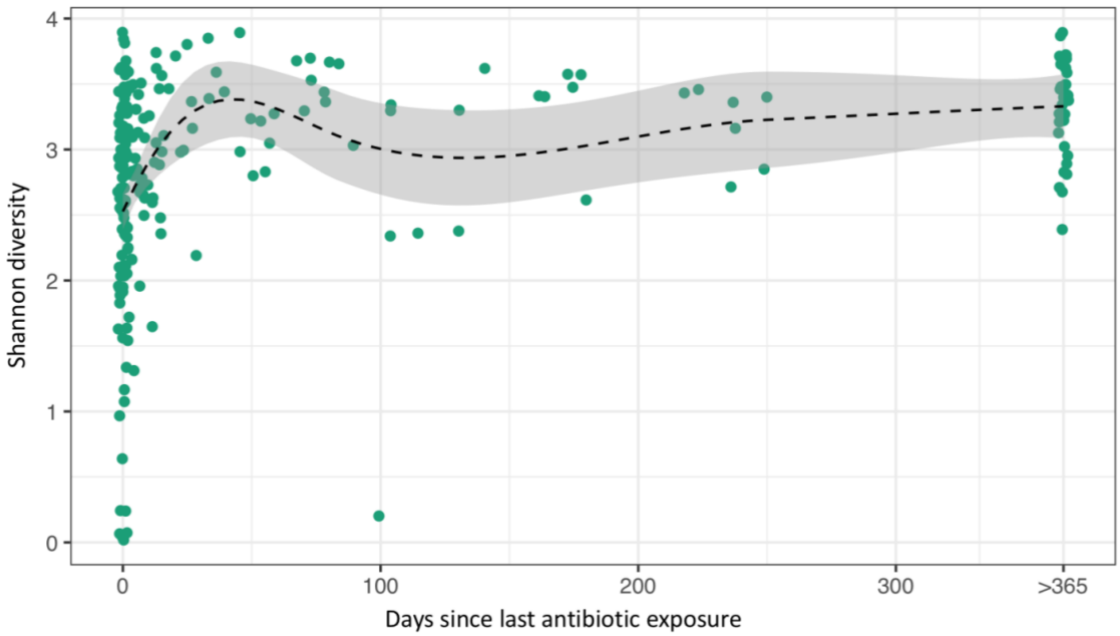

Figure 5.9 Most recent antibiotic use and gut microbiome diversity

Line of fit is locally estimated scatterplot smoothing, grey area is 95% confidence interval

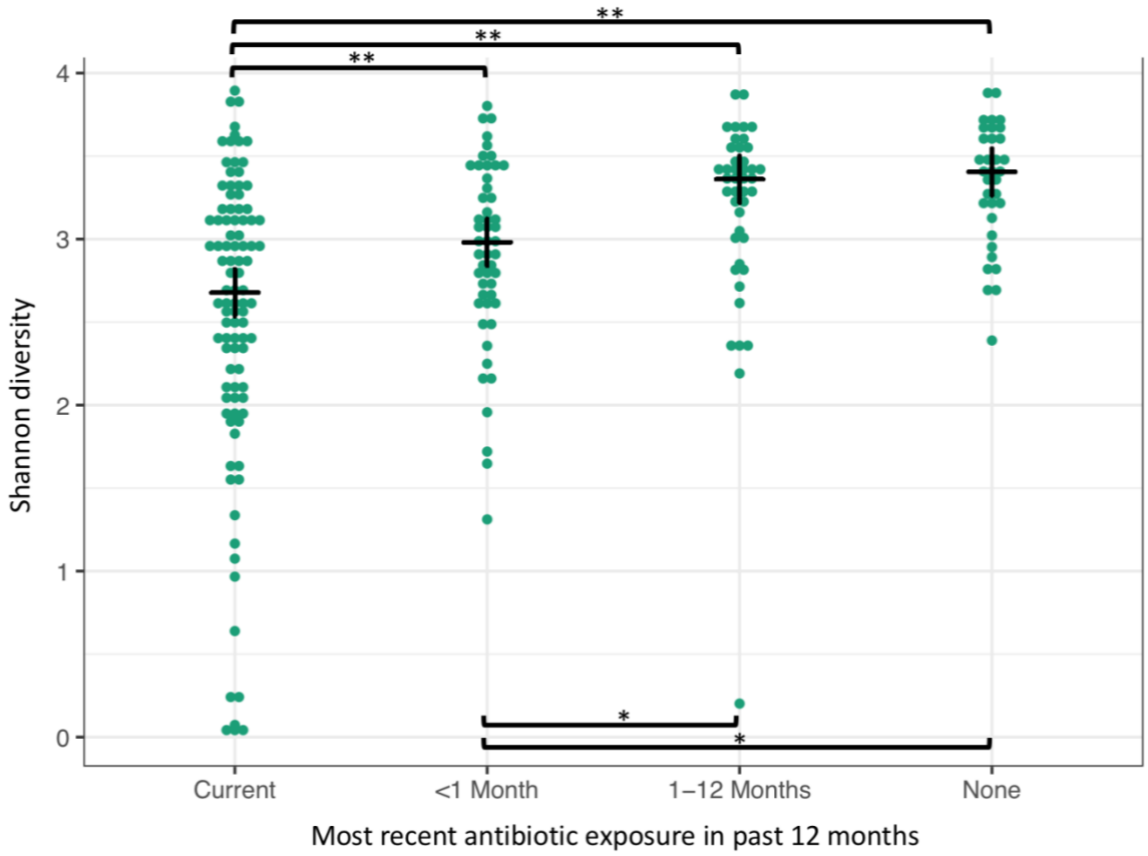

Figure 5.10 Most recent antibiotic use by category and gut microbiome diversity

Crosses indicate median values, ** = p <0.002, * = p < 0.05 by Welch's two sample t-test.

It was not possible to assess the effects of specific antibiotic exposures using similar mutually exclusive categories in univariable models, as few participants had had exposure to just one antimicrobial. Although 49 (22%) participants had had a single type of exposure in the past 28 days, no single exposure was shared by more than 11 (5%) participants, and only oral co-amoxiclav and oral co-trimoxazole were shared by more than 5 (2%). Assessing the effects of specific antimicrobial exposures required the use of an exposure measure that could be used in multiple regression.

5.3.3 Derivation of a measure of antimicrobial exposure

Eighteen different antimicrobials had been received by ≥10 individuals in the past year, which were used to assess the performance of the different exposure measures as described in the Methods above. Forty regression models were compared, each using a different type of exposure measure and window/half-life (5 types of measure with 8 different window/half-lives ranging from 7 to 365 days). Each model had 18 exposure variables, one for each antimicrobial, with Shannon diversity as the outcome variable. The goodness of fit of these regression models was compared using AIC in order to identify the optimal exposure measure to use in subsequent analysis, with lower AIC values indicating models that better fit the data. The results here do not include adjustment for potential confounders, but identical results (in terms of ordering of the different metrics) are obtained with adjustment for all covariates listed in section **5.2.2**.

The five measures that best fitted the data all included cumulative measures of exposure over the past 1-4 weeks (**Table** 5.2), four using the fading cumulative exposure measure (**Figure** 5.5). All models other than the top four had an AIC ≥12 greater than the model with the lowest AIC. For comparison, two models with the same number of variables and an AIC difference of 4 would have a relative likelihood of 7:1, with an AIC difference of 12 the relative likelihood would be 403:1, and with an AIC difference of 16 the relative likelihood would be 2,981:1 [227].

Exposure measure	Window/half-life	AIC
Fading (**Figure 5.5**)	21	464
Fading	28	468
Fading	14	469
Fading	7	473
Equal (**Figure 5.4**)	14	476
Exponential (**Figure 5.3**)	90	477
Equal	7	477
Fading	42	478
Exponential	42	479
Exponential	28	480

Table 5.2 Best fitting models using a single exposure measure type

Top 10 of 40 models shown

This suggests that significant recovery of diversity occurs over weeks following exposure, in keeping with previous work [136,137,145,153], supported by the fact that the best performing time windows using the "equal" exposure approach (**Figure** 5.4) were the shortest (7 and 14 days). It also suggests that cumulative exposure is an important determinant of microbiome disruption. However, because previous volunteer studies had shown that there are definite effects of antimicrobials beyond four weeks [136,137,148,150], further comparisons were made to assess whether the addition of a longer term measure of exposure would produce an improved exposure measure. Further multiple regression models were compared that included two different measures per antimicrobial exposure, one shorter term (5 types of measure with window/half-life ≤42 days) and one longer term (5 types of measure with window/half-life ≥90 days), for a total of 375 models. The best fitting models are shown below in **Table** 5.3. These showed an improved fit compared to a single measure models, with AIC up to 10 lower than those above, despite the penalty in the AIC for fitting 18 additional variables. Of note, the best

longer term metrics were all non-cumulative, possibly suggesting that longer term effects are more related to exposure per se, rather than cumulative exposure. The median correlation between the shorter and longer term measures for the same antimicrobial was 0.74 for the best fitting model, indicating a low risk of over-fitting due to collinearity.

Exposure measure 1	**Window/ half-life 1**	**Exposure measure 2**	**Window/ half-life 2**	**AIC**	**Median (range) correlation***
Fading (**Figure 5.5**)	28	Linear (**Figure** 5.1)	365	454	0.74 (0.60-0.92)
Fading	21	Linear (**Figure** 5.2)	365	454	0.73 (0.60-0.87)
Fading	28	Exponential **(Figure** 5.3**)**	365	454	0.74 (0.60-0.92)
Fading	28	Qualitative (**Figure** 5.1)	365	454	0.70 (0.53-0.91)
Fading	28	Exponential	180	455	0.74 (0.60-0.92)
Fading	21	Exponential	365	456	0.73 (0.60-0.87)
Fading	21	Exponential	180	456	0.73 (0.60-0.87)
Fading	21	Qualitative	180	456	0.74 (0.58-0.86)
Fading	28	Qualitative	180	456	0.78 (0.58-0.91)
Fading	21	Qualitative	365	457	0.66 (0.53-0.86)

Table 5.3 Best fitting models including two measures types

Top 10 of 375 models shown. *Spearman correlation between the two measures for the 18 antibiotics

However, these models have the significant disadvantage of including two measures per exposure (i.e. 36 in total per model). Because of this problem, combined measures were tested that consisted of the fading measure if there was recent exposure, or the linear measure if not. The relative weighting of the two components was allowed to vary to minimise AIC, with windows of ≤28 days tested for the fading measure, and ≥90 days for the linear measure. The best fitting of these combined measures consisted of:

i) Fading exposure over the past 7 days if recent exposure, or

ii) 0.2 * linear exposure over 365 days if no recent exposure

With this combined measure, full exposure represents ≥7 days of continuous antimicrobial use. Exposure falls rapidly in the 7 days after stopping, then slowly over the following year. This measure had an AIC of 444, ≥20 lower than all previous single-measure models (in **Table** 5.2). This was used as the principal exposure measure for subsequent analyses. Sensitivity analyses were also performed using the simpler measure of fading exposure over 21 days (the best non-combined single measure), and the simplest measure of qualitative exposure over the past 7 days. The distributions of the combined and fading 21-day exposure variables are shown in **Figure** 5.11 (qualitative exposures are 0 or 1 by definition).

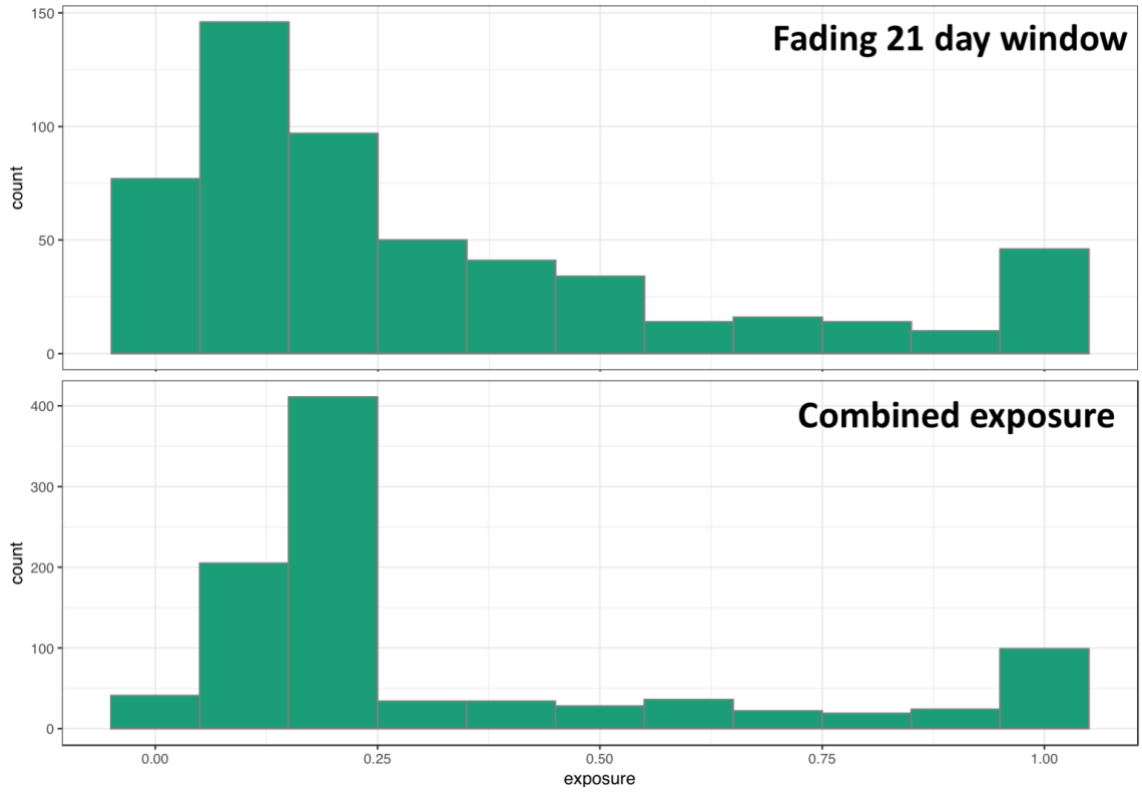

Figure 5.11 Distribution of values for different exposure variables

Exposures among 225 cross-sectional samples, with all antimicrobials counted separately. Combined exposure is derived from fading 7 day exposure and linear 365 day exposure, as described above. Zero-exposures, which would dominate both plots, are not shown.

5.3.4 Effects of antimicrobials on gut microbiome diversity

To compare the effects of specific antimicrobials, I created a multivariable model using the combined exposure measure for each drug taken by >5 patients (33 antimicrobials) whilst adjusting for the nine non-antibiotic potential confounders in **Table** 5.1. The results are shown in **Figure** 5.12. There was no evidence that any of the non-antibiotic covariates were associated with diversity after adjustment for antimicrobial exposure. Seven antibiotic exposures were significantly associated with reduction in Shannon diversity:

- oral clindamycin (effect size -1.7, 95% CI -2.6 to -0.9, p = 0.0001)
- oral ciprofloxacin (-1.6, 95% CI -2.4 to -0.7, p = 0.0005)
- piperacillin-tazobactam (-1.5, 95% CI -2.1 to -1.0, p < 0.0001)
- ceftazidime (-1.3, 95% CI -2.6 to -0.1, p = 0.04)
- meropenem (-1.2 95% CI -1.8 to -0.5, p = 0.0005)
- intravenous co-amoxiclav (-1.2, 95% CI -2.0 to -0.3, p = 0.006)
- oral co-amoxiclav (-0.6, 95% CI -1.1 to -0.2, p = 0.003)

Oral metronidazole, iv vancomycin, iv gentamicin, and oral clarithromycin were significantly associated with reduced diversity in a univariable model, but these effects largely disappeared in the multivariable model, suggesting confounding between antimicrobials. Oral azithromycin and doxycycline had the strongest evidence of minimal reductions in diversity, with neither effect consistent with a reduction in Shannon diversity of >0.5. No antiviral or antifungal was associated with a significant effect on diversity. Amoxicillin, co-amoxiclav and flucloxacillin were taken in both intravenous and oral formulations by at least 5 patients, but there was no evidence of heterogeneity in the effects of these drugs taken by different routes (all heterogeneity p > 0.1).

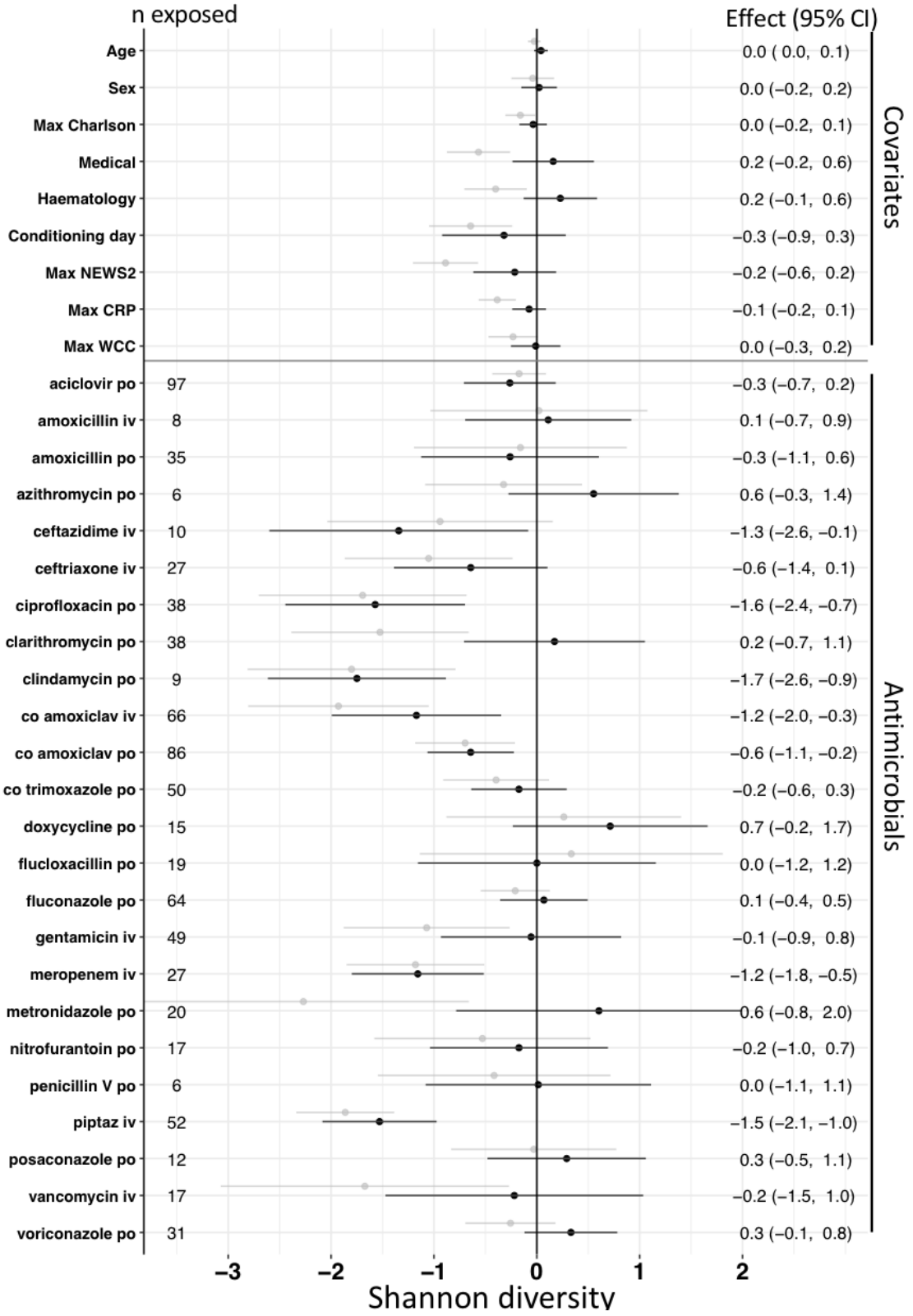

Figure 5.12 Effects of antimicrobials on Shannon diversity (combined exposure measure)

Multivariable estimates in black, univariable (unadjusted) estimates in grey. Results for 8 exposures (n = 6-13) not plotted as the range of 95% CI was >3; none differed significantly from zero. Estimates represent the effect of receiving the drug continually for ≥7 days. Sex is vs female, medical/haematology is vs healthy volunteers. Age, Charlson, NEWS2 and WCC are per 10 units higher, CRP is per 100 units higher, and conditioning is per 14 days.

Because of the risk that the combined exposure measure may be over-fitted to the data, a sensitivity analysis was performed using the fading 21 day exposure alone (**Figure** 5.13). This produced similar estimates to the main analysis, although in this model azithromycin was associated with a significant increase in gut microbiome diversity (effect +1.2, 95% CI +0.5 to +1.9, p = 0.001). Effects on diversity were generally larger than in the main analysis, as full exposure would represent 21 days of continuous use. Because this model only included exposure in the past 21 days, many fewer participant exposures were included, particularly for oral antibiotics, and standard errors around the effect estimates were larger. Further sensitivity analyses using species richness as an outcome, or using qualitative exposure over the past 7 days also had larger standard errors but were otherwise consistent with the main analysis (**Appendix 4 Figure** 12.1 and **Figure** 12.2 respectively).

A similar analysis was performed with the single combined exposure measure re-calculated by antimicrobial group, combining different agents and routes of administration but with the model otherwise the same (**Figure** 5.14). The uncertainty around the effect estimates was reduced by this approach, although this may combine agents with quite different effects. This showed that both broad-spectrum beta-lactams and quinolones caused significantly more reduction in diversity than antifolates, macrolides, tetracyclines and narrow-spectrum beta-lactams ($p < 0.01$ for all comparisons, see legend for group definitions).

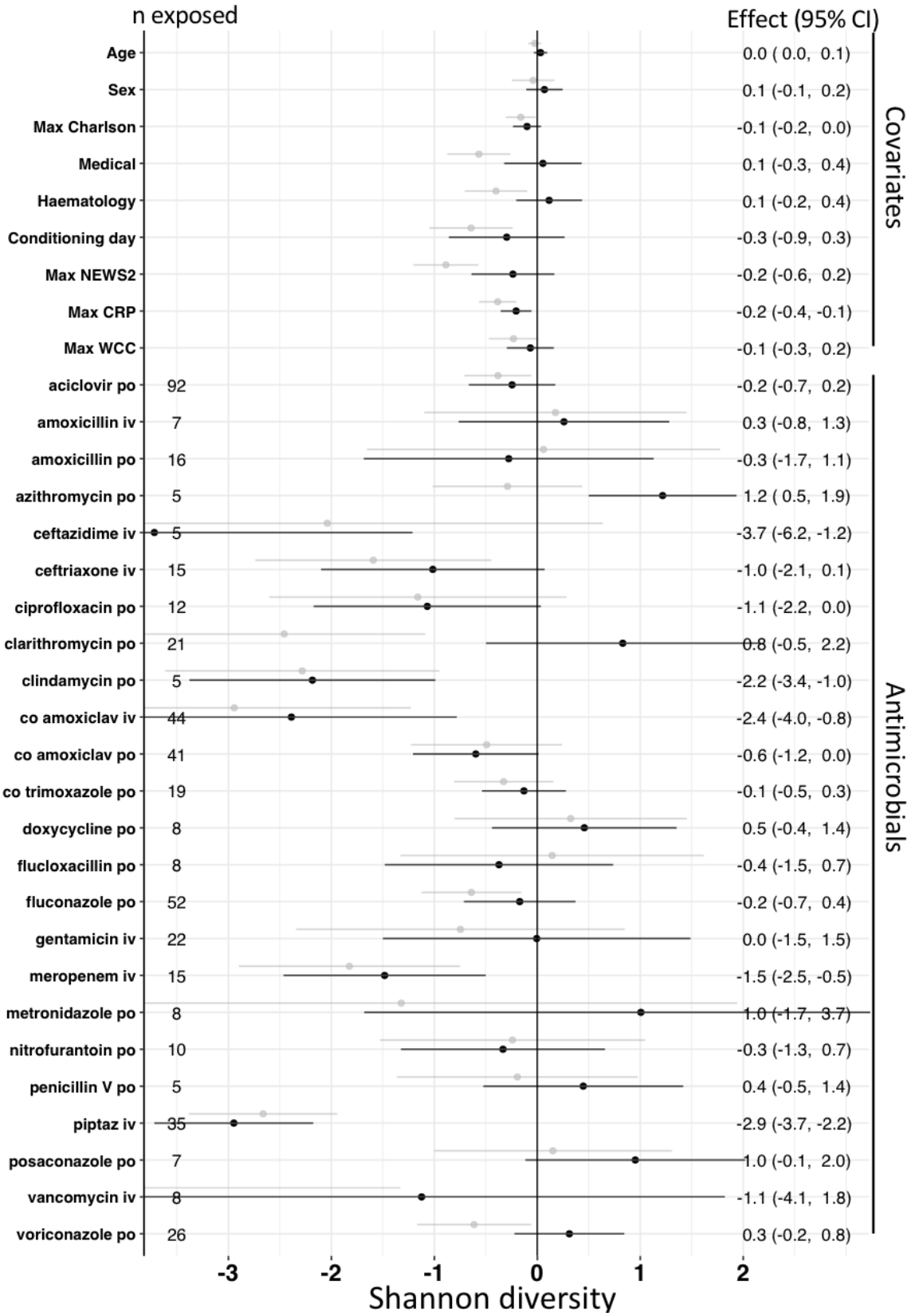

Figure 5.13 Effects of antimicrobials on Shannon diversity (fading 21 day exposure)

Multivariable estimates in black, univariable (unadjusted) estimates in grey. Antibiotic exposures and effects of non-antibiotic covariates are as in **Figure** 5.12. Estimates represent the effect of having received the drug continually for 21 days.

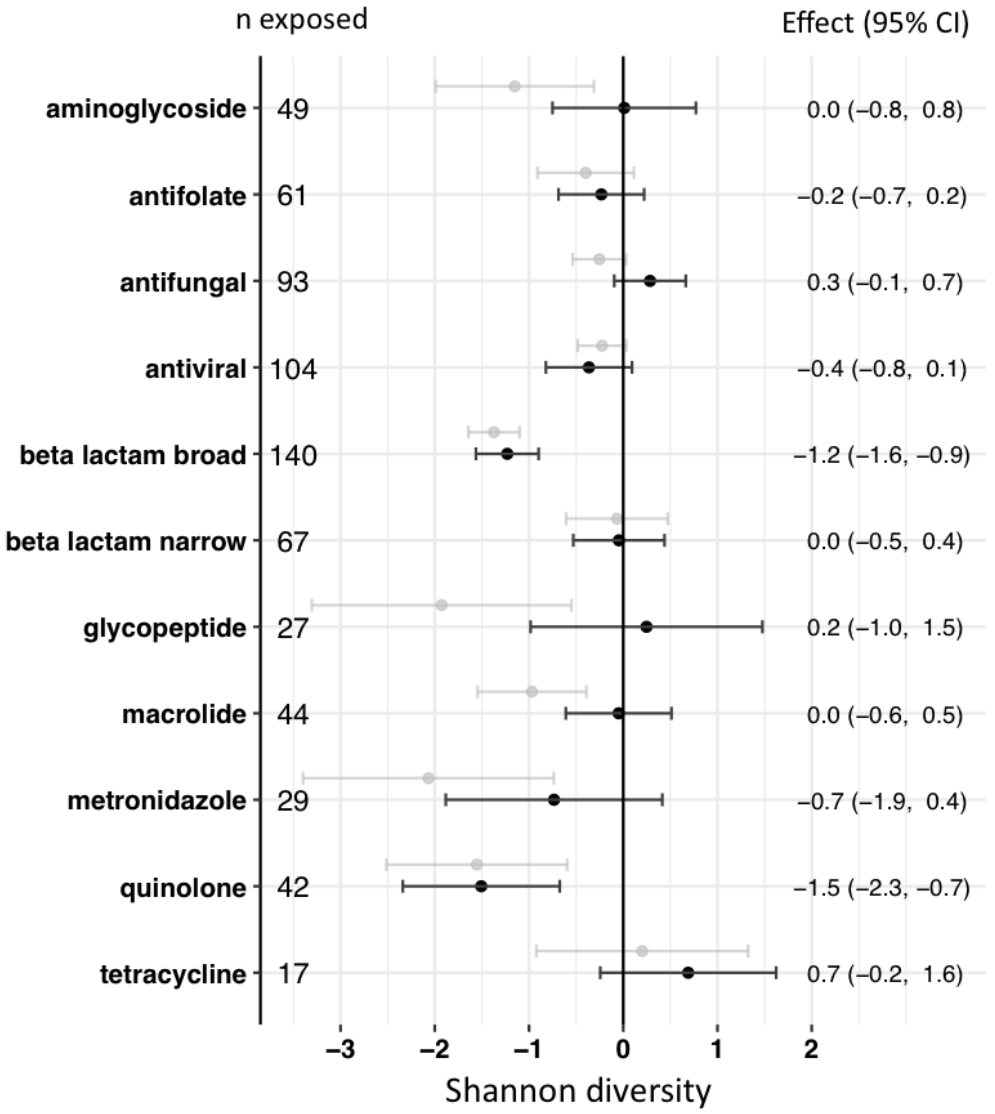

Figure 5.14 Effect of different antimicrobial groups on diversity

Combined exposure measure. 'Narrow' beta-lactams are penicillin, amoxicillin, flucloxacillin and first generation cephalosporins, all others are defined as 'broad'. Multivariable estimates in black, univariable (unadjusted) estimates in grey. Covariates are not shown, but were included in the model.

5.3.5 Effects of antimicrobials on selected taxa

The same multivariable model was used to explore the effects of antimicrobials on different constituents of the gut microbiome, using log relative abundance of specific taxa as the outcome variable (approximately normally distributed). The effects of antimicrobial exposure on five taxa were compared: three major groups of anaerobes (the phyla

Bacteroidetes and *Actinobacteria*, and the order *Clostridiales*) plus two groups of clinically important organisms (the *Enterobacteriaceae* and *E. faecium*). Together these groups accounted for 89% of the total abundance of organisms across all samples. Of note, because the outcome variable is relative, not absolute, abundance, this analysis is only sensitive to differential effects on abundance. A hypothetical antimicrobial that had similar effects on all major group of organisms could produce a large decrease in diversity but have no effects on the differential abundance of the groups themselves.

Uncertainty around the estimates of effect on different taxa was greater than for diversity (**Figure** 5.15). Three antibiotics significantly increased the relative abundance of *E. faecium*:

- meropenem (58.2 fold change, 95% CI 7.1 to 474.7, p = 0.0002)
- ceftriaxone (15.4 fold-change, 95% CI 1.3 to 176.5, p = 0.02)
- piperacillin-tazobactam (8.7 fold-change, 95% CI 1.4 to 53.4, p = 0.02)

No antibiotic caused a significant decrease in *E. faecium*. In contrast, several antibiotics caused large decreases in *Enterobacteriaceae*:

- ceftazidime (1.8×10^{-5} fold change, 95% CI 1.1×10^{-2} to 2.9×10^{-8}, p = 0.001)
- meropenem (5.6×10^{-3} fold-change, 95% CI 1.5×10^{-1} to 2.1×10^{-4}, p = 0.002)
- oral ciprofloxacin (7.4×10^{-3} fold-change, 95% CI 6.5×10^{-1} to 8.5×10^{-5}, p = 0.03)
- ceftriaxone (7.9×10^{-3} fold-change, 95% CI 3.6×10^{-1} to 1.8×10^{-4}, p = 0.01)
- piperacillin-tazobactam (3.3×10^{-2} fold-change, 95% CI 5.6×10^{-1} to 1.9×10^{-3}, p = 0.02)

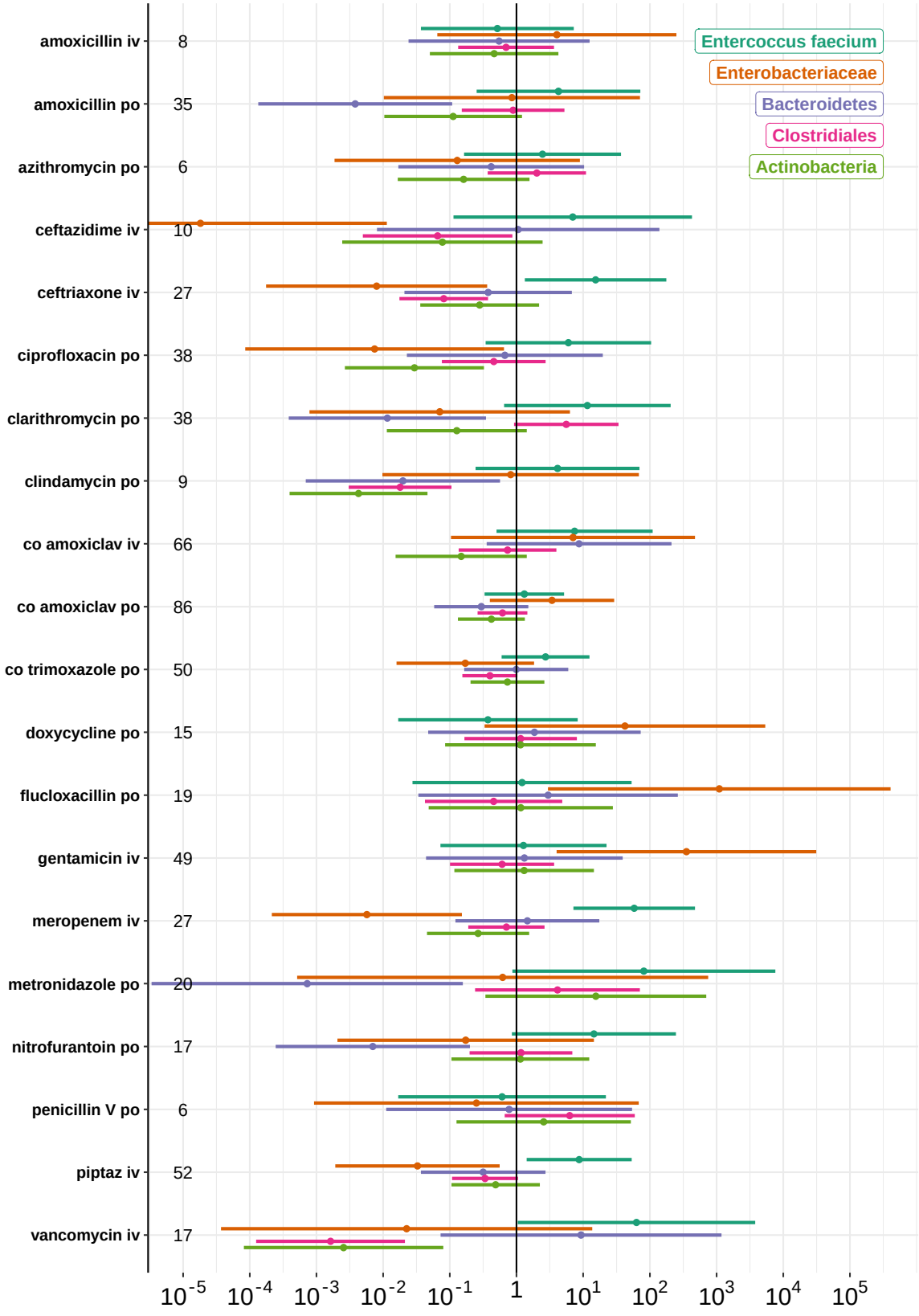

Figure 5.15 Effects of antimicrobial exposure on selected taxa

Relative change in abundance of selected taxa, using combined exposure measure. Error bars represent 95% confidence intervals. For clarity, results for antivirals, antifungals and covariates are not shown, but these are adjusted for in models.

Different effects were seen in the abundance of the major anaerobe groups. Large decreases in *Bacteroidetes* were observed with oral metronidazole ($7.2x10^{-4}$ fold change, 95% CI $1.5x10^{-1}$ to $3.3x10^{-6}$, p = 0.009) and nitrofurantoin ($7.0x10^{-3}$ fold-change, 95% CI $2.0x10^{-1}$ to $2.4x10^{-4}$, p = 0.004), but these antimicrobials appeared to have minimal effect on *Clostridiales* and *Actinobacteria*. Conversely, intravenous vancomycin caused the largest decreases in the *Clostridiales* ($1.6x10^{-3}$ fold-change, 95% CI $2.1x10^{-2}$ to $1.2x10^{-4}$, p < 0.0001) and *Actinobacteria* ($2.5x10^{-3}$ fold-change, 95% CI $8.0x\ 10^{-2}$ to $8.1x10^{-5}$, p < 0.0001), but had no significant effect on *Bacteroidetes*. Clindamycin caused significant decreases in all three anaerobe classes;

- *Bacteroidetes* ($2.0x10^{-2}$ fold-change, 95% CI $5.7x10^{-1}$ to $6.9x10^{-4}$, p = 0.02)
- *Clostridiales* ($1.8x10^{-2}$ fold-change, 95% CI $1.1x10^{-1}$ to $3.0x10^{-3}$, p < 0.0001)
- *Actinobacteria* ($4.3x10^{-3}$ fold-change, 95% CI $4.6x10^{-2}$ to $4.0x10^{-4}$, p < 0.0001)

5.3.6 Effects of antimicrobials on selected AMR genes

The effects on specific classes of AMR genes were analysed using the same model. As drug specific estimates had larger errors, exposure was classified by drug group (**Figure** 5.16). These groupings are the same as used above, other than the addition of clindamycin to the macrolide group because of shared resistance mechanisms. In several cases selection of specific resistance mechanisms by corresponding drug classes could be observed:

- Tetracyclines increased the abundance of tetracycline ribosomal protection proteins (18.2 fold-change, 95% CI 1.9 to 177.4, p = 0.01)
- Antifolate drugs increased the abundance of trimethoprim-resistant dihydrofolate reductase (4.2 fold-change, 95% CI 1.6 to 10.9, p = 0.003)

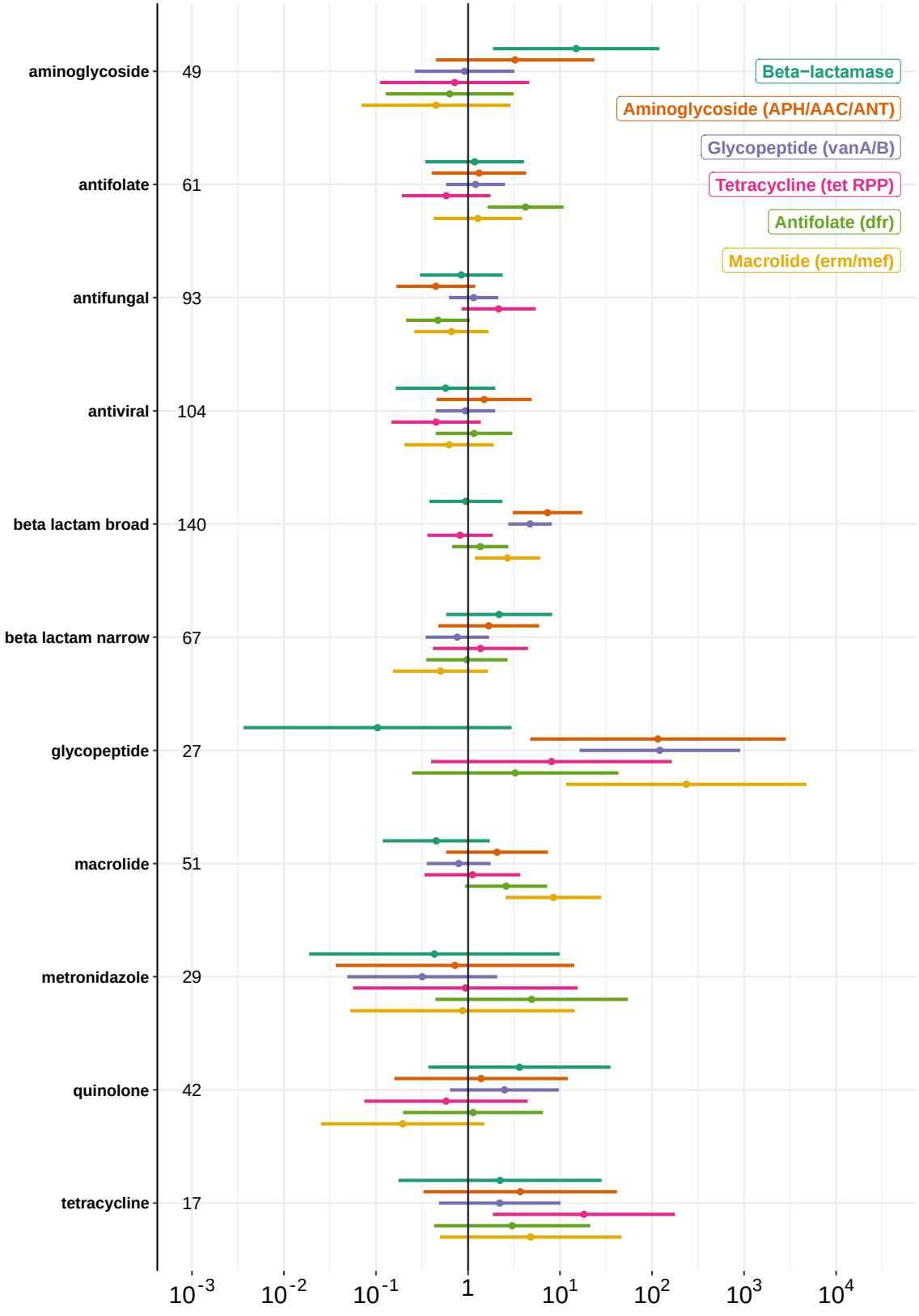

Figure 5.16 Effects of antimicrobials on selected AMR genes

Relative change in abundance of selected AMR genes, using combined exposure measure. Error bars represent 95% confidence intervals. Results are shown for the antibiotics in **Figure** 5.14, results for covariates are not shown but were adjusted for.

- Macrolides and clindamycin increased the abundance of *erm* 23S rRNA methyltransferases and *mef* macrolide efflux pumps (8.5 fold-change, 95% CI 2.6 to 28.0, p = 0.0005)
- The abundance of the *vanA* glycopeptide resistance gene was increased by both glycopeptides (123.2 fold-change, 95% CI 16.6 to 911.8, p <0.0001) and broad-spectrum beta-lactams (4.8 fold-change, 95% CI 2.8 to 8.3, p p <0.0001)
- Both of these groups also caused significant increases in aminoglycoside transferases and the above macrolide resistance genes.
- Only aminoglycosides had a significant effect on beta-lactamase abundance (15.0 fold-change, 95% CI 1.9 to 120, p = 0.01)

5.4 Discussion

Cross-sectional studies of the gut microbiome offer the possibility of recruiting large numbers of patients, allowing direct comparison of many different antimicrobials in a real clinical setting. In this chapter I have aimed to develop methods to analyse cross-sectional data to produce reliable, quantitative comparisons between different drugs to inform antimicrobial stewardship or generate hypotheses for interventional clinical studies. This has required consideration of some major problems associated with such studies.

The large degree of inter-individual variation in the gut microbiome increases the uncertainty around effect estimates compared to longitudinal sampling. Despite this, with a sample of 225 participants it was possible to observe clearly significant differences between specific antimicrobial exposures. Overall measures of diversity can be measured from every sample and vary less between individuals in health than do the abundance of specific taxa.

This makes them appealing as a surrogate marker for AMR selective pressure, and it was effects on overall diversity that produced the clearest differences between drugs. Variation was larger when analysing specific taxa and AMR genes, and although several significant effects were observed, most estimates had a large range of uncertainty. The magnitude of the errors themselves is of value in considering future studies, for example, one could predict that a similar study with 4,000 participants would have errors around ¼ as large.

Because antimicrobial exposure is so heterogeneous in most observational datasets, analysis requires the use of measures that can reflect differences in recency and amount of exposure and be incorporated into a multivariable model. I was not able to find previous studies to inform the choice of an exposure measure, so used the data to derive this, although this carries the risk of over-fitting and loss of generalisability. The fact that I was able to reproduce many expected associations mitigates this concern, at least in part, as does consistency with results from the sensitivity analysis using simpler exposure models. The only marked discrepancy between models was an increase in gut microbiome diversity associated with azithromycin in the fading 21 day model, which was not present in the combined exposure model. As increased in diversity with antimicrobial use seems unlikely, this could represent confounding with antibiotic use before the 21-day exposure window, highlighting a potential problem with any simpler exposure model that only reflects exposure in the past few weeks.

The combined exposure model derived from the data suggest that recent cumulative exposure to antibiotics has a large effect on diversity that mostly normalises within a few weeks, but that some residual longer term effects persist. This is supported by a study

published after this analysis was performed that found that gut microbiome diversity had largely returned to normal 16-21 days after treatment with moxifloxacin [228]. I explored relatively few simple exposure measures and parameters, as the dataset was not large enough for a more significant exploratory analysis. Separating my dataset into derivation and validation groups to determine measures of antimicrobial exposure was also not possible due to limited numbers, but would be ideal in a larger study. Also, although the exposure measure used was derived without reference to specific drugs, it will necessarily reflect the dynamics of the predominant disruptive antibiotics present in the data rather than rarer or less disruptive ones. It is unlikely that the dynamics of microbiome disruption are identical between different antibiotics, but using a single shared measure of exposure allows direct comparisons to be made between drugs. The multivariable model also assumed no statistical interaction between the effects of different drugs, which again is an assumption that could be tested in larger studies.

Adjustment for confounding is particularly important in cross-sectional studies, where different individuals are being compared with one another. Availability of EPR data meant that adjustment could be made with contemporaneous clinical records, including observations and blood results. A major concern is that acute infection itself could affect the gut microbiome and lead to antibiotic use, although in this analysis there was no evidence of an independent effect of illness, with the significant univariate effects of maximum recent NEWS2 score, CRP and WCC largely disappearing after adjustment for antibiotic exposure. An important source of residual confounding could be antimicrobials administered outside of OUH, particularly among HSCT patients who had had admissions to other hospitals trusts within in past few months and in whom it was not possible to obtain accurate full exposure

histories (although this was attempted). Adjusting for known antimicrobial use was also difficult because of the number of different exposures. Drugs received by only one or two individuals were excluded as their effects could only be estimated very imprecisely, introducing noise into other estimates in the model. However, this completely excluded some drugs that might be expected to have had substantial effects, such as ceftolozane-tazobactam, intravenous ciprofloxacin, and tigecycline. This would be less of a problem in larger studies where even uncommon exposures can be included in the model.

Microbiome disruption was clearest with clindamycin, ciprofloxacin, piperacillin-tazobactam, meropenem, ceftazidime and co-amoxiclav, consistent with previous microbiome studies of clindamycin [140], ciprofloxacin [137,140], co-amoxiclav [147,149], and piperacillin-tazobactam [159,160]. It is also largely in keeping with estimates of risk of *C. difficile* infection (CDI) following exposure to different antibiotic classes, which is highest with clindamycin, fluoroquinolones, carbapenems and third-generation cephalosporins [131,132]. The limited impact of doxycycline on diversity is also in keeping with the lower risk of CDI with tetracyclines. The decreased relative abundance of *Enterobacteriaceae* observed with piperacillin-tazobactam, ceftriaxone, meropenem, and ciprofloxacin correspond with reductions seen in culture based studies [125], and the significant decrease seen in *Clostridiales* and *Actinobacteria* with several antibiotic exposures is also in keeping with decreases in several culture-independent studies discussed in **Chapter 2**.

The observed increase in abundance of *Enterobacteriaceae* with intravenous gentamicin is surprising, as it is not excreted significantly into the bowel and would be expected to decrease *Enterobacteriaceae* abundance if it were. However, this may be a chance finding,

as one would expect at least one such extreme result (p = 0.01) by chance given the number of exposure-taxon comparisons. The reduction in *Clostridiales* and *Actinobacteria* seen with intravenous vancomycin is highly significant, and is out of keeping with one small longitudinal study that reported no effect of intravenous vancomycin on anaerobes [162]. However, intravenous vancomycin is excreted in significant amounts into the bile [229], and it is known from interventional studies that oral vancomycin significantly decreases the abundance of Gram positive anaerobes.

The effects on the abundance of AMR genes were more difficult to accurately estimate in this study, although there was a clear signal of selection of some resistance mechanisms by corresponding drug classes. The apparent effect of gentamicin on beta-lactamases may represent the same chance finding outlined above, as the effect was confined to beta-lactamases typically present in *Enterobacteriaceae*. The increase in *vanA* glycopeptide resistance genes, macrolide resistance genes and aminoglycoside resistance genes produced by exposure to both broad-spectrum beta-lactams and glycopeptides seems likely to be related to selection of VRE, as these genes are often present together in these organisms [230,231]. This is supported by the observed increase of *E. faecium* with exposure to these antimicrobial groups.

The consistency of these results with previous studies supports the use of cross-sectional observational data to compare the effects of specific antimicrobials. Although potential problems remain, particularly in identifying widely usable measures of exposure, the decreasing costs of sequencing and availability of electronic medical records make this an increasingly practical approach to studying the impact of antimicrobials on the gut flora.

6 Longitudinal analysis

6.1 Introduction

The appeal of longitudinal sampling is that it reduces problems related to confounding and inter-individual variation, as the outcome is the change between samples from the same individual rather than the difference between individuals. The relative stability of the gut microbiome in health makes longitudinal sampling particularly informative in interventional studies. For example, in one interventional study of three healthy volunteers with frequent sampling before, during and after ciprofloxacin, microbiome stability was followed by large, rapid reductions in diversity that were clearly attributable to the exposure [137]. Longitudinal sampling in observational studies is quite different, as it is likely to happen at a time of significant microbiome instability, when a patient may be acutely unwell, have recently received antimicrobials or other drugs, and will often be treated with several antimicrobials between samples. The duration and timing of antimicrobials in relation to the samples will also be very variable between patients. One option for analysis is to rely on pairs of samples that happen to be taken at similar intervals around an exposure of interest when no other antimicrobial changes occurred. However, this may represent a small minority of samples, for example only 212/2209 samples (9.6%, 106 pairs) could be used with this approach in one large study of HSCT patients [159].

An alternative approach is to use an exposure measure that can be applied to all sample pairs and used in multivariable analysis. In selecting a suitable measure for sample pairs, the same considerations apply that were previously discussed in relation to cross-sectional sampling, but in addition the measure should take into account the degree of exposure at

both timepoints. For example, differences between samples taken during long term antibiotic use would not be informative, and if the initial sample were taken just after stopping antibiotics then the subsequent sample might show an increase in diversity.

Confounding is less problematic with longitudinal rather than cross-sectional sampling, as all long term patient characteristics remain the same between samples. Acute changes that occur around the time of sampling could still cause biases, though, such as the development of new infection leading to antibiotic use. Adjustment for acute changes is made easier with the high resolution data available from EPR, such as clinical observations and blood results.

It is ideal to recruit patients as early as possible in their illness, before they have received any antimicrobials, but this is difficult in practice. The ARMORD study focussed longitudinal recruitment on HSCT patients, as this was the group in whom it was possible to reliably recruit participants before antimicrobial exposure and obtain regular samples afterwards. HSCT is primarily used for the treatment of haematological malignancy, although it is sometimes used to treat non-malignant diseases of bone marrow and non-haematological immune diseases [232]. There are two kinds of HSCT; autologous, where a patient's own haematopoietic stem cells are harvested and then infused after high dose chemotherapy with the aim of allowing treatment that would otherwise risk marrow failure, and allogeneic, where a patient receives matched haematopoietic stem cells from a donor. Allogeneic transplant is typically used for higher grade malignancies, as the use of a donor not only prevents reinfusion of malignant cells but also produces a graft versus tumour effect, whereby the new immune system actively eliminates malignant cells. However, a common complication is the development of graft versus host disease (GvHD), where this

immune response targets the host. GvHD alone is responsible for a mortality of 10-20% after transplant [159]. In both types of HSCT, patients are typically admitted for up to a week before stem cell infusion to receive conditioning chemotherapy that supresses the bone marrow and allows the new cells to engraft. A period of profound neutropenia is present for 1-2 weeks before engraftment, during which time nearly all patients develop infection and receive antibiotics, and a total admission of 3-5 weeks is typical in the absence of complications.

The gut microbiome of patients going through HSCT is of interest for broader reasons than just understanding the effects of antimicrobials. Infection is usually related to endogenous bacteria, particularly from the gastrointestinal tract because of neutropenia and mucositis related to chemotherapy. Enteric bacteria are the commonest organisms isolated in bloodstream infection after HSCT, and a better understanding of the dynamic changes that occur during transplant may help to allow the recognition of incipient infection. In one gut microbiome study in allogeneic HSCT, intestinal domination with enterococci or *Proteobacteria* (defined as a relative abundance >30%) was associated with a 9-fold and 5-fold increase in risk of subsequent bacteraemia with these organisms, respectively [126].

There is also evidence that the gut microbiome may be an important determinant of GvHD in allograft patients [198], and that the use of specific antibiotics may greatly increase the risk of developing GvHD [159,233]. Most data come from a single centre, where one report found that treatment with piperacillin-tazobactam or imipenem for neutropaenic sepsis was associated with a 60% increase in mortality from GvHD, with no increase seen with the use of cefepime or aztreonam [159].

In this chapter I analyse longitudinal data from HSCT patients in the ARMORD study to describe the impact of different antimicrobials on the gut microbiome and resistome, and compare these estimates to those from the cross-sectional data. I then describe results from a small number of paired samples available from the POETIC study of women receiving treatment for uncomplicated UTI. Following this, I present data on the gut microbiome during bloodstream infection and on organisms causing intestinal domination in all ARMORD samples. Finally, I present some exploratory data from all ARMORD and POETIC samples attempting to infer the host organism of AMR genes detected by sequencing.

6.2 Methods

6.2.1 ARMORD recruitment and sampling

Participants admitted to the haematology ward or attending the day unit for HSCT were recruited to the study as early as possible and asked to provide their next stool sample, then one every other day until discharge. First and last samples were sequenced from all patients with ≥2 samples, as were samples in close proximity to bacteraemia. Additional samples were sequenced where these were immediately before or immediately after specific antibiotic exposures. Analysis was based on sequential pairs separated by 2-30 days, so a sample could act as the second member of one pair and the first member of another pair (with different exposures between the pairs).

6.2.2 Measurement of antimicrobial exposure in ARMORD

No attempt was made to derive an exposure measure from the longitudinal data, and instead the single combined antimicrobial exposure measure used in the cross-sectional

analysis was calculated for each sample (maximum of fading 7 day or 0.2*linear 365 day exposure). The exposure for sample pairs was then calculated as the value of sample 2 minus sample 1. This creates the possibility of negative exposure values, for example if sample 1 was taken during a course of antibiotics that had stopped before sample 2 was taken. Illustrative examples from participants with multiple antimicrobial exposures are shown in **Figure** 6.1. As the concept of negative antimicrobial exposure may be unintuitive, a sensitivity analysis was also performed without negative values, in which the minimum exposure value was truncated at 0.

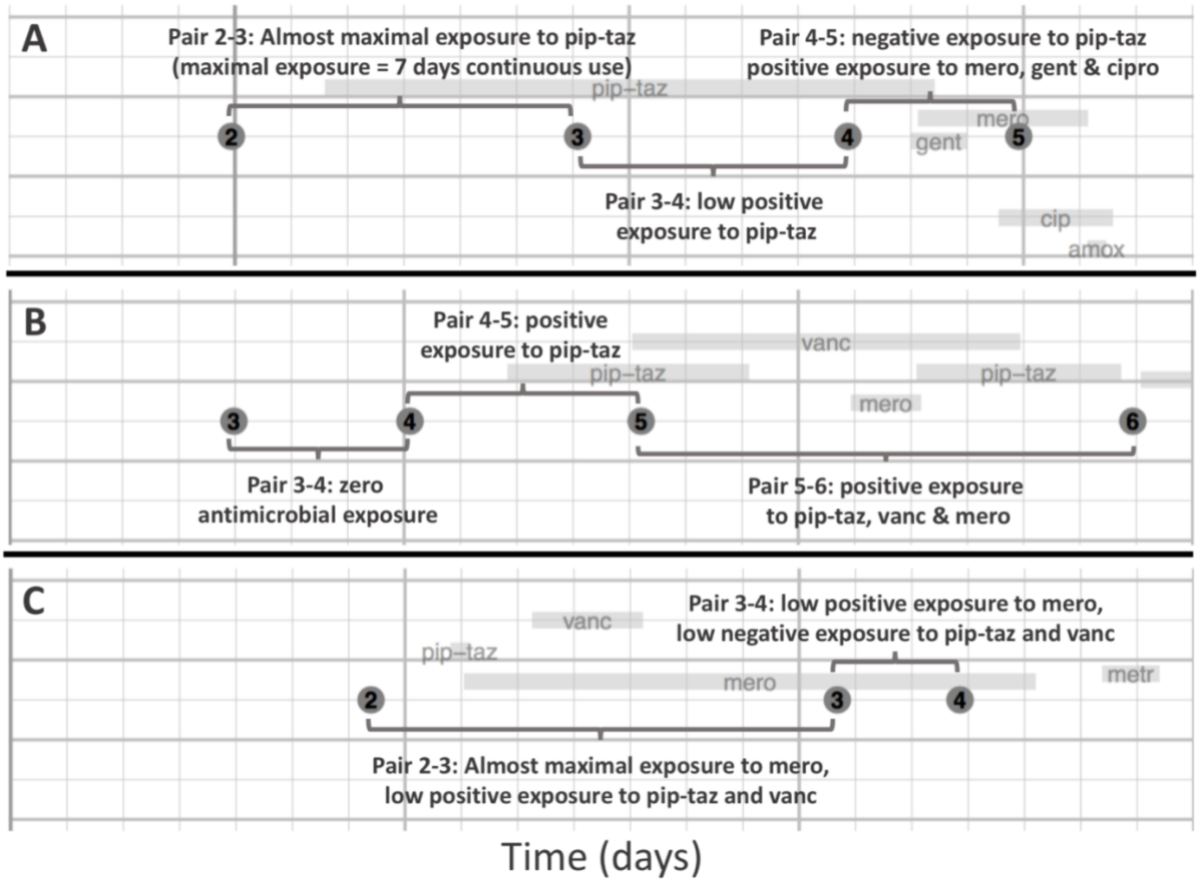

Figure 6.1 Illustrative examples of sample pair exposures in three HSCT patients

Numbered circles represent sequenced samples, grey bars are antimicrobial exposures.

6.2.3 ARMORD multiple regression model

Multiple regression was used to estimate the effects of antimicrobials on the gut microbiome and resistome, with each consecutive sample pair representing one observation. The statistical methods were kept as similar as possible to those in the cross-sectional analysis so the results could be compared, but in most cases the exposure and outcome variables represent *changes* between the two samples. Change in Shannon diversity was the primary outcome for comparing exposures. Other outcomes were change in relative abundance of specific bacterial taxa and in specific classes of AMR genes, both analysed on a log scale. Where a particular taxon or gene was not detected its presence was conservatively imputed at the lower limit of detection of 10^{-6} or 10^{-5}, respectively. The normality of outcome variables was assessed by visual inspection of histograms and inverse-normal plots.

Covariates used to adjust for potential confounding are shown below. In rare cases where EPR data were missing, values were imputed as indicated.

- Age (years)
- Sex: male or female (reference)
- Transplant type: autologous or allogeneic (reference)
- Baseline value of the outcome in first sample from pair (Shannon diversity or log relative abundance of specific bacterial taxa or AMR genes)
- Days from start of chemotherapy to the first sample
- Days between samples
- Difference in maximum NEWS2 track & trigger score in the 14 days before each sample (individual scores imputed as 0 if missing)

- Difference in the maximum CRP in the 14 days before each sample (individual scores imputed as 0 if missing)
- New high WCC (WCC >11x10^9/L in the 14 days before the second sample but not the first)
- New low WCC (WCC <0.5 x10^9/L in the 14 days before the second sample but not the first)

Note that change in WCC was not used as a continuous covariate as both extremes (neutropenia and neutrophilia) should be adjusted for.

All antimicrobial exposures which were observed in >2 participants were included in the model to allow for confounding, but only those with >5 participants are presented below, as results from exposures with 3-5 observations were considered unreliable. Because pairs of samples from the same individual cannot be assumed to be independent, robust estimates of standard error were used with lmtest (v0.9.37) and multiwaycov (v 1.2.3) packages in R, allowing for clustering within individual patients.

6.2.4 Analysis of POETIC samples

Too few sample pairs were available from POETIC participants for analysis using a regression model, and no data were available regarding participant characteristics or previous exposures to allow adjustment for confounding. Changes in Shannon diversity and abundance of *Enterobacteriaceae* were therefore compared by type of antimicrobial exposure, but no formal statistical testing was performed.

6.3 Results

6.3.1 ARMORD participants and samples

Eighty HSCT patients had ≥2 samples successfully sequenced and so were included in the longitudinal analysis. Half received allogeneic HSCT (41/80, 51%) and the remainder received autologous HSCT, with 67/80 (84%) having a diagnosis of haematological malignancy. Median age was 61 (range 37-75) years and 33 (41%) patients were female. Only 2/80 (2%) patients did not receive antibiotic treatment for infection during admission. Including prophylaxis, the mean number of different antimicrobial drugs received during admission was 3.8 antibiotics, 1.5 antifungals, and 1.0 antivirals.

Recruitment occurred a median 1 day after the start of conditioning (range -3 to +7). A total of 507 samples were collected from the 80 longitudinal patients, with a median 6 samples per participant. Median duration of admission was 23 days with mean sampling frequency of one sample per 3.6 days of admission. Of these samples, 281 (55%) were selected for sequencing and successfully sequenced, producing 173 sample pairs for analysis in the multiple regression model. At the time the first sample was taken, 29 (36%) of the 80 included patients were receiving antibiotics, including prophylaxis, and 47 (59%) had received an antibiotic within the past 28 days.

6.3.2 Effects of antimicrobials on gut microbiome diversity in ARMORD

The results of the multivariable model are shown in **Figure** 6.2. The only non-antibiotic covariate significantly associated with change in Shannon diversity was baseline diversity, although there was a borderline effect of greater reductions in diversity with autologous transplants (p=0.05).

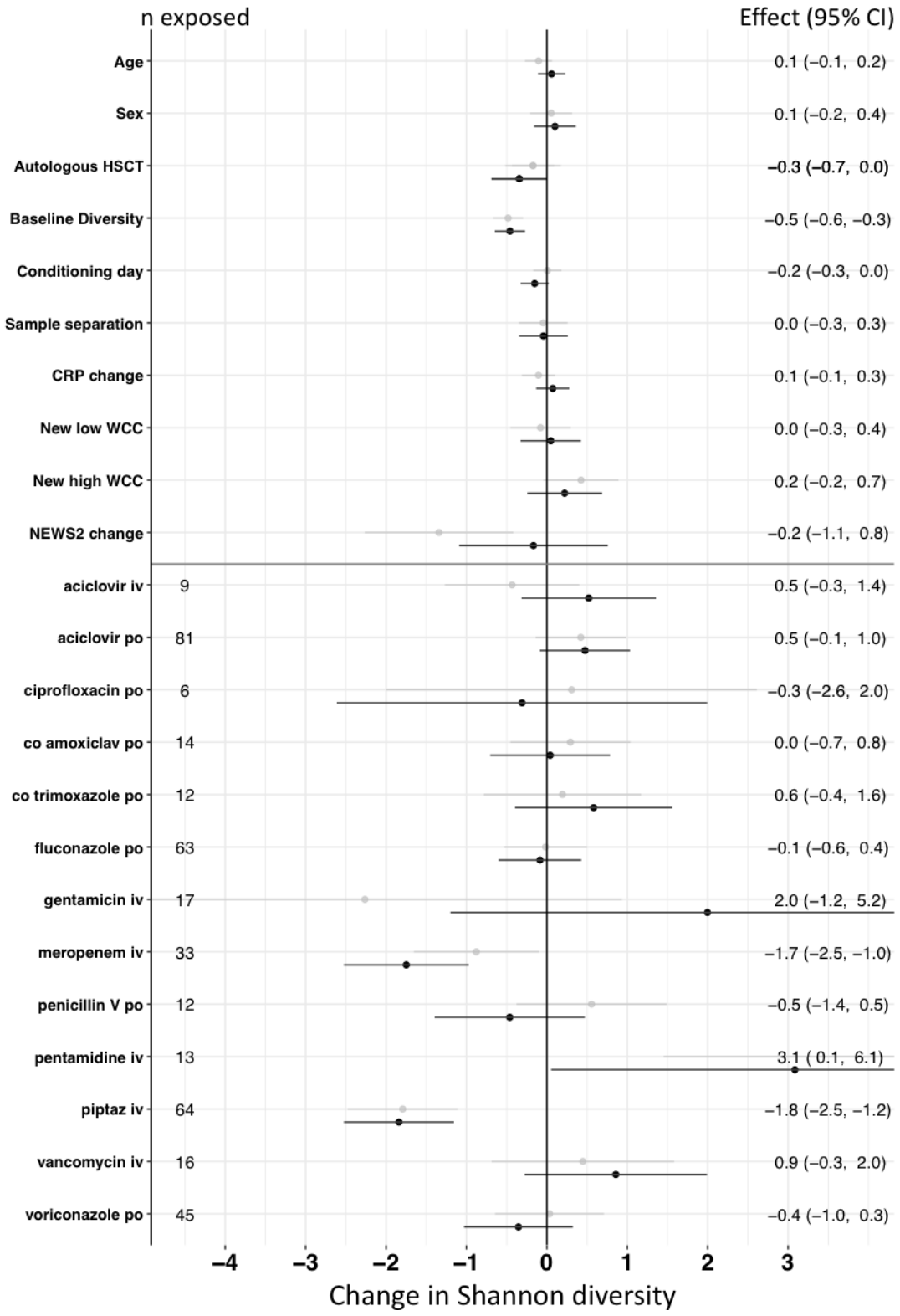

Figure 6.2 Effects of antimicrobial exposure on Shannon diversity

Combined exposure measure. Multivariable estimates in black, univariable (unadjusted) estimates in grey. Estimates represent the effect of having received the drug continually for ≥7 days. Sex effect is vs female, autologous effect is vs allogeneic. Age, Charlson, NEWS2 and WCC effects are per 10 units higher, CRP effect is per 100 units higher, and conditioning effect is per 14 days (all covariates scaled to aid visibility).

Two antibiotic exposures were significantly associated with a reduction in Shannon diversity between the first and second sample in each pair: piperacillin-tazobactam (-1.8, 95% CI -2.5 to -1.2, $p < 0.0001$) and meropenem (-1.7, 95% CI -2.5 to -1.0, $p < 0.0001$). Oral co-trimoxazole and intravenous vancomycin had the strongest evidence for lack of detrimental impact on the gut microbiome, with both having a 95% CI that lay above -0.5. Intravenous pentamidine was associated with a marginally significant increase in diversity (3.1, 95% CI 0.1 to 6.1, $p = 0.05$) but no other antifungal or antiviral had a significant effect. Sensitivity analyses using fading 21 day exposure alone or excluding negative exposures produced similar estimates (**Appendix 4 Figure** 12.3 and **Figure** 12.4).

Comparison of the estimates of effect of the same drug/route from the longitudinal and cross-sectional studies showed that, although the estimates from the longitudinal study had larger errors for most exposures, in all cases 95% confidence intervals were consistent with the same effect in both analyses (95% CI intersecting the line of identity in **Figure** 6.3). Importantly, several antimicrobials in the cross-sectional study were used in too few patients in the longitudinal study to be compared.

6.3.3 Effects of antimicrobials on selected taxa in ARMORD

The longitudinal data were used to estimate the effects of antimicrobials on changes in relative abundance of the five groups of organisms compared in **Chapter 5**; *E. faecium*, *Enterobacteriaceae*, *Bacteroidetes*, *Clostridiales* and *Actinobacteria* (**Figure** 6.4).

Abundance of *E. faecium* was significantly increased with meropenem use (32 fold change, 95% CI 1.7 to 599, $p = 0.02$).

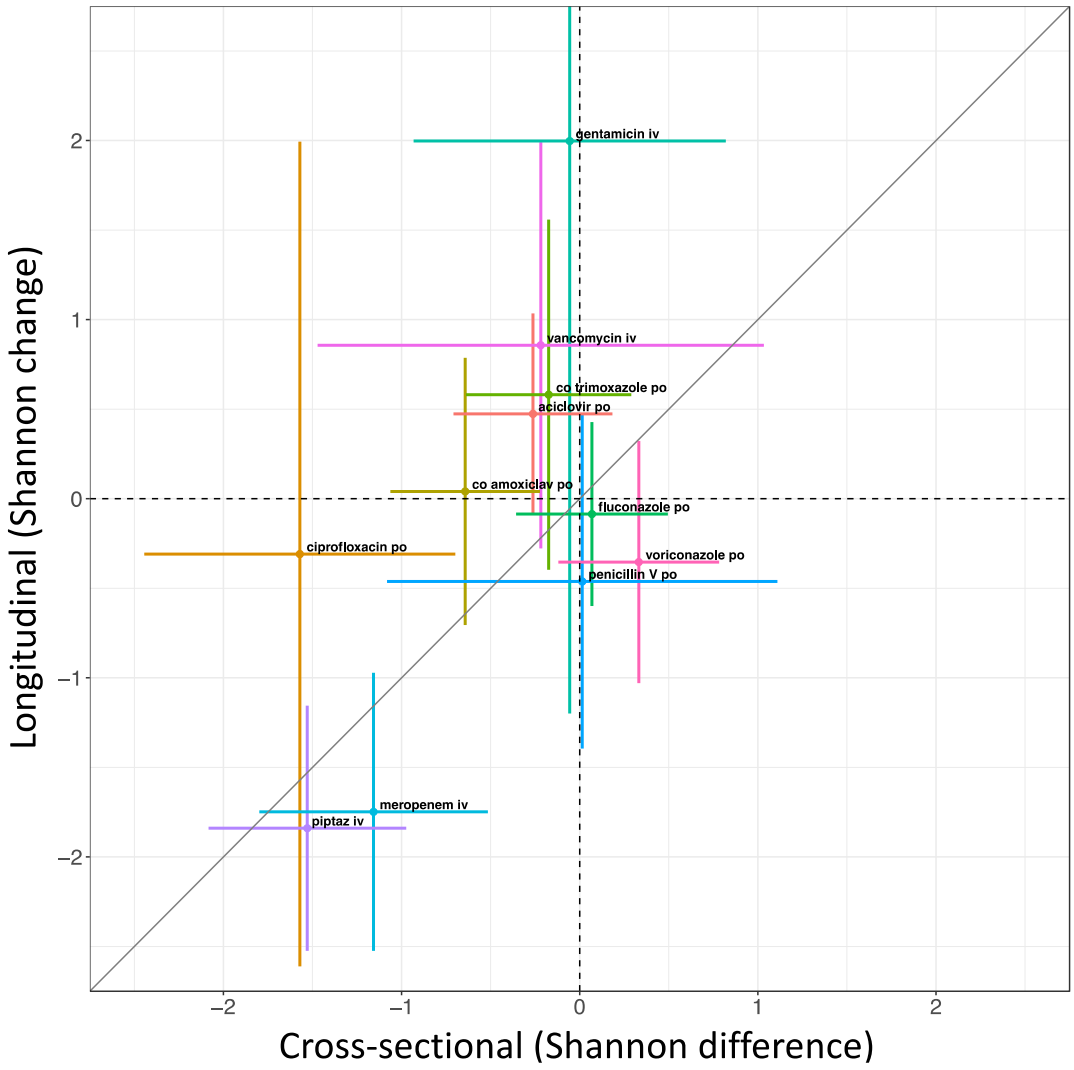

Figure 6.3 Comparison of the effects of antimicrobials in cross-sectional and longitudinal studies

Combined exposure measure. Diagonal line represents the same effect in both analyses.

Enterobacteriaceae abundance was decreased by:

- meropenem (3.7×10^{-4} fold change, 95% CI 5.1×10^{-3} to 2.7×10^{-5}, p <0.0001)
- ciprofloxacin (2.9×10^{-3} fold change, 95% CI 8.8×10^{-2} to 9.8×10^{-5}, p = 0.001)
- piperacillin-tazobactam (4.1×10^{-2} fold change, 95% CI 4.7×10^{-1} to 3.6×10^{-3}, p = 0.01)

Meropenem and piperacillin-tazobactam also caused significant decreases in all three groups of anaerobes. In contrast, intravenous vancomycin was associated with increased abundance of *Enterobacteriaceae* (51 fold change, 95% CI 3.7 to 708, p = 0.004) as was pentamidine (1.6×10^{5} fold change, 95% CI 28 to 9×10^{8}, p = 0.008).

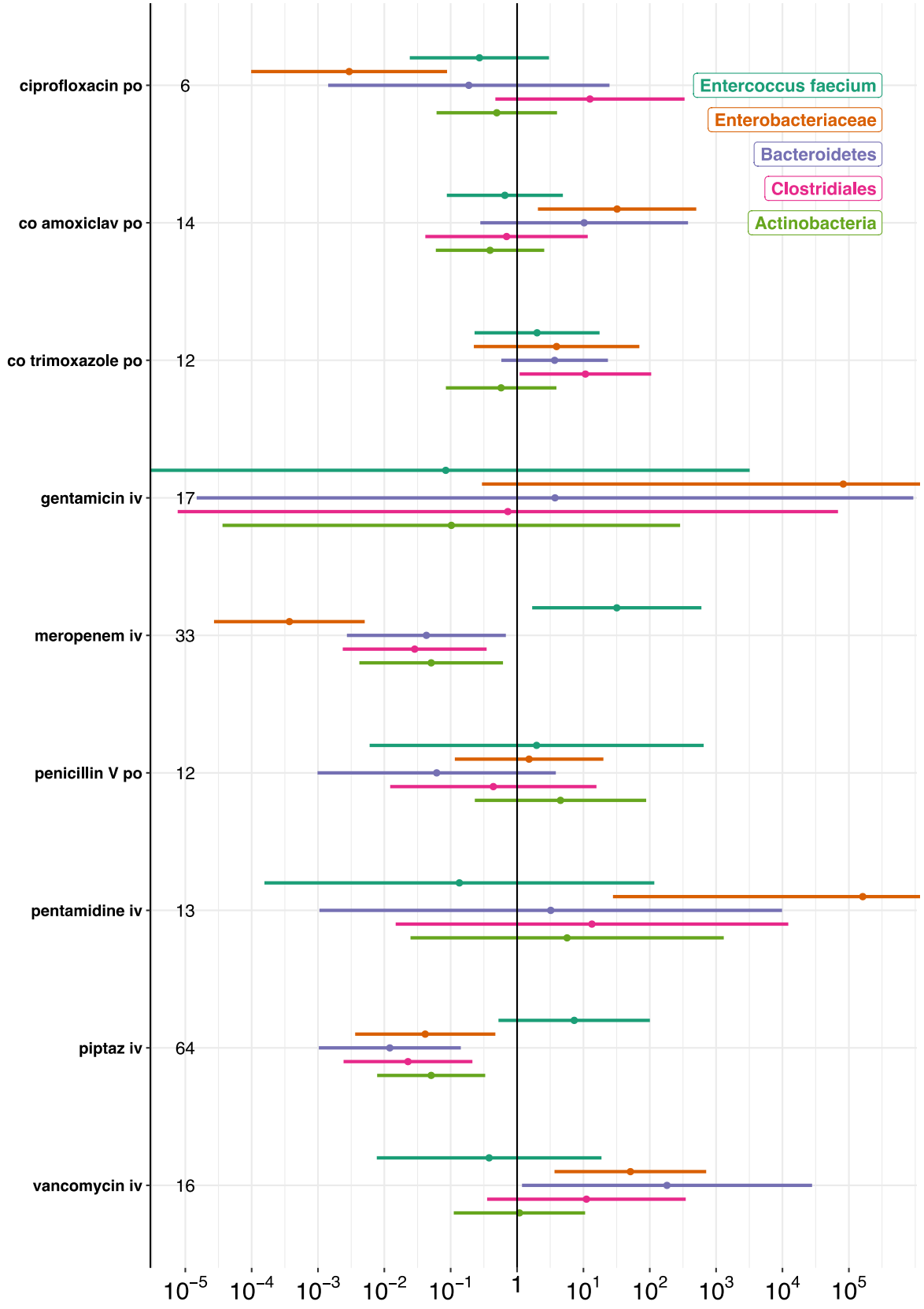

Figure 6.4 Effects of antimicrobials on selected taxa

Relative change in abundance of selected taxa, using combined exposure measure. Error bars are 95% confidence intervals. For clarity, results for antivirals, antifungals and covariates are not shown, but are adjusted for in models.

6.3.4 Effects of antimicrobials on selected AMR genes in ARMORD

The longitudinal data were used to estimate the effects of antimicrobials on changes in relative abundance of the six groups of AMR genes compared in **Chapter 5** (**Figure** 6.5). As previously, analysis was performed by antimicrobial group due to greater standard errors when comparing specific drugs.

A decrease in beta-lactamase resistance gene abundance was seen with broad-spectrum beta-lactam use ($7x10^{-2}$ fold change, 95% CI $2.7x10^{-1}$ to $2.0x10^{-2}$, p = 0.0001).

Broad spectrum beta-lactams were associated with increased abundance of:

- *vanA* (5.1 fold change, 95% CI 1.5 to 17, p = 0.009) and
- Aminoglycoside transferase genes (9.9 fold change, 95% CI 2.0 to 49, p = 0.005).

Macrolide and clindamycin use was associated with increased abundance of:

- erm 23S rRNA methyltransferases and *mef* macrolide efflux pumps (36 fold change, 95% CI 4.1 to 324, p = 0.001),
- Aminoglycoside transferases genes (50 fold change, 95% CI 3.5 to 722, p = 0.005) and
- Trimethoprim resistant dihydrofolate reductase (23 fold change, 95% CI 2.5 to 218, p = 0.006).

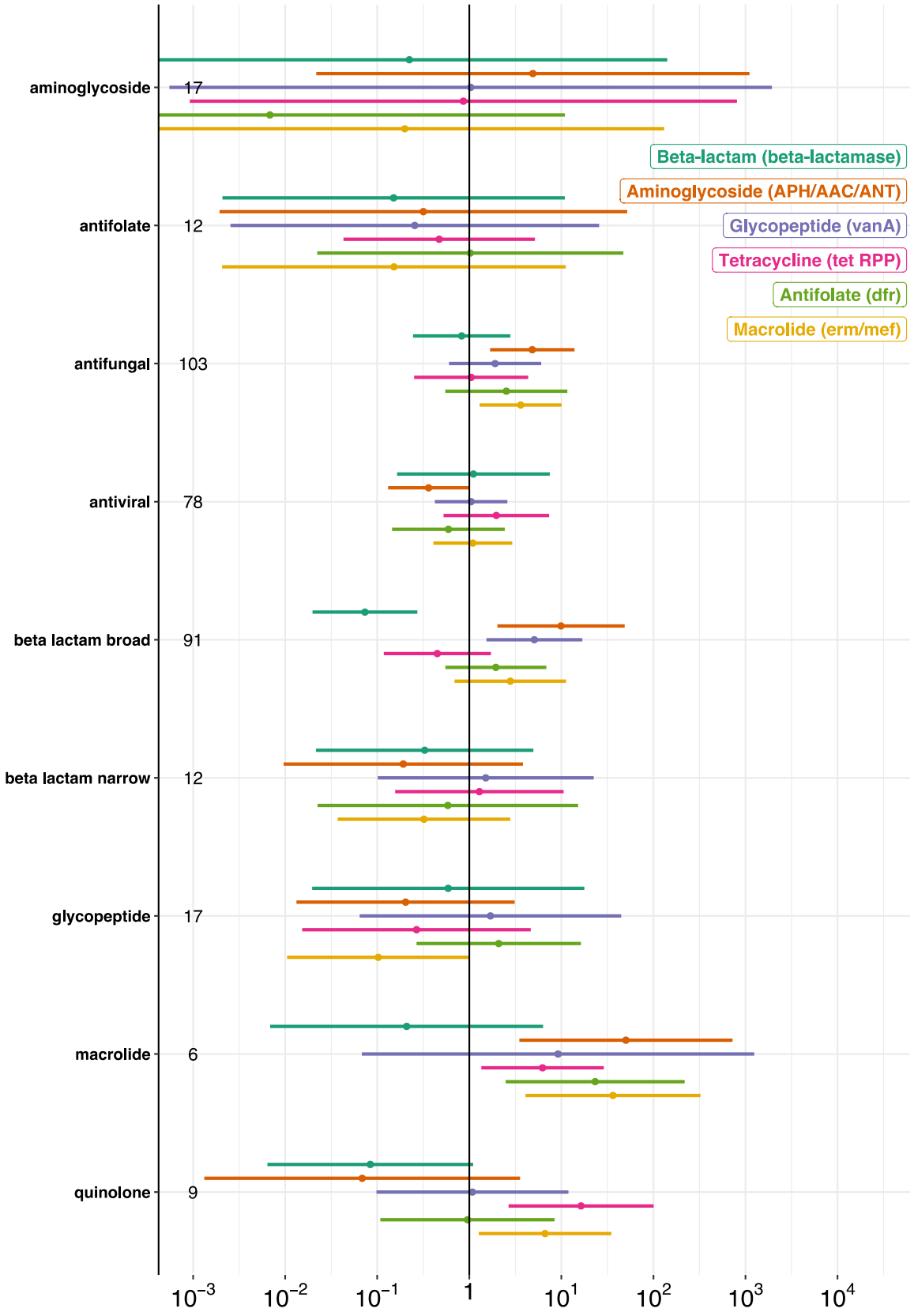

Figure 6.5 Effects of antimicrobials on selected AMR genes (combined exposure measure)

Relative change in abundance of selected AMR genes, using combined exposure measure. Error bars represent 95% confidence intervals. For clarity, results for covariates are not shown, but are adjusted for.

6.3.5 Abundance of organisms causing bacteraemia in the gut during HSCT

Bacteraemia was diagnosed in 30/101 (30%) of HSCT patients during admission, excluding skin flora such as coagulase negative staphylococci that often represent contaminants. *Enterobacteriaceae* and *E. faecium* were the commonest organisms identified, isolated from 14 and 6 patients, respectively. The gut abundance of selected groups of organisms from all sequenced samples in patients developing bacteraemia are shown in **Figure** 6.6.

Enterobacteriaceae bacteraemia typically occurred during the initial period of neutropenia (median 16 days after admission, range 10-30 days). In 16/17 (94%) cases, including 3 patients with two different species from the same culture, the infecting species was detected in the gut around the time of infection. However, the median peak abundance was 2.9%, and in only 3/17 (18%) cases was intestinal domination observed (relative abundance >30%). *E. faecium* bacteraemia often occurred later in admission (median 42 days after admission, range 15-58 days), and was associated with intestinal domination in 5/6 (83%) of patients, with a peak abundance >95% in each. In the sixth patient *E. faecium* was present at an abundance of 14%, but only a single sample was taken 12 days before bacteraemia, so later intestinal domination before the bacteraemia may have been missed. *Pseudomonas aeruginosa* bacteraemia developed in three patients, but in no sample was the organism detected in the gut at an abundance above 0.3%.

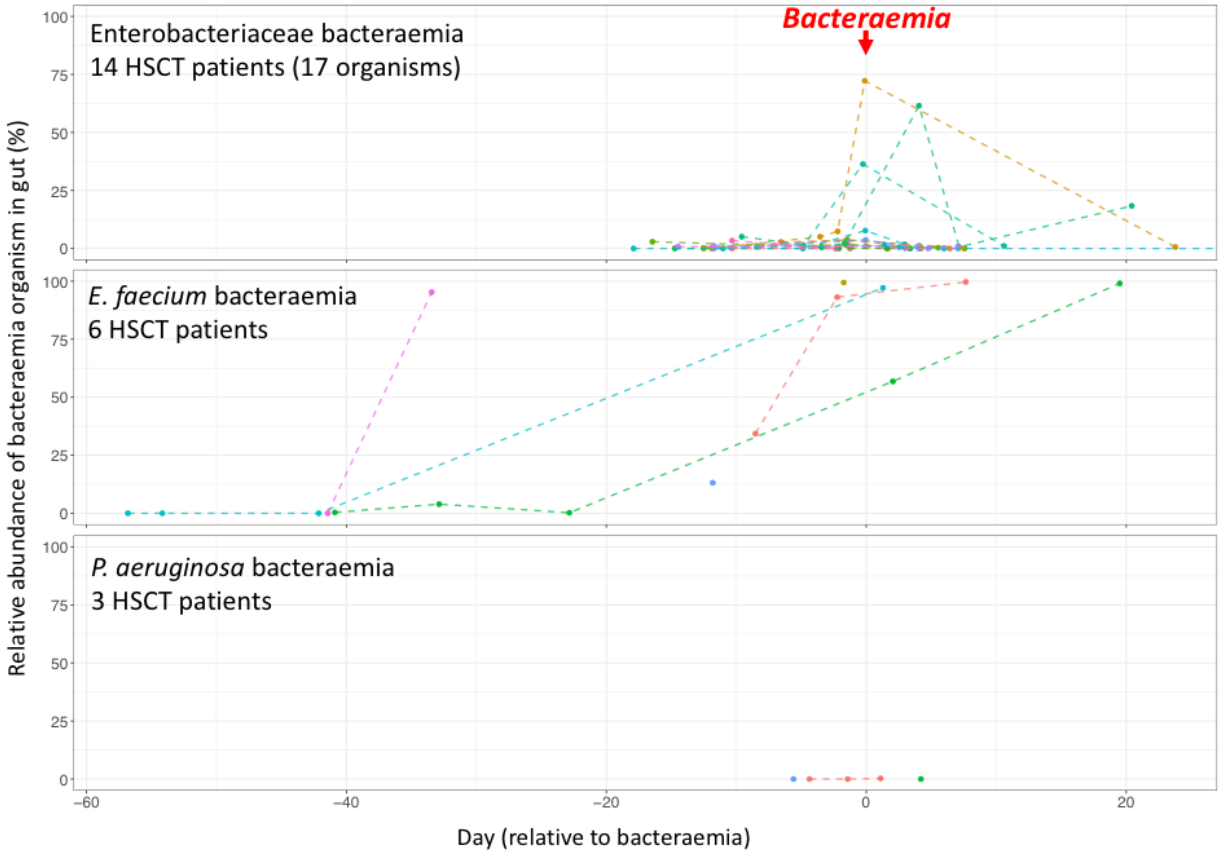

Figure 6.6 Abundance of selected organisms causing bacteraemia in the gut

Colours represent different patients, dashed lines join samples from the same patient. Note, in three patients two different *Enterobacteriaceae* species were isolated simultaneously, each represented by separate line.

6.3.6 Intestinal domination during antibiotic use in ARMORD

Intestinal domination with a single species was studied among all ARMORD participants, including non-HSCT patients. Domination with a relative abundance ≥30% was observed in only 5/33 (15%) healthy volunteers (*Prevotella copri* in 4 and *Bifidobacterium adolescentis* in 1) (**Table** 6.1). Among participants receiving antibiotics at the time of at least one sample, intestinal domination was observed in 78/135 (58%), with quite different organisms being responsible (**Figure** 6.7). *E. faecium* was by far the commonest cause of domination, responsible for 43 (55%) of the 78 domination occurrences, with 38/43 (89%) of these occurrences seen in HSCT patients. Despite this, only 5 (13%) HCST patients with *E. faecium*

domination developed *E. faecium* bacteraemia as described above. *E. faecium* was not a significant component of the gut microbiome of healthy volunteers, being detectable in only 1/33 (3%) participants, in whom its abundance was 0.005%.

Participant group	**Healthy Volunteers** (sample >24h after abs)	**General Medicine** (sample >24h after abs)	**General Medicine** (sample <24h after abs)	**HSCT** (no samples <24h after abs)	**HSCT** (≥1 sample taken <24h after abs)
No. participants (samples)	33 (33)	36 (36)	55 (55)	21 (40)	80 (262)
Domination (≥30%) by any organism	5 (15%)	4 (11%)	20 (36%)	9/21 (43%)	59/80 (74%)
Domination (≥30%) by *E. faecium*	0 (0%)	0 (0%)	5 (9%)	0/21 (0%)	38/80 (48%)
Detection of *E. faecium*	1 (3%)	15 (42%)	33 (60%)	4/21 (19%)	61/80 (76%)

Table 6.1 Intestinal domination in ARMORD samples

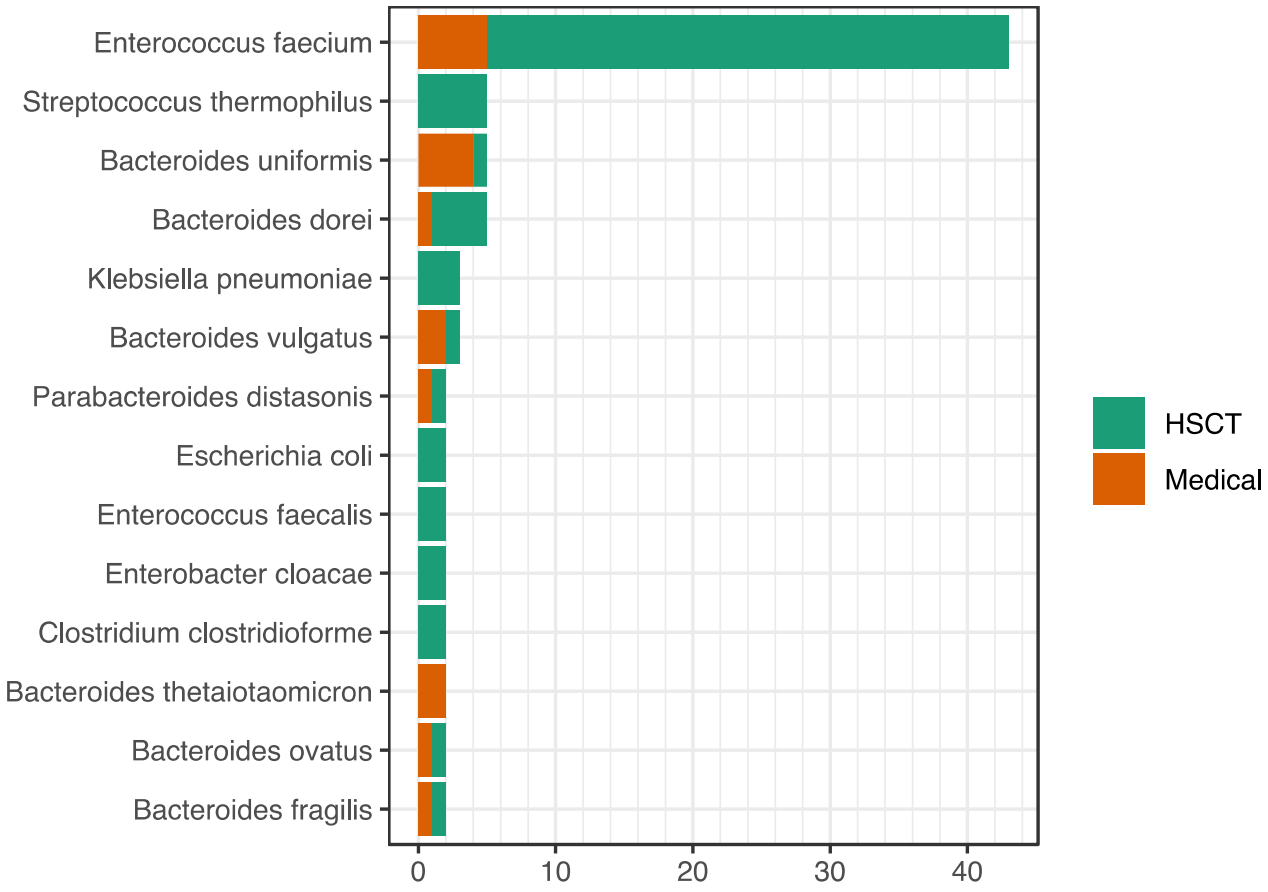

Figure 6.7 Organisms causing intestinal domination in participants on antibiotics

Organisms causing domination in >1 participant are shown. Each organisms is counted once per participant even if domination was present in multiple longitudinal samples.

6.3.7 Effect of antimicrobials in the POETIC study

Fifteen sample pairs were successfully sequenced from 14 women attending primary care with uncomplicated cystitis in the POETIC study (one was recruited twice with a three month interval). Antibiotic exposures observed were cefalexin (n = 4), ciprofloxacin (4), nitrofurantoin (2), and trimethoprim alone or with co-amoxiclav or nitrofurantoin (1 each). Two women did not receive any antibiotics between samples. Mean time between samples was 13 days (range 7-20) and mean duration treatment for those receiving antibiotics was 7 days (range 3-16). In two cases women were still receiving antibiotics at the time the second sample was collected, and in all others antibiotics had stopped 6-12 days beforehand.

No consistent changes in diversity were seen between antibiotic exposures, and in most cases diversity was similar in the first and second samples (**Figure** 6.8). The two pairs with the largest decrease in diversity were those still receiving antibiotics when sample 2 was taken, one of whom received ciprofloxacin and one cefalexin. In one woman receiving ciprofloxacin there was a large increase in diversity between pairs, and in this case sample 1 was notable for a high relative abundance of *Candida albicans* at 8%.

Analysis of the change in specific taxa showed that *Enterobacteriaceae* became undetectable in two women who had received ciprofloxacin (**Figure** 6.9). In contrast, the woman still receiving ciprofloxacin at the time of the second sample had developed intestinal domination with *E. coli*. In this sample the AAC(6')-Ib-cr bifunctional aminoglycoside and fluoroquinolone resistance gene was assembled with 100% identity at an abundance of 178 Read per Kilobase per Million, indicating its probable presence in the dominant *E. coli*.

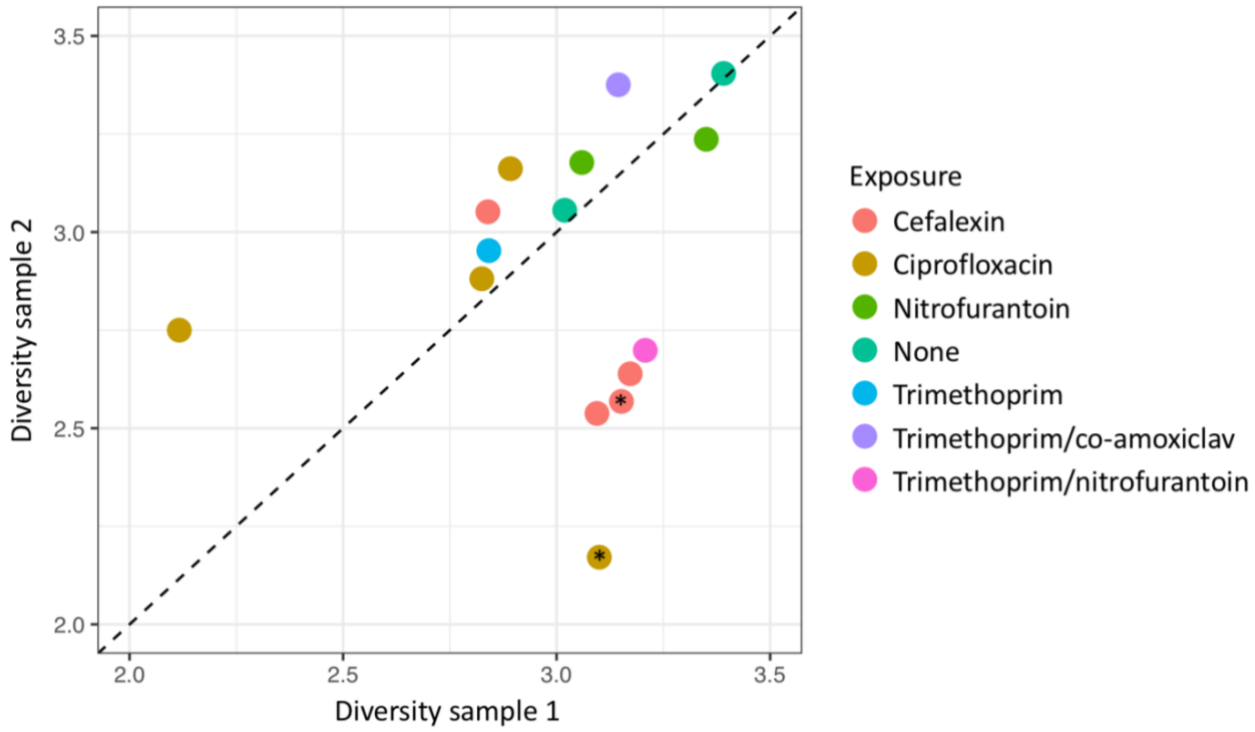

Figure 6.8 Change in Shannon diversity in POETIC sample pairs

Dashed line indicates no change in diversity between samples, starred samples are those still receiving antibiotics at sample 2.

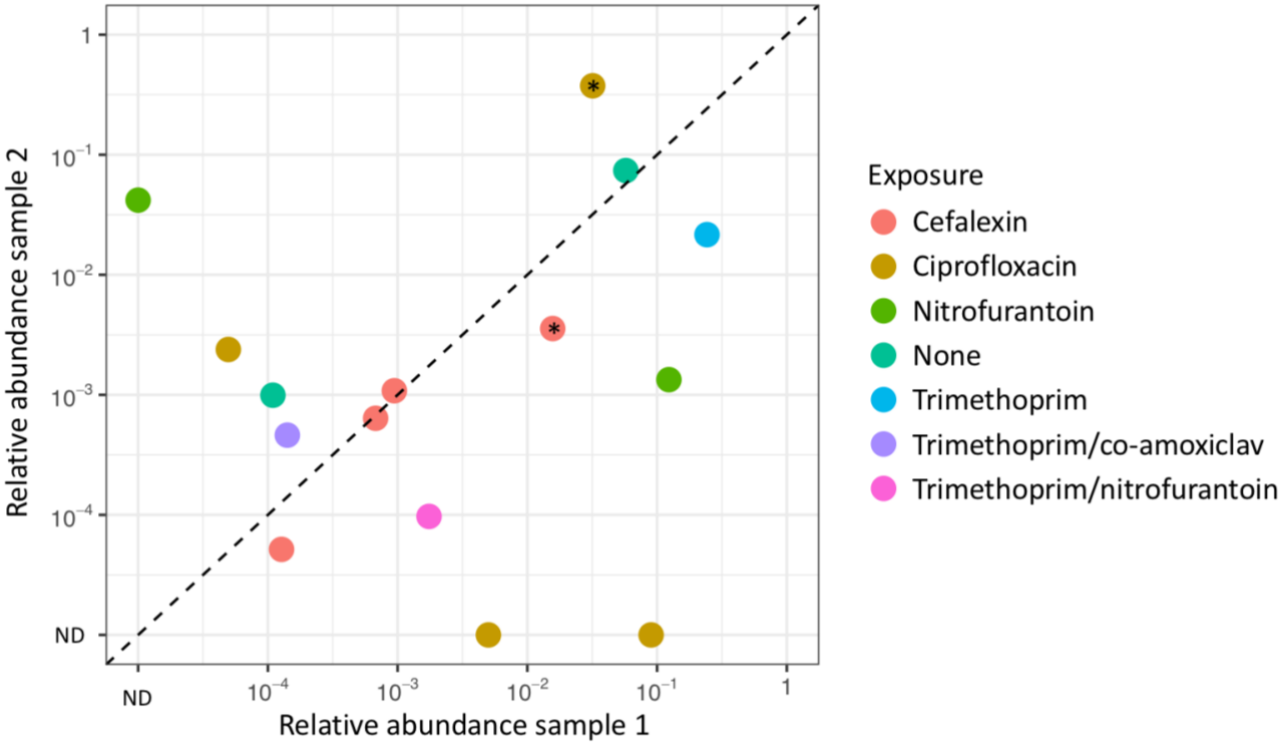

Figure 6.9 Change in abundance of *Enterobacteriaceae* in POETIC sample pairs

Dashed line indicates no change in diversity between samples, starred samples are those still receiving antibiotics at sample 2. ND = not detected.

6.3.8 Inferring host organism of AMR genes

If a bacterium with a 5Mb genome were present at a relative abundance of 1 (i.e. 100%), then the expected relative abundance of reads mapping to a single-copy 1kb AMR gene would be expected to be around $2x10^{-4}$. If the same AMR gene were present at 10 copies/cell it would be expected to have a relative abundance of around $2x10^{-3}$. These ratios (1:$2x10^{-4}$ organism:gene if single copy per cell, 1:$2x10^{-3}$ organism:gene if 10 copies per cell) should stay fixed as the relative abundance of the organism decreases, and can be used to make some limited inferences about the possible host organism of particular AMR genes. The relative abundance of selected organisms and AMR genes in all 478 sequenced ARMORD and POETIC samples are shown in **Figure** 6.10, with dashed lines indicating the approximate ranges above. The presence of an AMR gene much higher than the upper dashed line implies that it is likely to be present in a different organism. Panel A shows the abundance of *E. faecium* and *vanA*, and suggests that this gene was not present in any other abundant organism. In most cases where *vanA* was detected, VRE appeared to be the dominant *E. faecium* strain, but in some samples (circled) VRE probably represented a subpopulation of *E. faecium* (since the ratio suggested it was present on average at under 1 copy/cell). Unlike *vanA*, the circled samples in panel B provide evidence that *vanB*, *vanC*, *vanD* and *vanG* were present in non-enterococci.

Detection of the *E. coli* ampC, shown in panel C, was as expected for a single copy chromosomal gene in most cases, but the circle indicates some samples in which the predominant *E. coli* appears to lack this gene. Panel D shows abundance of the CTX-M beta-lactamase, which appeared to be present in both *E. coli* and *K. pneumoniae* with no evidence of its presence in other organisms. The TEM beta-lactamase, shown in panel E,

appeared almost entirely present in *Enterobacteriaceae*, but the OXA beta-lactamase appeared to be present in some non-*Proteobacteria* (circled).

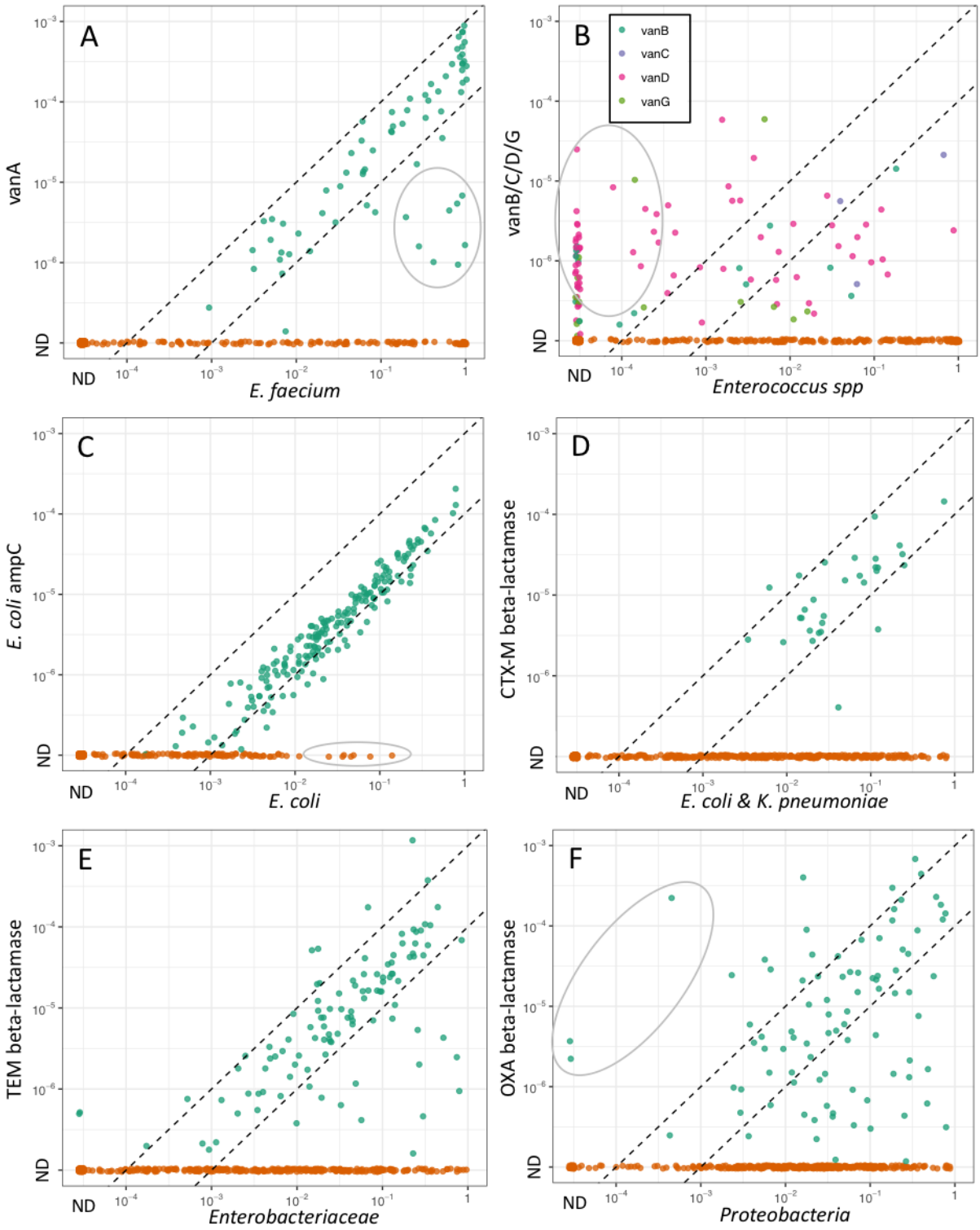

Figure 6.10 Abundance of selected organisms and AMR genes

Dashed lines represent expected range for AMR gene abundance if present in the specified host organism at 1-10 copies/cell.

6.4 Discussion

The estimated effects of antimicrobials on diversity in the longitudinal ARMORD analysis are independent of those from the cross-sectional analysis, but are generally similar for exposures with sufficient data for comparison. This suggests that these approaches can be complementary in observational studies. Despite the decision to use longitudinal sampling to reduce the errors in effect estimates, these were actually larger than in the cross-sectional analysis. This is partly because the number of participants was smaller, but also because treatment was dominated by the two antibiotics commonly used for neutropaenic sepsis, reducing power to detect effects of other antimicrobial exposures. Excluding prophylaxis, few data were available for any antibiotics other than piperacillin-tazobactam and meropenem.

The effects on specific taxa and AMR genes had substantial uncertainty in the longitudinal study, but were largely consistent with estimates from the cross-sectional study. Ciprofloxacin, meropenem and piperacillin-tazobactam were associated with reductions in *Enterobacteriaceae*, and *E. faecium* increased significantly with meropenem and non-significantly with piperacillin-tazobactam. Large decreases in *Clostridiales* and *Actinobacteria* were also seen with these two drugs, effects which were not significant in the cross-sectional data although similar in magnitude. There were some inconsistencies, however, in particular the lack of any reduction in *Clostridiales* and *Actinobacteria* with intravenous vancomycin use in the longitudinal study. This could represent differences in the context in which vancomycin was given, for example, if it was often used following other antibiotics in HSCT then the remaining *Clostridiales* and *Actinobacteria* at baseline for the

sample pairs may differ from those typically present in samples in the cross-sectional analysis. Alternatively, the difference could represent residual confounding in either model.

HSCT patients were recruited because of this was one of very few groups of inpatients where it was possible to obtain samples prior to almost certain antibiotic use. The strategy for recruitment was successful, and this longitudinal cohort is one of the largest for this purpose to date. However, because of the choice of patient population many participants had recently received antibiotics by the time the first sample was collected. Although the gut microbiome of patients undergoing HSCT is of particular interest with regards to infection and GvHD, when studying the impact of antimicrobials the use of chemotherapy and antimicrobial prophylaxis causes some issues of generalisability. For example, intestinal domination with *E. faecium* seems particularly common in HSCT patients, and it is not clear whether this would necessarily be true in other patients with similar antimicrobial exposure.

Intestinal domination with *E. faecium* and *Proteobacteria* has previously been associated with risk of bacteraemia [126]. In this study *E. faecium* domination tended to occur late in admission and frequently preceded bacteraemia, but 38% of the HSCT patients developed *E. faecium* domination at some point and most of these did not develop bacteraemia. Thus while the overall association between domination and bacteraemia is relatively large, its use as a predictor of subsequent bacteraemia may be limited, with positive predictive value only 13%. *Enterobacteriaceae* bacteraemia was more common and was only occasionally associated with intestinal domination, but the species causing bacteraemia was usually detected in the bowel at levels higher than would be typical in health. In contrast,

P. aeruginosa was not detected in the bowel to any significant extent around the time of bacteraemia, suggesting that it is unlikely to have been the source of infecting organisms.

It would be of great interest to discover benign commensal bacteria that are able to dominate the bowel during antibiotics in the manner of *E. faecium*, as these could have potential use as probiotics to reduce the risk of infection or the selection of AMR. Unfortunately, no such organism was apparent in the ARMORD participants, with *E. faecium* seeming to be unique in its ability to dominate the bowel flora during antibiotic exposure. Almost all other bacteria that were observed to cause domination of the intestinal flora during antibiotic use were either common pathogens or were in the same genus as common pathogens, making them unattractive for use as a probiotic.

The POETIC results, which were actually analysed before the main ARMORD results and informed the ARMORD analysis plan, highlight the difficulty of analysing a dataset with small sample size and few data on possible confounders. The limited number of sample pairs sequenced from the POETIC study meant that a similar analysis to that used for ARMORD was impossible, and no clear effect of antibiotics was observed. The use of categorical exposure meant that the amount and recency of exposure was not incorporated into the analysis, although it was notable that the largest decreases in diversity were seen in the two women still receiving antibiotics when the second sample was taken. Although trial participants should not have received antibiotics in the previous four weeks, in one case there may have been undocumented antibiotic exposure at the time of the initial sample as it contained abundant *C. albicans*, which is rare in health and often selected for by antibiotics [234,235].

One reason for using culture independent approaches to analyse AMR genes is the concern that significant resistance mechanisms could exist in organisms that are not routinely culturable. However, although detecting AMR genes in metagenomic data is relatively straightforward, identifying the host organism is difficult, particularly using short read data. No comparative culture data are presented here, but purely metagenomic analysis allowed some inference about the host organisms of important AMR genes. The data suggest that *vanA* and CTX-M genes were only present in typical host organisms, and that nearly all of the TEM genes present were in *Enterobacteriaceae*. OXA beta-lactamases are clinically important AMR genes found primarily in *Acinetobacter*, *Pseudomonas*, *Enterobacteriaceae*, and other *Proteobacteria* [236], yet in this case there was evidence that of some OXA beta-lactamases were present in non-*Proteobacteria*. Relatively little data are available on the host range of most AMR genes outside of pathogens, yet this is likely to become important context for interpreting metagenomic sequencing data if they are to be used routinely in clinical practice. The approach presented here could easily be used in large, publicly available, gut metagenome datasets and may allow better definition of AMR gene host range in non-pathogens.

7 Conclusions and future directions

Increasing AMR in gut organisms is a pressing global health problem, and its control could be greatly aided by reliable, quantitative measures of the impact of different antimicrobials on the gut microbiome. Many studies have described the profound microbiome disruption that can occur with antibiotic use, with recent ones using culture-independent techniques to provide a far more comprehensive view than was previously possible. However, these studies have generally not led to simple measures for each antimicrobial that can be taken into account alongside clinical spectrum, side effects, cost, and other considerations when prescribing or deciding policy. A major aim should be to differentiate antibiotics that might be given for the same clinical indication but have different effects on the microbiome and AMR selection. The wide variation in antibiotic choice for the same indication between different clinicians, different hospitals, and different countries implies that if important differences in AMR selection became clear it could lead to widespread changes practice. For example, treatment of neutropaenic sepsis may variously be with piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, or aztreonam [159,237]. Little published data exist describing the effects of these antibiotics on the gut microbiome, but given the scale of antibiotic use and the AMR problem, even moderate differences in the extent to which these select for resistance would be important, and it is even possible that large differences exist that are currently unrecognised.

In this thesis I have aimed to address the lack of comparative data on antimicrobials. Direct measurement of AMR genes present in the gut microbiome provides an important insight into selection, but metagenomic sequencing is limited in its inability to detect AMR genes

present at low abundance. In **Chapter 3** I explored methods to lower the limit of detection of important AMR genes in a metagenomic sequencing assay. Plasmid purification and culture-enrichment were tested, and although both techniques decreased the limit of detection for selected resistance mechanisms, neither was suitable for use in a quantitative assay. The use of target capture with a custom library of probes provides a promising alternative approach, and two studies published since I conducted my experiments have used this method to successfully sequence scarce AMR genes from direct DNA extracts [173,197]. It is not clear, though, whether this method can provide reliable quantification, and for the most important AMR genes, which are in *Enterobacteriaceae* and enterococci, PCR or quantitative culture may still be preferable. Target capture also remains relatively expensive, making it less attractive to use in the larger gut microbiome studies that are needed in future.

In the remaining work, in **Chapters 5 and 6**, I analysed observational data from the ARMORD gut microbiome study. In doing so I addressed two of the major obstacles to analysing observational data; how to adequately adjust for possible confounding, particularly from different antimicrobial exposures, and how to estimate antimicrobial exposure in a way that can be used in a multiple regression model. The measure of antimicrobial exposure that I used was derived from the cross-sectional dataset, and this suggested that cumulative exposure over the past week had the greatest impact on diversity, but that longer term effects were also relevant. Analysis of the cross-sectional data allowed estimation of the impact of different antimicrobials on overall diversity, abundance of specific taxa and abundance of specific AMR genes. This clearly distinguished many highly disruptive antibiotics, in particular broad-spectrum beta-lactams, ciprofloxacin and clindamycin, from

less disruptive ones, such as azithromycin, doxycycline and low dose co-trimoxazole. This is in keeping with CDI risk and data from some previous culture-based and culture-independent studies, but, as far as I am aware this is the first such analysis to provide direct, quantitative comparisons for a wide range of antimicrobial exposures in the same study. By using similar methods to analyse the longitudinal data I produced estimates consistent with those from the cross-sectional data, and demonstrated that these approaches can be complementary.

The ARMORD data do have some important limitations. Along with age and sex, several markers of acute illness and comorbidity were included in the model to adjust for potential confounding, but there may have been some residual confounding related to factors not included in the model. The antimicrobial exposure measure that I used is almost certainly not optimal, and would ideally be assessed in a validation dataset. Also, because the study is observational, no data are available on some potentially interesting drugs that are not commonly used at OUH, such as imipenem, cefepime and aztreonam. Finally, although this analysis demonstrates how exposure-specific effects can be estimated, the errors are too large to provide measures for practical use. For example, the cross-sectional data are consistent with an identical average reduction in diversity with piperacillin-tazobactam, meropenem, ceftazidime, ceftriaxone, intravenous co-amoxiclav, ciprofloxacin and clindamycin. The data are also consistent with large, clinically important differences between these drugs.

All of these problems could be addressed in a larger observational study. A similar study that was around sixteen times as large, with 4,000 participants, would have standard errors a

quarter the size, and so would be much more likely to demonstrate important differences between broad-spectrum antibiotics. Although microbiome diversity is a useful surrogate for AMR selective pressure, a larger study would allow clearer estimates of many other outcomes as well, including higher resolution assessment of effects on specific taxa and AMR genes. It would also provide a far more robust dataset to understand the dynamics of antimicrobial mediated disruption and establish the optimal antimicrobial exposure measures. Having fewer antimicrobial exposures that are observed in only one or two patients would improve adjustment for confounding, and recruitment from a variety of different settings and centres could mean that data were available from a wider range of drugs.

Although such a study would be relatively straightforward to perform compared to a similar sized interventional trial, it would have to be significantly more streamlined than ARMORD. Accurate data on exposure and potential confounders is important, and the availability of hospital EPR data makes a large study much more practicable, although merging data from different EPR systems could be problematic. Electronic prescribing data from primary care was not available for ARMORD, and instead this relied mainly on patient recall. Large scale linkage of electronic data on primary care prescribing with secondary care EPR could avoid this problem and make a large study much more feasible.

Focussing recruitment on hospital inpatients, as in ARMORD, is likely to be the most efficient recruitment strategy. As well as a baseline sample, longitudinal samples could also be taken opportunistically from any willing participants who remain in hospital, rather than by targeting a particular patient group for longitudinal sampling. In ARMORD, HSCT patients

were chosen for longitudinal recruitment because of the difficulty of identifying any other patients who were about to receive antibiotics, and particularly obtaining any samples before the initial antibiotic was received. However, recruitment of HSCT patients took the majority of my time and produced less precise estimates than the cross-sectional data. Despite the attempt to obtain samples before antibiotics, many of the HSCT patients had had recent exposure at the time the first sample was taken. These patients also have the disadvantage of frequently receiving multiple antimicrobials simultaneously as well as chemotherapy. This focus on a quasi-experimental design, i.e. aiming to collect samples around an anticipated course of antibiotics, may the wrong approach to longitudinal sampling, as it is unnecessary when using a robust measures of antimicrobial exposure in a multivariable model.

As well as larger observational studies, it would be useful if the primary data from different studies could be combined, but this is not possible with previous publications in the field. Uploading metagenomic sequence data to public databases has become common practice, and has allowed some striking combined analyses [37,41]. If gut microbiome studies also released data on antimicrobial exposure and a minimum set of covariates, it would allow meta-analysis and maximise their value to other researchers.

Finally, although results from future observational studies may be clear enough to directly inform practice, one of their roles will be to provide hypotheses to inform interventional studies, particularly randomised controlled trials. Currently, there are little data on microbiome disruption or AMR selection from trials, but they provide the ideal setting to produce reliable comparisons. Almost any trial comparing different treatment strategies

could incorporate microbiome outcomes along with clinical ones, adding another dimension to the results. Because differences in microbiome impact are likely to be larger than differences in clinical outcome, these measures may only be needed from a subgroup of patients. If the use of microbiome and resistome measures became routine in future trials, it would rapidly produce large amounts of data to inform antimicrobial stewardship and aid the control of AMR.

8 References

1. Baillie L, Hawe E. Causes of Death: A Study of a Century of Change in England and Wales. Office for Health Economics. 2012 Oct.

2. Roth GA, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet. 2018;392: 1736–1788. doi:10.1016/S0140-6736(18)32203-7

3. O'Neill J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. 2016 May.

4. Godfrey C, Thigpen MC, Crawford KW, Jean-Phillippe P, Pillay D, Persaud D, et al. Global HIV Antiretroviral Drug Resistance. J Infect Dis. 2017;216: S798–S800. doi:10.1093/infdis/jix137

5. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of Artemisinin Resistance in Plasmodium falciparumMalaria. N Engl J Med. 2014;371: 411–423. doi:10.1056/NEJMoa1314981

6. World Health Organization. WHO | Global tuberculosis report 2018. WHO. World Health Organization. Available: https://www.who.int/tb/publications/global_report/en/

7. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486: 207–214. doi:10.1038/nature11234

8. National Institute for Clinical Excellence (NICE). Antimicrobial stewardship: systems and processes for effective antimicrobial medicine use, 2015 [Internet]. 2015 Aug. Available: https://www.nice.org.uk/guidance/ng15

9. Jensen A, Valdórsson O, Frimodt-Møller N, Hollingshead S, Kilian M. Commensal Streptococci Serve as a Reservoir for β-Lactam Resistance Genes in Streptococcus pneumoniae. Antimicrob Agents Chemother. 2015;59: 3529–3540. doi:10.1128/AAC.00429-15

10. Smyth DS, Wong A, Robinson DA. Cross-species spread of SCCmec IV subtypes in staphylococci. Infection, Genetics and Evolution. 2011;11: 446–453. doi:10.1016/j.meegid.2010.12.005

11. Cantón R, González-Alba JM, Galán JC. CTX-M Enzymes: Origin and Diffusion. Front Microbiol. 2012;3. doi:10.3389/fmicb.2012.00110

12. Drasar BS, Shiner M, McLeod GM. Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. Gastroenterology. 1969;56: 71–79.

13. Gerhardt NB, Iglewski WJ. Alterations in the Fecal Flora of an Individual Frequently Lacking Coliforms. J Infect Dis. 1976;134: 490–494. doi:10.1093/infdis/134.5.490

14. Bennet JE, Dolin R, Blaser MJ. Mandell, Douglas and Bennet's Principles and practice of infectious diseases, 8th edition. 2015.

15. Ruppé E, Woerther P-L, Barbier F. Mechanisms of antimicrobial resistance in Gram-negative bacilli. Ann Intensive Care. Springer Paris; 2015;5: 61. doi:10.1186/s13613-015-0061-0

16. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. - PubMed - NCBI. The Lancet Infectious Diseases. 2010;10: 597–602. doi:10.1016/S1473-3099(10)70143-2

17. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet Infectious Diseases. 2016;16: 161–168. doi:10.1016/S1473-3099(15)00424-7

18. Werner G, Coque TM, Franz CMAP, Grohmann E, Hegstad K, Jensen L, et al. Antibiotic resistant enterococci—Tales of a drug resistance gene trafficker. International Journal of Medical Microbiology. 2013;303: 360–379. doi:10.1016/j.ijmm.2013.03.001

19. Wernli D, Jørgensen PS, Harbarth S, Carroll SP, Laxminarayan R, Levrat N, et al. Antimicrobial resistance: The complex challenge of measurement to inform policy and the public. PLoS Med. Public Library of Science; 2017;14: e1002378. doi:10.1371/journal.pmed.1002378

20. Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, et al. Antibiotic resistance—the need for global solutions. The Lancet Infectious Diseases. Elsevier; 2013;13: 1057–1098. doi:10.1016/S1473-3099(13)70318-9

21. Investigators of the Delhi Neonatal Infection Study (DeNIS) collaboration. Characterisation and antimicrobial resistance of sepsis pathogens in neonates born in tertiary care centres in Delhi, India: a cohort study. The Lancet Global Health. 2016;4: e752–e760. doi:10.1016/S2214-109X(16)30148-6

22. Gandra S, Mojica N, Klein EY, Ashok A, Nerurkar V, Kumari M, et al. Trends in antibiotic resistance among major bacterial pathogens isolated from blood cultures tested at a large private laboratory network in India, 2008–2014. International Journal of Infectious Diseases. 2016;50: 75–82. doi:10.1016/j.ijid.2016.08.002

23. The European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe 2017 [Internet]. 2017 Jan. Available: https://ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2017

24. Public Health England. English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) report. 2018. Available: https://www.gov.uk/government/publications/english-surveillance-programme-antimicrobial-utilisation-and-resistance-espaur-report

25. Decraene V, Phan HTT, George R, Wyllie DH, Akinremi O, Aiken Z, et al. A Large, Refractory Nosocomial Outbreak of Klebsiella pneumoniaeCarbapenemase-Producing Escherichia coliDemonstrates Carbapenemase Gene Outbreaks Involving Sink Sites Require Novel Approaches to Infection Control. Antimicrob Agents Chemother. 2018;62: 785. doi:10.1128/AAC.01689-18

26. Forslund K, Sunagawa S, Coelho LP, Bork P. Metagenomic insights into the human gut resistome and the forces that shape it. BioEssays. 2014;36: 316–329. doi:10.1002/bies.201300143

27. Forslund K, Sunagawa S, Kultima JR, Mende DR, Arumugam M, Typas A, et al. Country-specific antibiotic use practices impact the human gut resistome. Genome Res. Cold Spring Harbor Lab; 2013;23: 1163–1169. doi:10.1101/gr.155465.113

28. Yang Z, Guo Z, Qiu C, Li Y, Feng X, Liu Y, et al. Preliminary analysis showed country-specific gut resistome based on 1,267 feces samples. Gene. 2016;581: 178–182. doi:10.1016/j.gene.2016.01.043

29. World Health Organization. Global antimicrobial surveillance system (GLASS) report 2017-2018 [Internet]. 2018 Jan. Available: https://www.who.int/glass/resources/publications/early-implementation-report-2017-2018/en/

30. Chief Medical Officer. Annual report of the chief medical officer 2011: volume two [Internet]. Available: https://www.gov.uk/government/publications/chief-medical-officer-annual-report-volume-2

31. Public Health England. Annual epidemiological commentary: Gram-negative bacteraemia, MRSA bacteraemia, MSSA bacteraemia and C. difficile infections, up to and including financial year April 2018 to March 2019 [Internet]. 2019. Available: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/815449/Annual_epidemiological_commentary_April_2017_to_March_2019.pdf

32. Public Health England. UK One Health Report Joint report on antibiotic use and antibiotic resistance, 2013–2017 [Internet]. 2019 Jan. Available: https://www.gov.uk/government/publications/uk-one-health-report-antibiotic-use-and-antibiotic-resistance-in-animals-and-humans

33. Donskey CJ. The Role of the Intestinal Tract as a Reservoir and Source for Transmission of Nosocomial Pathogens. Clin Infect Dis. 2004;39: 219–226. doi:10.1086/422002

34. Ruppé E, Burdet C, Grall N, de Lastours V, Lescure FX, Andremont A, et al. Impact of antibiotics on the intestinal microbiota needs to be re-defined to optimize antibiotic

usage. Clinical Microbiology and Infection. Elsevier; 2018;24: 3–5. doi:10.1016/j.cmi.2017.09.017

35. Woerther P-L, Lepeule R, Burdet C, Decousser J-W, Ruppé E, Barbier F. Carbapenems and alternative β-lactams for the treatment of infections due to extended-spectrum β-lactamase-producing Enterobacteriaceae: What impact on intestinal colonisation resistance? Int J Antimicrob Agents. 2018;52: 762–770. doi:10.1016/j.ijantimicag.2018.08.026

36. Donskey CJ. Antibiotic regimens and intestinal colonization with antibiotic-resistant gram-negative bacilli. Clin Infect Dis. 2nd ed. 2006;43 Suppl 2: S62–9. doi:10.1086/504481

37. Pasolli E, Asnicar F, Manara S, Zolfo M, Karcher N, Armanini F, et al. Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. Cell. Cell Press; 2019;176: 649–662.e20. doi:10.1016/j.cell.2019.01.001

38. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLOS Biol. Public Library of Science; 2016;14: e1002533. doi:10.1371/journal.pbio.1002533

39. Rajilić-Stojanović M, de Vos WM. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiology Reviews. 2014;38: 996–1047. doi:10.1111/1574-6976.12075

40. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308: 1635–1638. doi:10.1126/science.1110591

41. Almeida A, Mitchell AL, Boland M, Forster SC, Gloor GB, Tarkowska A, et al. A new genomic blueprint of the human gut microbiota. Nature. Nature Publishing Group; 2019;568: 499–504. doi:10.1038/s41586-019-0965-1

42. Consortium THMP. A framework for human microbiome research. Nature. Nature Research; 2012;486: 215–221. doi:10.1038/nature11209

43. Li K, Bihan M, Yooseph S, Methé BA. Analyses of the microbial diversity across the human microbiome. Xu P, editor. PLoS ONE. Public Library of Science; 2012;7: e32118. doi:10.1371/journal.pone.0032118

44. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. - PubMed - NCBI. Science. 2013;341: 1237439. doi:10.1126/science.1237439

45. Stojanović MR, Heilig HGHJ, Tims S, Zoetendal EG, de Vos WM. Long-term monitoring of the human intestinal microbiota composition. Environmental Microbiology. John Wiley & Sons, Ltd (10.1111); 2013;15: 1146–1159. doi:10.1111/1462-2920.12023

46. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464: 59–65. doi:10.1038/nature08821

47. Zimmer J, Lange B, Frick J-S, Sauer H, Zimmermann K, Schwiertz A, et al. A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. European Journal of Clinical Nutrition 2011 66:1. Nature Publishing Group; 2012;66: 53–60. doi:10.1038/ejcn.2011.141

48. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555: 623–628. doi:10.1038/nature25979

49. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. - PubMed - NCBI. Science. 2016;352: 565–569. doi:10.1126/science.aad3369

50. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449: 811–818. doi:10.1038/nature06245

51. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol. 2012;9: 577–589. doi:10.1038/nrgastro.2012.156

52. Gentile CL, Weir TL. The gut microbiota at the intersection of diet and human health. Science. American Association for the Advancement of Science; 2018;362: 776–780. doi:10.1126/science.aau5812

53. Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nature Immunology 2013 14:7. Nature Publishing Group; 2013;14: 668–675. doi:10.1038/ni.2635

54. Fulde M, Hornef MW. Maturation of the enteric mucosal innate immune system during the postnatal period. Immunol Rev. 2014;260: 21–34. doi:10.1111/imr.12190

55. Sommer F, Bäckhed F. The gut microbiota — masters of host development and physiology. Nat Rev Microbiol. Nature Publishing Group; 2013;11: 227–238. doi:10.1038/nrmicro2974

56. Lynch SV, Pedersen O. The Human Intestinal Microbiome in Health and Disease. Phimister EG, editor. N Engl J Med. 2016;375: 2369–2379. doi:10.1056/NEJMra1600266

57. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut. BMJ Publishing Group Ltd and British Society of Gastroenterology; 2016;65: 330–339. doi:10.1136/gutjnl-2015-309990

58. Dobell C. The Discovery of the Intestinal Protozoa of Man. Proc R Soc Med. 1920;13: 1–15.

59. Thiel R, Blaut M. An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. Journal of Microbiological Methods. Elsevier; 2005;61: 369–379. doi:10.1016/j.mimet.2004.12.014

60. Fraher MH, O'Toole PW, Quigley EMM. Techniques used to characterize the gut microbiota: a guide for the clinician. Nat Rev Gastroenterol Hepatol. 2012;9: 312–322. doi:10.1038/nrgastro.2012.44

61. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol. 1999;65: 4799–4807.

62. Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics: paradigm shift in the human gut microbiome study. Clinical Microbiology and Infection. Elsevier; 2012;18: 1185–1193. doi:10.1111/1469-0691.12023

63. Rettedal EA, Gumpert H, Sommer MOA. Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. Nat Commun. 2014;5: 4714. doi:10.1038/ncomms5714

64. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing of “unculturable” human microbiota reveals novel taxa and extensive sporulation. Nature. 2016;533: 543–546. doi:10.1038/nature17645

65. Lagier J-C, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of previously uncultured members of the human gut microbiota by culturomics. Nat Microbiol. 2016;1: 136. doi:10.1038/nmicrobiol.2016.203

66. Lau JT, Whelan FJ, Herath I, Lee CH, Collins SM, Bercik P, et al. Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. - PubMed - NCBI. Genome Med. 2016;8: 1635. doi:10.1186/s13073-016-0327-7

67. Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. Nat Rev Microbiol. 2018;16: 540–550. doi:10.1038/s41579-018-0041-0

68. Kitahara K, Miyazaki K. Revisiting bacterial phylogeny. Mobile Genetic Elements. 2014;3: e24210. doi:10.4161/mge.24210

69. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology. Nature Publishing Group; 2019;339: 1. doi:10.1038/s41587-019-0209-9

70. Sleator RD, Shortall C, Hill C. Metagenomics. Letters in Applied Microbiology. John Wiley & Sons, Ltd (10.1111); 2008;47: 361–366. doi:10.1111/j.1472-765X.2008.02444.x

71. Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nature Methods. Nature Publishing Group; 2012;9: 811–814. doi:10.1038/nmeth.2066

72. Staley C, Kaiser T, Khoruts A. Clinician Guide to Microbiome Testing. Dig Dis Sci. Springer US; 2018;63: 3167–3177. doi:10.1007/s10620-018-5299-6

73. Hillmann B, Al-Ghalith GA, Shields-Cutler RR, Zhu Q, Gohl DM, Beckman KB, et al. Evaluating the Information Content of Shallow Shotgun Metagenomics. Rawls JF, editor. mSystems. American Society for Microbiology Journals; 2018;3: e00069–18. doi:10.1128/mSystems.00069-18

74. Truong DT, Tett A, Pasolli E, Huttenhower C, Segata N. Microbial strain-level population structure and genetic diversity from metagenomes. Genome Res. Cold Spring Harbor Lab; 2017;27: 626–638. doi:10.1101/gr.216242.116

75. Mu A, Kwong JC, Isles NS, da Silva AG, Schultz MB, Ballard SA, et al. Reconstruction of the Genomes of Drug-Resistant Pathogens for Outbreak Investigation through Metagenomic Sequencing. Gales AC, editor. mSphere. American Society for Microbiology Journals; 2019;4: e00529–18. doi:10.1128/mSphere.00529-18

76. Flynn KJ, Ruffin MT IV, Turgeon DK, Schloss PD. Spatial Variation of the Native Colon Microbiota in Healthy Adults. Cancer Prev Res. 2018;11: 393–402. doi:10.1158/1940-6207.CAPR-17-0370

77. Tropini C, Earle KA, Huang KC, Sonnenburg JL. The Gut Microbiome: Connecting Spatial Organization to Function. Cell Host Microbe. 2017;21: 433–442. doi:10.1016/j.chom.2017.03.010

78. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. Moschetta A, editor. PLoS ONE. Public Library of Science; 2012;7: e39743. doi:10.1371/journal.pone.0039743

79. Tang MS, Poles J, Leung JM, Wolff MJ, Davenport M, Lee SC, et al. Inferred metagenomic comparison of mucosal and fecal microbiota from individuals undergoing routine screening colonoscopy reveals similar differences observed during active inflammation. Gut Microbes. 2015;6: 48–56. doi:10.1080/19490976.2014.1000080

80. Bassis CM, Moore NM, Lolans K, Seekatz AM, Weinstein RA, Young VB, et al. Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiology 2017 17:1. BioMed Central; 2017;17: 78. doi:10.1186/s12866-017-0983-9

81. Biehl LM, Garzetti D, Farowski F, Ring D, Koeppel MB, Rohde H, et al. Usability of rectal swabs for microbiome sampling in a cohort study of hematological and oncological patients. Alekseyenko AV, editor. PLoS ONE. Public Library of Science; 2019;14: e0215428. doi:10.1371/journal.pone.0215428

82. Tedjo DI, Jonkers DMAE, Savelkoul PH, Masclee AA, van Best N, Pierik MJ, et al. The Effect of Sampling and Storage on the Fecal Microbiota Composition in Healthy and Diseased Subjects. Favia G, editor. PLoS ONE. Public Library of Science; 2015;10: e0126685. doi:10.1371/journal.pone.0126685

83. Vandeputte D, Tito RY, Vanleeuwen R, Falony G, Raes J. Practical considerations for large-scale gut microbiome studies. FEMS Microbiology Reviews. 2017;41: S154–S167. doi:10.1093/femsre/fux027

84. Carroll IM, Ringel-Kulka T, Siddle JP, Klaenhammer TR, Ringel Y. Characterization of the Fecal Microbiota Using High-Throughput Sequencing Reveals a Stable Microbial Community during Storage. Neufeld J, editor. PLoS ONE. Public Library of Science; 2012;7: e46953. doi:10.1371/journal.pone.0046953

85. Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Doré J, et al. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiology 2017 17:1. BioMed Central; 2012;12: 158. doi:10.1186/1471-2180-12-158

86. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. Nature Publishing Group; 2015;5: 16350. doi:10.1038/srep16350

87. Shaw AG, Sim K, Powell E, Cornwell E, Cramer T, McClure ZE, et al. Latitude in sample handling and storage for infant faecal microbiota studies: the elephant in the room? Microbiome. BioMed Central; 2016;4: 40. doi:10.1186/s40168-016-0186-x

88. Wesolowska-Andersen A, Bahl MI, Carvalho V, Kristiansen K, Sicheritz-Ponten T, Gupta R, et al. Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. Microbiome. BioMed Central; 2014;2: 19. doi:10.1186/2049-2618-2-19

89. Costea PI, Zeller G, Sunagawa S, Pelletier E, Alberti A, Levenez F, et al. Towards standards for human fecal sample processing in metagenomic studies. Nature Biotechnology. 2017;35: 1069–1076. doi:10.1038/nbt.3960

90. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of Methods for the Extraction and Purification of DNA from the Human Microbiome. Gilbert JA, editor. PLoS ONE. Public Library of Science; 2012;7: e33865. doi:10.1371/journal.pone.0033865

91. Zaheer R, Noyes N, Polo RO, Cook SR, Marinier E, Van Domselaar G, et al. Impact of sequencing depth on the characterization of the microbiome and resistome. Sci Rep. Nature Publishing Group; 2018;8: 5890. doi:10.1038/s41598-018-24280-8

92. Satinsky BM, Gifford SM, Crump BC, Moran MA. Use of internal standards for quantitative metatranscriptome and metagenome analysis. Meth Enzymol. 2013;531: 237–250. doi:10.1016/B978-0-12-407863-5.00012-5

93. Stämmler F, Gläsner J, Hiergeist A, Holler E, Weber D, Oefner PJ, et al. Adjusting microbiome profiles for differences in microbial load by spike-in bacteria. Microbiome. BioMed Central; 2016;4: 28. doi:10.1186/s40168-016-0175-0

94. Hardwick SA, Chen WY, Wong T, Kanakamedala BS, Deveson IW, Ongley SE, et al. Synthetic microbe communities provide internal reference standards for metagenome sequencing and analysis. Nat Commun. Nature Publishing Group; 2018;9: 3096. doi:10.1038/s41467-018-05555-0

95. Breitwieser FP, Lu J, Salzberg SL. A review of methods and databases for metagenomic classification and assembly. Brief Bioinformatics. 2017. doi:10.1093/bib/bbx120

96. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biology 2014 12:1. BioMed Central; 2014;12: 87. doi:10.1186/s12915-014-0087-z

97. Mathers AJ, Stoesser N, Chai W, Carroll J, Barry K, Cherunvanky A, et al. Chromosomal Integration of the Klebsiella pneumoniae Carbapenemase Gene, blaKPC, in Klebsiella Species Is Elusive but Not Rare. Antimicrob Agents Chemother. American Society for Microbiology Journals; 2017;61: e01823–16. doi:10.1128/AAC.01823-16

98. Souverein D, Euser SM, van der Reijden WA, Herpers BL, Kluytmans J, Rossen JWA, et al. Clinical sensitivity and specificity of the Check-Points Check-Direct ESBL Screen for BD MAX, a real-time PCR for direct ESBL detection from rectal swabs. J Antimicrob Chemother. 2017;72: 2512–2518. doi:10.1093/jac/dkx189

99. Naas T, Ergani A, Carrër A, Nordmann P. Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples. Antimicrob Agents Chemother. American Society for Microbiology; 2011;55: 4038–4043. doi:10.1128/AAC.01734-10

100. Viau R, Frank KM, Jacobs MR, Wilson B, Kaye K, Donskey CJ, et al. Intestinal Carriage of Carbapenemase-Producing Organisms: Current Status of Surveillance Methods. - PubMed - NCBI. Clin Microbiol Rev. 2015;29: 1–27. doi:10.1128/CMR.00108-14

101. Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. PNAS. 2013;110: 3435–3440. doi:10.1073/pnas.1222743110

102. Lu N, Hu Y, Zhu L, Yang X, Yin Y, Lei F, et al. DNA microarray analysis reveals that antibiotic resistance-gene diversity in human gut microbiota is age related. Sci Rep. Nature Publishing Group; 2014;4: 4302. doi:10.1038/srep04302

103. Card RM, Warburton PJ, MacLaren N, Mullany P, Allan E, Anjum MF. Application of microarray and functional-based screening methods for the detection of antimicrobial

resistance genes in the microbiomes of healthy humans. Aziz RK, editor. PLoS ONE. Public Library of Science; 2014;9: e86428. doi:10.1371/journal.pone.0086428

104. Ruppé E, Chatelier EL, Pons N, Andremont A, Ehrlich SD. Dissemination of the mcr-1 colistin resistance gene. The Lancet Infectious Diseases. 2016;16: 290–291. doi:10.1016/S1473-3099(16)00066-9

105. Stalder T, Press MO, Sullivan S, Liachko I, Top EM. Linking the resistome and plasmidome to the microbiome. ISME J. Nature Publishing Group; 2019;9: e00105. doi:10.1038/s41396-019-0446-4

106. Grall N, Lazarevic V, Gaïa N, Couffignal C, Laouénan C, Ilic-Habensus E, et al. Unexpected persistence of extended-spectrum β-lactamase-producing Enterobacteriaceae in the faecal microbiota of hospitalised patients treated with imipenem. Int J Antimicrob Agents. 2017;50: 81–87. doi:10.1016/j.ijantimicag.2017.02.018

107. Levy SB, Marshall B, Schluederberg S, Rowse D, Davis J. High frequency of antimicrobial resistance in human fecal flora. Antimicrob Agents Chemother. American Society for Microbiology Journals; 1988;32: 1801–1806. doi:10.1128/AAC.32.12.1801

108. GUINEY DG, DAVIS CE. Identification of a conjugative R plasmid in Bacteroides ochraceus capable of transfer to Escherichia coli. Nature. Nature Publishing Group; 1978;274: 181–182. doi:10.1038/274181a0

109. Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. Evidence for Extensive Resistance Gene Transfer among Bacteroides spp. and among Bacteroides and Other Genera in the Human Colon. Appl Environ Microbiol. 2001;67: 561–568. doi:10.1128/AEM.67.2.561-568.2001

110. Sheppard AE, Stoesser N, Wilson DJ, Sebra R, Kasarskis A, Anson LW, et al. Nested Russian Doll-Like Genetic Mobility Drives Rapid Dissemination of the Carbapenem Resistance Gene blaKPC. Antimicrob Agents Chemother. American Society for Microbiology; 2016;60: 3767–3778. doi:10.1128/AAC.00464-16

111. Rasmussen BA, Gluzman Y, Tally FP. Cloning and sequencing of the class B beta-lactamase gene (ccrA) from Bacteroides fragilis TAL3636. Antimicrob Agents Chemother. American Society for Microbiology Journals; 1990;34: 1590–1592. doi:10.1128/aac.34.8.1590

112. Rasmussen BA, Gluzman Y, Tally FP. Escherichia coli chromosomal mutations that permit direct cloning of the Bacteroides fragiiis metallo-β-lactamase gene, ccrA. Molecular Microbiology. John Wiley & Sons, Ltd (10.1111); 1991;5: 1211–1219. doi:10.1111/j.1365-2958.1991.tb01895.x

113. van Schaik W. The human gut resistome. Philos Trans R Soc Lond, B, Biol Sci. The Royal Society; 2015;370: 20140087. doi:10.1098/rstb.2014.0087

114. Penders J, Stobberingh EE, Savelkoul PHM, Wolffs PFG. The human microbiome as a reservoir of antimicrobial resistance. Front Microbiol. Frontiers; 2013;4: 87. doi:10.3389/fmicb.2013.00087

115. Sommer MOA, Church GM, Dantas G. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence. 2014;1: 299–303. doi:10.4161/viru.1.4.12010

116. Patterson AJ, Rincon MT, Flint HJ, Scott KP. Mosaic Tetracycline Resistance Genes Are Widespread in Human and Animal Fecal Samples. Antimicrob Agents Chemother. 2007;51: 1115–1118. doi:10.1128/AAC.00725-06

117. Seville LA, Patterson AJ, Scott KP, Mullany P, Quail MA, Parkhill J, et al. Distribution of Tetracycline and Erythromycin Resistance Genes Among Human Oral and Fecal Metagenomic DNA. Microbial Drug Resistance. 2009;15: 159–166. doi:10.1089/mdr.2009.0916

118. Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat Commun. Nature Publishing Group; 2013;4: 2151. doi:10.1038/ncomms3151

119. Santiago-Rodriguez TM, Fornaciari G, Luciani S, Dowd SE, Toranzos GA, Marota I, et al. Gut Microbiome of an 11th Century A.D. Pre-Columbian Andean Mummy. Wilson BA, editor. PLoS ONE. Public Library of Science; 2015;10: e0138135. doi:10.1371/journal.pone.0138135

120. Santiago-Rodriguez TM, Fornaciari G, Luciani S, Toranzos GA, Marota I, Giuffra V, et al. Gut Microbiome and Putative Resistome of Inca and Italian Nobility Mummies. Genes 2017, Vol 8, Page 310. Multidisciplinary Digital Publishing Institute; 2017;8: 310. doi:10.3390/genes8110310

121. Nordahl Petersen T, Rasmussen S, Hasman H, Carøe C, Bælum J, Schultz AC, et al. Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. Sci Rep. 2015;5: 11444. doi:10.1038/srep11444

122. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. The structure and diversity of human, animal and environmental resistomes. Microbiome. BioMed Central; 2016;4: 54. doi:10.1186/s40168-016-0199-5

123. Ma L, Xia Y, Li B, Yang Y, Li L-G, Tiedje JM, et al. Metagenomic Assembly Reveals Hosts of Antibiotic Resistance Genes and the Shared Resistome in Pig, Chicken, and Human Feces. Environ Sci Technol. 2016;50: 420–427. doi:10.1021/acs.est.5b03522

124. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The shared antibiotic resistome of soil bacteria and human pathogens. Science. American Association for the Advancement of Science; 2012;337: 1107–1111. doi:10.1126/science.1220761

125. Sullivan Å, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. The Lancet Infectious Diseases. Elsevier; 2001;1: 101–114. doi:10.1016/S1473-3099(01)00066-4

126. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis. Oxford University Press; 2012;55: 905–914. doi:10.1093/cid/cis580

127. Ruppé E, Lixandru B, Cojocaru R, Büke Ç, Paramythiotou E, Angebault C, et al. Relative Fecal Abundance of Extended-Spectrum-β-Lactamase-Producing Escherichia coli Strains and Their Occurrence in Urinary Tract Infections in Women. Antimicrob Agents Chemother. American Society for Microbiology; 2013;57: 4512–4517. doi:10.1128/AAC.00238-13

128. Raymond F, Ouameur AA, Déraspe M, Iqbal N, Gingras H, Dridi B, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics. - PubMed - NCBI. ISME J. 2015;10: 707–720. doi:10.1038/ismej.2015.148

129. Rashid M-U, Weintraub A, Nord CE. Effect of new antimicrobial agents on the ecological balance of human microflora. Anaerobe. Academic Press; 2012;18: 249–253. doi:10.1016/j.anaerobe.2011.11.005

130. Donskey CJ, Chowdhry TK, Hecker MT, Hoyen CK, Hanrahan JA, Hujer AM, et al. Effect of antibiotic therapy on the density of vancomycin-resistant enterococci in the stool of colonized patients. N Engl J Med. 2000;343: 1925–1932. doi:10.1056/NEJM200012283432604

131. Slimings C, Riley TV. Antibiotics and hospital-acquired Clostridium difficile infection: update of systematic review and meta-analysis. J Antimicrob Chemother. 2013;69: 881–891. doi:10.1093/jac/dkt477

132. Deshpande A, Pasupuleti V, Thota P, Pant C, Rolston DDK, Sferra TJ, et al. Community-associated Clostridium difficile infection and antibiotics: a meta-analysis. J Antimicrob Chemother. 2013;68: 1951–1961. doi:10.1093/jac/dkt129

133. Brown KA, Khanafer N, Daneman N, Fisman DN. Meta-Analysis of Antibiotics and the Risk of Community-Associated Clostridium difficile Infection. Antimicrob Agents Chemother. 2013;57: 2326–2332. doi:10.1128/AAC.02176-12

134. Wilcox MH, Chalmers JD, Nord CE, Freeman J, Bouza E. Role of cephalosporins in the era of Clostridium difficileinfection. J Antimicrob Chemother. 2016;72: 1–18. doi:10.1093/jac/dkw385

135. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. Nature Publishing Group; 2007;1: 56–66. doi:10.1038/ismej.2007.3

136. Dethlefsen L, Huse S, Sogin ML, Relman DA. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. Eisen JA, editor. PLOS Biol. Public Library of Science; 2008;6: e280. doi:10.1371/journal.pbio.0060280

137. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci USA. National Acad Sciences; 2011;108 Suppl 1: 4554–4561. doi:10.1073/pnas.1000087107

138. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. Journal of Hepatology. Elsevier; 2014;60: 824–831. doi:10.1016/j.jhep.2013.11.034

139. Raymond F, Déraspe M, Boissinot M, Bergeron MG, Corbeil J. Partial recovery of microbiomes after antibiotic treatment. Gut Microbes. 2016;7: 428–434. doi:10.1080/19490976.2016.1216747

140. Zaura E, Brandt BW, Teixeira de Mattos MJ, Buijs MJ, Caspers MPM, Rashid M-U, et al. Same Exposure but Two Radically Different Responses to Antibiotics: Resilience of the Salivary Microbiome versus Long-Term Microbial Shifts in Feces. mBio. American Society for Microbiology; 2015;6: e01693–15. doi:10.1128/mBio.01693-15

141. Rashid M-U, Zaura E, Buijs MJ, Keijser BJF, Crielaard W, Nord CE, et al. Determining the Long-term Effect of Antibiotic Administration on the Human Normal Intestinal Microbiota Using Culture and Pyrosequencing Methods. Clin Infect Dis. 10 ed. 2015;60 Suppl 2: S77–84. doi:10.1093/cid/civ137

142. Abeles SR, Jones MB, Santiago-Rodriguez TM, Ly M, Klitgord N, Yooseph S, et al. Microbial diversity in individuals and their household contacts following typical antibiotic courses. Microbiome. BioMed Central; 2016;4: 39. doi:10.1186/s40168-016-0187-9

143. Isaac S, Scher JU, Djukovic A, Jiménez N, Littman DR, Abramson SB, et al. Short- and long-term effects of oral vancomycin on the human intestinal microbiota. J Antimicrob Chemother. 2016;72: 128–136. doi:10.1093/jac/dkw383

144. Reijnders D, Goossens GH, Hermes GDA, Neis EPJG, van der Beek CM, Most J, et al. Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial. Cell Metabolism. Cell Press; 2016;24: 63–74. doi:10.1016/j.cmet.2016.06.016

145. de Gunzburg J, Ghozlane A, Ducher A, Le Chatelier E, Duval X, Ruppé E, et al. Protection of the Human Gut Microbiome From Antibiotics. - PubMed - NCBI. J Infect Dis. 2017;217: 628–636. doi:10.1093/infdis/jix604

146. Doan T, Arzika AM, Ray KJ, Cotter SY, Kim J, Maliki R, et al. Gut Microbial Diversity in Antibiotic-Naive Children After Systemic Antibiotic Exposure: A Randomized Controlled Trial. Clin Infect Dis. 2017;64: 1147–1153. doi:10.1093/cid/cix141

147. Kabbani TA, Pallav K, Dowd SE, Villafuerte-Galvez J, Vanga RR, Castillo NE, et al. Prospective randomized controlled study on the effects of Saccharomyces boulardiiCNCM I-745 and amoxicillin-clavulanate or the combination on the gut microbiota of healthy volunteers. Gut Microbes. 2017;8: 17–32. doi:10.1080/19490976.2016.1267890

148. Haak BW, Lankelma JM, Hugenholtz F, Belzer C, de Vos WM, Wiersinga WJ. Long-term impact of oral vancomycin, ciprofloxacin and metronidazole on the gut microbiota in healthy humans. J Antimicrob Chemother. 2018;74: 782–786. doi:10.1093/jac/dky471

149. MacPherson CW, Mathieu O, Tremblay J, Champagne J, Nantel A, Girard S-A, et al. Gut Bacterial Microbiota and its Resistome Rapidly Recover to Basal State Levels after Short-term Amoxicillin-Clavulanic Acid Treatment in Healthy Adults. Sci Rep. Nature Publishing Group; 2018;8: 11192. doi:10.1038/s41598-018-29229-5

150. Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. Recovery of gut microbiota of healthy adults following antibiotic exposure. Nat Microbiol. Nature Publishing Group; 2018;3: 1255–1265. doi:10.1038/s41564-018-0257-9

151. Oldenburg CE, Sié A, Coulibaly B, Ouermi L, Dah C, Tapsoba C, et al. Effect of Commonly Used Pediatric Antibiotics on Gut Microbial Diversity in Preschool Children in Burkina Faso: A Randomized Clinical Trial. Open Forum Infectious Diseases. 2018;5: 1147. doi:10.1093/ofid/ofy289

152. Oldenburg CE, Hinterwirth A, Sié A, Coulibaly B, Ouermi L, Dah C, et al. Gut resistome after oral antibiotics in preschool children in Burkina Faso: A randomized controlled trial. Clin Infect Dis. 2019. doi:10.1093/cid/ciz455

153. Burdet C, Grall N, Linard M, Bridier-Nahmias A, Benhayoun M, Bourabha K, et al. Ceftriaxone and Cefotaxime Have Similar Effects on the Intestinal Microbiota in Human Volunteers Treated by Standard-Dose Regimens. Antimicrob Agents Chemother. 2019;63: 675.

154. Wei S, Mortensen MS, Stokholm J, Brejnrod AD, Thorsen J, Rasmussen MA, et al. Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial. EBioMedicine. 2018;38: 265–272. doi:10.1016/j.ebiom.2018.11.035

155. Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-Term Antibiotic Treatment Has Differing Long-Term Impacts on the Human Throat and Gut Microbiome. Ratner AJ, editor. PLoS ONE. Public Library of Science; 2010;5: e9836. doi:10.1371/journal.pone.0009836

156. Panda S, khader El I, Casellas F, López Vivancos J, García Cors M, Santiago A, et al. Short-Term Effect of Antibiotics on Human Gut Microbiota. Cotter PD, editor. PLoS ONE. 2014;9: e95476. doi:10.1371/journal.pone.0095476

157. Stewardson AJ, Gaïa N, François P, Malhotra-Kumar S, Delémont C, Martinez de Tejada B, et al. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients

with urinary tract infections: a culture-free analysis of gut microbiota. Clinical Microbiology and Infection. Elsevier; 2015;21: 344.e1–344.e11. doi:10.1016/j.cmi.2014.11.016

158. Vervoort J, Xavier BB, Stewardson A, Coenen S, Godycki-Cwirko M, Adriaenssens N, et al. Metagenomic analysis of the impact of nitrofurantoin treatment on the human faecal microbiota. J Antimicrob Chemother. 2015;8: 183. doi:10.1093/jac/dkv062

159. Shono Y, Docampo MD, Peled JU, Perobelli SM, Velardi E, Tsai JJ, et al. Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. Sci Transl Med. American Association for the Advancement of Science; 2016;8: 339ra71–339ra71. doi:10.1126/scitranslmed.aaf2311

160. Pettigrew MM, Gent JF, Kong Y, Halpin AL, Pineles L, Harris AD, et al. Gastrointestinal Microbiota Disruption and Risk of Colonization With Carbapenem-resistant Pseudomonas aeruginosain Intensive Care Unit Patients. Clin Infect Dis. 2018;69: 405. doi:10.1093/cid/ciy936

161. Martín-Núñez GM, Cornejo-Pareja I, Coin-Aragüez L, Roca-Rodríguez MDM, Muñoz-Garach A, Clemente-Postigo M, et al. H. pylori eradication with antibiotic treatment causes changes in glucose homeostasis related to modifications in the gut microbiota. Virolle M-J, editor. PLoS ONE. 2019;14: e0213548. doi:10.1371/journal.pone.0213548

162. Morjaria S, Schluter J, Taylor BP, Littmann ER, Carter RA, Fontana E, et al. Antibiotic-induced shifts in fecal microbiota density and composition during hematopoietic stem cell transplantation. Infect Immun. 2019. doi:10.1128/IAI.00206-19

163. Korpela K, Salonen A, Virta LJ, Kekkonen RA, Forslund K, Bork P, et al. Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. Nat Commun. Nature Publishing Group; 2016;7: 10410. doi:10.1038/ncomms10410

164. Wipperman MF, Fitzgerald DW, Juste MAJ, Taur Y, Namasivayam S, Sher A, et al. Antibiotic treatment for Tuberculosis induces a profound dysbiosis of the microbiome that persists long after therapy is completed. Sci Rep. 2017;7: 2. doi:10.1038/s41598-017-10346-6

165. Meletiadis J, Turlej-Rogacka A, Lerner A, Adler A, Tacconelli E, Mouton JW. Amplification of Antimicrobial Resistance in Gut Flora of Patients Treated with Ceftriaxone. Antimicrob Agents Chemother. American Society for Microbiology Journals; 2017;61: S2. doi:10.1128/AAC.00473-17

166. Bernard J, Armand-Lefèvre L, Luce E, Mniai El A, Chau F, Casalino E, et al. Impact of a short exposure to levofloxacin on faecal densities and relative abundance of total and quinolone-resistant Enterobacteriaceae. Clinical Microbiology and Infection. Elsevier; 2016;22: 646.e1–646.e4. doi:10.1016/j.cmi.2016.04.015

167. Arcilla MS, van Hattem JM, Haverkate MR, Bootsma MCJ, van Genderen PJJ, Goorhuis A, et al. Import and spread of extended-spectrum β-lactamase-producing

Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study. The Lancet Infectious Diseases. 2016. doi:10.1016/S1473-3099(16)30319-X

168. Sjlund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-Term Persistence of Resistant EnterococcusSpecies after Antibiotics To Eradicate Helicobacter pylori. Ann Intern Med. 2003;139: 483. doi:10.7326/0003-4819-139-6-200309160-00011

169. Spellberg B. The New Antibiotic Mantra—"Shorter Is Better." JAMA Intern Med. American Medical Association; 2016;176: 1254–1255. doi:10.1001/jamainternmed.2016.3646

170. Ashiru-Oredope D, Sharland M, Charani E, McNulty C, Cooke J, ARHAI Antimicrobial Stewardship Group. Improving the quality of antibiotic prescribing in the NHS by developing a new Antimicrobial Stewardship Programme: Start Smart--Then Focus. J Antimicrob Chemother. 2012;67 Suppl 1: i51–63. doi:10.1093/jac/dks202

171. Buelow E, González TDJB, Fuentes S, de Steenhuijsen Piters WAA, Lahti L, Bayjanov JR, et al. Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects. Microbiome. BioMed Central; 2017;5: 88. doi:10.1186/s40168-017-0309-z

172. Andersen H, Connolly N, Bangar H, Staat M, Mortensen J, Deburger B, et al. Use of Shotgun Metagenome Sequencing To Detect Fecal Colonization with Multidrug-Resistant Bacteria in Children. Burnham CAD, editor. J Clin Microbiol. American Society for Microbiology Journals; 2016;54: 1804–1813. doi:10.1128/JCM.02638-15

173. Lanza VF, Baquero F, Martínez JL, Ramos-Ruíz R, González-Zorn B, Andremont A, et al. In-depth resistome analysis by targeted metagenomics. Microbiome. BioMed Central; 2018;6: 11. doi:10.1186/s40168-017-0387-y

174. Shintani M, Sanchez ZK, Kimbara K. Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front Microbiol. Frontiers; 2015;6: 242. doi:10.3389/fmicb.2015.00242

175. Hoffmann P, Hoffmann C, Weimann A. Choice of plasmid preparation kit influences sequencing results. Biotechnol Lett. 2009;32: 421–422. doi:10.1007/s10529-009-0157-4

176. Stadler J, Lemmens R, Nyhammar T. Plasmid DNA purification. - PubMed - NCBI. J Gene Med. 2004;6: S54–S66.

177. Tan SC, Yiap BC. DNA, RNA, and Protein Extraction: The Past and The Present. Journal of Biomedicine and Biotechnology. 2009;2009: 1–10. doi:10.1155/2009/574398

178. Ghanem A, Healey R, Adly FG. Current trends in separation of plasmid DNA vaccines: A review. Analytica Chimica Acta. 2013;760: 1–15. doi:10.1016/j.aca.2012.11.006

179. Abdulrahman A, Ghanem A. Recent advances in chromatographic purification of plasmid DNA for gene therapy and DNA vaccines: A review. Analytica Chimica Acta. 2018;1025: 41–57. doi:10.1016/j.aca.2018.04.001

180. Zhang T, Zhang X-X, Ye L. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. PLoS ONE. Public Library of Science; 2011;6: e26041. doi:10.1371/journal.pone.0026041

181. Pankhurst L, Macfarlane-Smith L, Buchanan J, Anson L, Davies K, O'Connor L, et al. Can rapid integrated polymerase chain reaction-based diagnostics for gastrointestinal pathogens improve routine hospital infection control practice? A diagnostic study. Health Technol Assess. 2014;18: 1–167. doi:10.3310/hta18530

182. Blane B, Brodrick HJ, Gouliouris T, Ambridge KE, Kidney AD, Ludden CM, et al. Comparison of 2 chromogenic media for the detection of extended-spectrum β-lactamase producing Enterobacteriaceae stool carriage in nursing home re... - PubMed - NCBI. Diagnostic Microbiology and Infectious Disease. 2016;84: 181–183. doi:10.1016/j.diagmicrobio.2015.11.008

183. Stoesser N, Giess A, Batty EM, Sheppard AE, Walker AS, Wilson DJ, et al. Genome sequencing of an extended series of NDM-producing Klebsiella pneumoniae isolates from neonatal infections in a Nepali hospital characterizes the extent of community- versus hospital-associated transmission in an endemic setting. Antimicrob Agents Chemother. American Society for Microbiology; 2014;58: 7347–7357. doi:10.1128/AAC.03900-14

184. Jett BD, Hatter KL, Huycke MM, Gilmore MS. Simplified agar plate method for quantifying viable bacteria. BioTechniques. 1997;23: 648–650. doi:10.2144/97234bm22

185. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. BioMed Central; 2014;15: R46. doi:10.1186/gb-2014-15-3-r46

186. Babraham Bioinformatics. FastQC. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Internet]. Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

187. Bushnell B. BBMap. http://sourceforge.net/projects/bbmap/. Available: http://sourceforge.net/projects/bbmap/

188. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nature Methods. Nature Publishing Group; 2015;12: 902–903. doi:10.1038/nmeth.3589

189. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25: 1754–1760. doi:10.1093/bioinformatics/btp324

190. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25: 2078–2079. doi:10.1093/bioinformatics/btp352

191. Arredondo-Alonso S, Willems RJ, van Schaik W, Schürch AC. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. Microbial Genomics. Microbiology Society; 2017;3. doi:10.1099/mgen.0.000128

192. Jitwasinkul T, Suriyaphol P, Tangphatsornruang S, Hansen MA, Hansen LH, Sørensen SJ, et al. Plasmid metagenomics reveals multiple antibiotic resistance gene classes among the gut microbiomes of hospitalised patients. - PubMed - NCBI. Journal of Global Antimicrobial Resistance. 2016;6: 57–66. doi:10.1016/j.jgar.2016.03.001

193. Brown Kav A, Benhar I, Mizrahi I. A method for purifying high quality and high yield plasmid DNA for metagenomic and deep sequencing approaches. Journal of Microbiological Methods. 2013;95: 272–279. doi:10.1016/j.mimet.2013.09.008

194. Li A-D, Li L-G, Zhang T. Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants. Front Microbiol. Frontiers; 2015;6: 1025. doi:10.3389/fmicb.2015.01025

195. Orlek A, Phan H, Sheppard AE, Doumith M, Ellington M, Peto T, et al. A curated dataset of complete Enterobacteriaceae plasmids compiled from the NCBI nucleotide database. Data Brief. 2017;12: 423–426. doi:10.1016/j.dib.2017.04.024

196. Raymond F, Boissinot M, Ouameur AA, Déraspe M, Plante P-L, Kpanou SR, et al. Culture-enriched human gut microbiomes reveal core and accessory resistance genes. Microbiome. 2019;7: 894. doi:10.1186/s40168-019-0669-7

197. Noyes NR, Weinroth ME, Parker JK, Dean CJ, Lakin SM, Raymond RA, et al. Enrichment allows identification of diverse, rare elements in metagenomic resistome-virulome sequencing. Microbiome. BioMed Central; 2017;5: 142. doi:10.1186/s40168-017-0361-8

198. Jenq RR, Taur Y, Devlin SM, Ponce DM, Goldberg JD, Ahr KF, et al. Intestinal Blautia Is Associated with Reduced Death from Graft-versus-Host Disease. Biology of Blood and Marrow Transplantation. 2015;21: 1373–1383. doi:10.1016/j.bbmt.2015.04.016

199. Papanicolas LE, Gordon DL, Wesselingh SL, Rogers GB. Not Just Antibiotics: Is Cancer Chemotherapy Driving Antimicrobial Resistance? Trends Microbiol. 2018;26: 393–400. doi:10.1016/j.tim.2017.10.009

200. Thompson WA Jr, Endriss J. The Required Sample Size When Estimating Variances. The American Statistician. 1961;15: 22–23.

201. Bates J, Thomas-Jones E, Pickles T, Kirby N, Gal M, Bongard E, et al. Point of care testing for urinary tract infection in primary care (POETIC): protocol for a randomised controlled trial of the clinical and cost effectiveness of FLEXICULT™ informed

management of uncomplicated UTI in primary care. BMC Fam Pract. 3rd ed. 2014;15: 131. doi:10.1186/s12875-014-0187-4

202. Butler CC, Francis NA, Thomas-Jones E, Longo M, Wootton M, Llor C, et al. Point-of-care urine culture for managing urinary tract infection in primary care: a randomised controlled trial of clinical and cost-effectiveness. Br J Gen Pract. British Journal of General Practice; 2018;68: e268–e278. doi:10.3399/bjgp18X695285

203. Lagier J-C, Million M, Hugon P, Armougom F, Raoult D. Human Gut Microbiota: Repertoire and Variations. Front Cell Inf Microbio. 2012;2. doi:10.3389/fcimb.2012.00136

204. Drengenes C, Wiker HG, Kalananthan T, Nordeide E, Eagan TML, Nielsen R. Laboratory contamination in airway microbiome studies. - PubMed - NCBI. BMC Microbiology 2017 17:1. 2019;19: e8578.

205. Minich JJ, Sanders JG, Amir A, Humphrey G, Gilbert JA, Knight R. Quantifying and Understanding Well-to-Well Contamination in Microbiome Research. Segata N, editor. mSystems. 2019;4: 457. doi:10.1128/mSystems.00186-19

206. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32: 3047–3048. doi:10.1093/bioinformatics/btw354

207. Lindgreen S, Adair KL, Gardner PP. An evaluation of the accuracy and speed of metagenome analysis tools. Sci Rep. 2016;6: 376. doi:10.1038/srep19233

208. Seppey M, Manni M, Zdobnov EM. LEMMI: A Live Evaluation of Computational Methods for Metagenome Investigation. bioRxiv. Cold Spring Harbor Laboratory; 2019;7: 507731. doi:10.1101/507731

209. Kim B-R, Shin J, Guevarra RB, Lee JH, Kim DW, Seol K-H, et al. Deciphering Diversity Indices for a Better Understanding of Microbial Communities. Journal of Microbiology and Biotechnology. 2017;27: 2089–2093. doi:10.4014/jmb.1709.09027

210. Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J, Keane JA, et al. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. Microbial Genomics. 2017;3: 113. doi:10.1099/mgen.0.000131

211. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. 2017;45: D566–D573. doi:10.1093/nar/gkw1004

212. Zankari E, Hasman H, Kaas RS, Seyfarth AM, Agersø Y, Lund O, et al. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. J Antimicrob Chemother. Oxford University Press; 2013;68: 771–777. doi:10.1093/jac/dks496

213. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother. 2014;58: 212–220. doi:10.1128/AAC.01310-13

214. Lakin SM, Dean C, Noyes NR, Dettenwanger A, Ross AS, Doster E, et al. MEGARes: an antimicrobial resistance database for high throughput sequencing. Nucleic Acids Res. 2017;45: D574–D580. doi:10.1093/nar/gkw1009

215. Yin X, Jiang X-T, Chai B, Li L, Yang Y, Cole JR, et al. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. Wren J, editor. Bioinformatics. 2018;34: 2263–2270. doi:10.1093/bioinformatics/bty053

216. Pierce KE, Peter H, Bachmann TT, Volpe C, Mistry R, Rice JE, et al. Rapid Detection of TEM-Type Extended-Spectrum β-Lactamase (ESBL) Mutations Using Lights-On/Lights-Off Probes with Single-Stranded DNA Amplification. The Journal of Molecular Diagnostics. 2013;15: 291–298. doi:10.1016/j.jmoldx.2013.02.002

217. Rowe W, Baker KS, Verner-Jeffreys D, Baker-Austin C, Ryan JJ, Maskell D, et al. Search Engine for Antimicrobial Resistance: A Cloud Compatible Pipeline and Web Interface for Rapidly Detecting Antimicrobial Resistance Genes Directly from Sequence Data. PLoS ONE. Public Library of Science; 2015;10: e0133492. doi:10.1371/journal.pone.0133492

218. Yang Y, Jiang X, Chai B, Ma L, Li B, Zhang A, et al. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. Bioinformatics. 2016;32: 2346–2351. doi:10.1093/bioinformatics/btw136

219. Arango-Argoty G, Garner E, Pruden A, Heath LS, Vikesland P, Zhang L. DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. Microbiome. BioMed Central; 2018;6: 23. doi:10.1186/s40168-018-0401-z

220. Gweon HS, Shaw LP, Swann J, De Maio N, AbuOun M, Hubbard ATM, et al. The impact of sequencing depth on the inferred taxonomic composition and AMR gene content of metagenomic samples. bioRxiv. Cold Spring Harbor Laboratory; 2019;18: 593301. doi:10.1101/593301

221. Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J, Keane JA, et al. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. bioRxiv. Cold Spring Harbor Labs Journals; 2017;: 118000. doi:10.1101/118000

222. Inouye M, Dashnow H, Raven L-A, Schultz MB, Pope BJ, Tomita T, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. BioMed Central; 2014;6: 90. doi:10.1186/s13073-014-0090-6

223. Phan H. reistType. https://github.com/hangphan/resistType. Available: https://github.com/hangphan/resistType

224. Rowe WPM, Winn MD. Indexed variation graphs for efficient and accurate resistome profiling. - PubMed - NCBI. Hancock J, editor. Bioinformatics. 2018;34: 3601–3608. doi:10.1093/bioinformatics/bty387

225. Versporten A, Zarb P, Caniaux I, Gros M-F, Drapier N, Miller M, et al. Antimicrobial consumption and resistance in adult hospital inpatients in 53 countries: results of an internet-based global point prevalence survey. The Lancet Global Health. 2018;6: e619–e629. doi:10.1016/S2214-109X(18)30186-4

226. Wagenmakers E-J, Farrell S. AIC model selection using Akaike weights. Psychon Bull Rev. 2004;11: 192–196.

227. Murtaugh PA. In defense of P values. Ecology. John Wiley & Sons, Ltd; 2014;95: 611–617. doi:10.1890/13-0590.1

228. Burdet C, Nguyen TT, Duval X, Ferreira S, Andremont A, Guedj J, et al. Impact of Antibiotic Gut Exposure on the Temporal Changes in Microbiome Diversity. Antimicrob Agents Chemother. 2019;63: 1449. doi:10.1128/AAC.00820-19

229. Currie BP, Lemos-Filho L. Evidence for Biliary Excretion of Vancomycin into Stool during Intravenous Therapy: Potential Implications for Rectal Colonization with Vancomycin-Resistant Enterococci. Antimicrob Agents Chemother. 2004;48: 4427–4429. doi:10.1128/AAC.48.11.4427-4429.2004

230. Hershberger E, Donabedian S, Konstantinou K, Zervos MJ. Quinupristin-dalfopristin resistance in gram-positive bacteria: mechanism of resistance and epidemiology. - PubMed - NCBI. Clin Infect Dis. 2004;38: 92–98. doi:10.1086/380125

231. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. Expert Review of Anti-infective Therapy. 2014;12: 1221–1236. doi:10.1586/14787210.2014.956092

232. Copelan EA. Hematopoietic Stem-Cell Transplantation. N Engl J Med. 2006;354: 1813–1826. doi:10.1056/NEJMra052638

233. Weber D, Jenq RR, Peled JU, Taur Y, Hiergeist A, Koestler J, et al. Microbiota Disruption Induced by Early Use of Broad-Spectrum Antibiotics Is an Independent Risk Factor of Outcome after Allogeneic Stem Cell Transplantation. Biology of Blood and Marrow Transplantation. 2017;23: 845–852. doi:10.1016/j.bbmt.2017.02.006

234. Samonis G, Gikas A, Anaissie EJ, Vrenzos G, Maraki S, Tselentis Y, et al. Prospective evaluation of effects of broad-spectrum antibiotics on gastrointestinal yeast colonization of humans. Antimicrob Agents Chemother. 1993;37: 51–53.

235. Samonis G, Dassiou M, Anastassiadou H. Antibiotics affecting gastrointestinal colonization of mice by yeasts. J Chemother. 1994;6: 50–52.

236. Evans BA, Amyes SGB. OXA -Lactamases. Clin Microbiol Rev. 2014;27: 241–263. doi:10.1128/CMR.00117-13

237. Paul M, Yahav D, Bivas A, Fraser A, Leibovici L. Anti-pseudomonal beta-lactams for the initial, empirical, treatment of febrile neutropenia: comparison of beta-lactams. Cochrane Gynaecological, Neuro-oncology and Orphan Cancer Group, editor. Cochrane Database of Systematic Reviews. 2010;84: 581. doi:10.1002/14651858.CD005197.pub3

9 Appendix 1 – Search strategy for literature review

PubMed was searched using the following terms up to 1st July 2019
1) (microbiome[title] OR microbiota[title] OR microflora[title] OR resistome[title] OR resistan*[title]) AND
2) (faeces OR faecal OR feces OR fecal OR stool OR gut OR bowel OR intestine* OR gastrointestinal OR colon) AND
3) (antibiotic[title] OR antibiotics[title] OR antimicrobial[title] OR antimicrobials[title] OR amikacin[title] OR aminoglycoside*[title] OR amoxicillin[title] OR ampicillin[title] OR azithromycin[title] OR aztreonam[title] OR carbapenem*[title] OR cefalexin[title] OR cefoxitin[title] OR ceftazidime[title] OR ceftolozane[title] OR ceftriaxone[title] OR cephalosporin*[title] OR chloramphenicol[title] OR ciprofloxacin[title] OR clarithromycin[title] OR clindamycin[title] OR co-amoxiclav[title] OR co-trimoxazole[title] OR colistin[title] OR daptomycin[title] OR doxycycline[title] OR ertapenem[title] OR erythromycin[title] OR ethambutol[title] OR ethambutol[title] OR fidaxomicin[title] OR flucloxacillin[title] OR fluoroquinolone*[title] OR fosfomycin[title] OR gentamicin[title] OR glycopeptide*[title] OR isoniazid[title] OR levofloxacin[title] OR linezolid[title] OR macrolide*[title] OR meropenem[title] OR metronidazole[title] OR moxifloxacin[title] OR neomycin[title] OR nitrofurantoin[title] OR penicillin[title] OR piperacillin[title] OR pivmecillinam[title] OR pyrazinamide[title] OR pyrazinamide[title] OR quinolone*[title] OR rifampicin[title] OR streptomycin[title] OR teicoplanin[title] OR temocillin [title] OR tetracycline*[title] OR tigecycline[title] OR tobramycin[title] OR trimethoprim[title] OR vancomycin[title])

Studies that met the following criteria were reviewed, including reference searching for additional relevant studies:
1) Contain primary data about the effect of antibiotics on the gut microbiome or resistome
2) Utilise culture-independent methods of microbiome/resistome analysis (excluding microbiome methods limited to a few specific organisms)
3) Report data from >1 human (i.e. excluding case reports and animal/*in vitro* studies)
4) Report data in a form that can usefully be compared with results from other studies
5) Do not only report on specific groups likely to have atypical gut microbiome, e.g. infants (<1 year), patients with diarrhoea, colectomy or bowel transplant patients

10 Appendix 2 – Consent form

Oxford University Hospitals NHS
NHS Foundation Trust

Nuffield Department of Medicine

University of Oxford

Chief investigator: Professor Derrick Crook
Consultant in Microbiology and Infectious Diseases
Tel: 01865 221226

Centre: John Radcliffe Hospital Study Number: PID:11371-TMA REC: 15/EM/0270

Participant Identification Number for this study: _____

Antibiotic Resistance in the Microbiome OxfoRd (ARMORd) Study

CONSENT FORM Version 4.0, 13th July 2016 Author: Dr N Fawcett

Name of Researcher: _____

Please initial box

1. I confirm that I have read the information sheet dated 13th July 2016 (version 4.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I consider these samples as a gift to the University of Oxford and I understand I will not gain any direct personal benefit from this. ☐

4. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the University of Oxford, from regulatory Authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I understand that the DNA sequences from the bacteria in my poo may be shared in public not-for-profit scientific databases to support other scientific research in the future. ☐

6. (Optional) I agree for the poo sample and bacterial DNA I give to be stored anonymously for a period of up to 10 years after the study has ended for use in future research, as mentioned in the information sheet. ☐

7. (Optional) I am happy to be contacted about future research projects by this group looking at antibiotic resistance. ☐

8. (Optional) I would like to receive a report of my gut microbe profile at the end of the study. ☐

9. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person receiving consent	Date	Signature

11 Appendix 3 – Case record form

Oxford University Hospitals NHS
NHS Foundation Trust

Nuffield Department of Medicine

University of Oxford

Antibiotic Resistance in the Microbiome OxfoRD (ARMORD) Study – Study Questionnaire for investigator completion

version 3.0 (25th November 2015) Author: N Fawcett

THIS IS A RESEARCH STUDY OFFICIAL DOCUMENT AND IS HIGHLY CONFIDENTIAL. IF YOU ARE NOT A MEMBER OF THE ARMORd RESEARCH TEAM AND HAVE OBTAINED THIS DOCUMENT IN ERROR, PLEASE INFORM THE RESEARCH TEAM IMMEDIATELY ON: armord@ndm.ox.ac.uk /01865 222194, or hand it in to the Microbiology Department on Level 7 of the John Radcliffe Hospital (for attention of Prof Crook Group). We will pick it up and review our handling of this document.

Please confirm with the participant this statement: "Whilst this information is of great help to us in understanding antibiotic resistance and the microbiome, you are free to choose which questions to answer, and if you require clarification or prefer not to answer, let us know".

Study Number: _____ **Date of Questionnaire: _____**

Researcher: _____

1) Tell me about any antibiotics you may have received in the last year:

Antibiotics in past month? Y / N
Antibiotics in the past year? Y / N
If yes to either then use additional data sheet

2) Tell me anything you can recall about your antibiotic use throughout your life. For example - we would like to find out if you have a condition like asthma and may have taken many courses of antibiotics every year, or if you are someone who virtually never takes antibiotics.

Antibiotic use: More than 10 courses in the last 5 years.....[ ]
Antibiotic use: 1 – 10 courses in the last 5 years.....[ ]
Antibiotic use: No antibiotics in the last 5 years.....[ ]
Notes:

3) Have you had any infections (such as urinary tract infections, pneumonia or skin infections) in the last 3 years?

Had an infection, think it was resistant.....[ ]
Had an infection, think it was sensitive.....[ ]
Had an infection, but don't know the resistance information.....[ ]
No infection.....[ ]
Details (what do you know about it?):

4) Tell me about any travel in the last three years:

No Travel outside UK.....[ ]
Travel outside UK.....[ ] (use additional data sheet)

5) What best describes your diet?

No dietary restrictions [ ]
Vegetarian (eats eggs & milk) [ ]
Vegan (no eggs or milk products) [ ]

6) How often do you touch or handle the following food products?

	Never	Occasionally/once a month or less	Once a week to once a month	Multiple times a week
Raw chicken meat				
Other raw meat:				
Raw shellfish: (including prawns)				

7) Do you work in the food industry?

No [ ] Animal farm [ ] Plant farm [ ] Abattoir [ ]

8) Do you work in the healthcare profession?

No [ ]
Hospital, direct physical contact with patients [ ]
Hospital, no direct contact with patients [ ]
Hospital including patient bodily hygiene and contact with waste matter [ ]
GP surgery/outpatient clinic* only, direct contact with patients [ ]
GP surgery/clinic*, no direct contact with patients [ ]
*a healthcare site which doesn't have patients to stay overnight for care

9) How often have you been to hospital in the last year?

No contact [ ]
As a patient:
Contact, only outpatients/day case [ ]
Contact, inpatient [ ]
Last date of contact:

How much?	1 day [ ]	2-14 days [ ]	>14 days [ ]

Details:
Do you visit others in hospital regularly?
Notes (place/duration):

10) Have you been admitted to hospital overnight or more in the last 5 years?

No [ ] Yes [ ] (use additional data sheet)

11) How often have you been in contact with residential/nursing care in the last year?

As a resident:
Never [ ] Previous contact, not current [ ] Current [ ]
Notes (last contact, type of care, total approx. duration):

As a visitor:

How often as a visitor?	Never	Occasionally/once a month or less	Once a week to once a month	Multiple times a week	Approx. date of last contact
Nursing Care					
Residential care					

12) How often do you provide childcare for babies or children <2 years?

	Never	Occasionally/once a month or less	Once a week to once a month	Multiple times a week	Approx. date of last contact
Any care					
Care, including personal hygiene for child (body washing and/or handling of nappies)					

13) Do you have any pets in your household?

Cat [ ]
Dog [ ]
Other(s) [ ] (please specify):
None [ ]
I clean up their poo Y / N

14) Have you had any episodes of diarrhoea in the last 2 months?

No [ ]
1+ episode of diarrhoea [ ]
Loose stools on daily basis [ ]
Notes (cause if known, timing):

15) Have you been treated with chemotherapy, radiotherapy or medication which affects the immune system* in the last 5 years?

No [ ]
Yes [ ]
Don't know [ ]
Notes (What was it for? How long did you take it for? How did you get given it? e.g. tablets/injections):

* You may have received this if you had an autoimmune disease (overactive immune system) This includes steroids, methotrexate, hydroxychloroquine, sulfasalazine, leflunomide, and injections of anti-TNFa/IL-2R treatment for conditions like rheumatoid arthritis.

16) Have you taken any of the following in the last month?

Medication *(if in doubt, you can discuss at the visit)*	Never	Yes, regularly	Occasionally	Don't know
Proton-pump inhibitors (e.g. Omeprazole, lansoprazole)				
Non-steroidal anti-inflammatory drugs (e.g. ibuprofen, aspirin, diclofenac, naproxen)				
Vitamin, herbal or dietary supplements (including fibre)				

Notes (What was it called? What was it for? How long did you take it for? How did you get given it? e.g. tablets/injections):

17) How often do you eat the following foods?

(if you are tired or short of time, we can leave it or go through this at another visit)

Type of food (approx. serving size)	Never	Less than once a week	Once a week	2-4 times a week	5-6 times a week	Once or more daily	If more than once daily – how many times?
High fibre food – brown bread, brown rice, high fibre cereal (one handful/piece of bread)							
Other cereals, breads potatoes, rice, pasta (one handful/piece of bread)							
Fresh fruit (one apple size)							
Fresh vegetables (one apple size)							
Milk, cheese and yoghurt products (one glass/ small yoghurt pot)							
Meat, fish and poultry (one handful)							
Non-animal meat alternatives (Quorn, soy mince) (one handful)							
Eggs							
Oil and butter (one tablespoon)							
Chocolate, cake and sweets (one chocolate bar/handful)							
Ready meals/pre-packaged meals							

18) Have you ever smoked as much as one cigarette a day for as long as a year?

No (non-smoker)..... [ ]

Yes:

I have smoked this amount previously but have not smoked a cigarette in the last year ..... [ ]

I have smoked less than one cigarette a day in the last year ..... [ ]

I currently smoke one cigarette or more, on average, daily ..... [ ]

Notes:

19) How many units a week do you drink of alcohol (on average in the last year)?

(pint of beer = 2 units, small glass of wine = 1 unit, large glass of wine = 2 units, shot of spirits = 1 unit)

Units/week:

Never drink alcohol [ ]

20) Do you have anything else of relevance you think we should know?

ARMORD additional data sheet for investigator (enter approximate values if unknown)

Antimicrobial (non-OUH in past year, U = unknown)	Route	Start date	Stop date or duration (d/w/m)	Indication (blank if unknown)

Hospital (admissions outside OUH in past 5 years)	Admission	Discharge or duration (d/w/m)	Reason (blank if unknown)

Country (past 3 years, record only last visit to each country)	Start	End or duration (d/w/m)

12 Appendix 4 – Sensitivity analyses

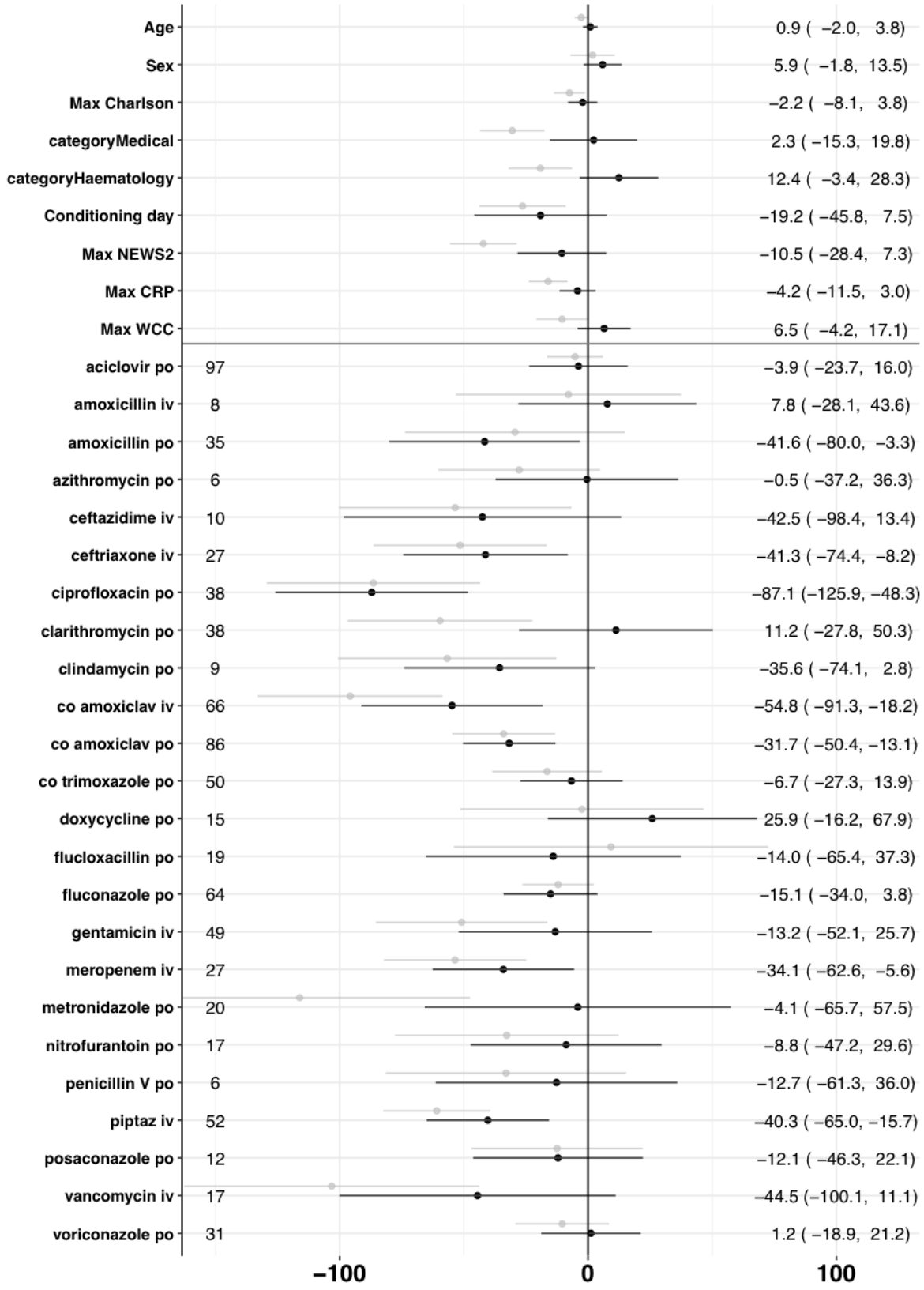

Figure 12.1 Sensitivity analysis: species richness as outcome

Cross-sectional analysis, combined exposure measure

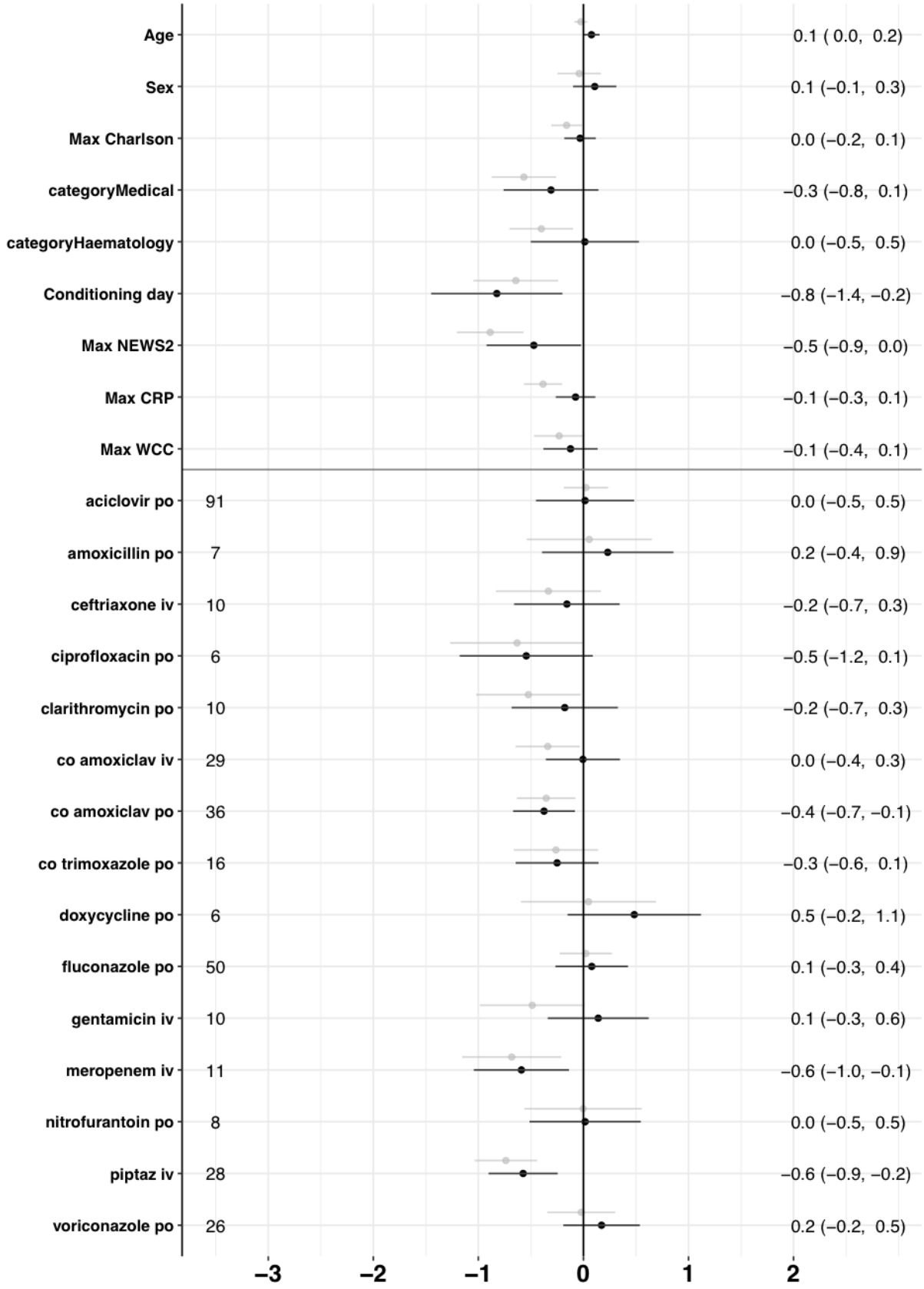

Figure 12.2 Sensitivity analysis: qualitative exposure over 7 days

Cross-sectional analysis. Note effect sizes are smaller as cumulative exposure is not taken into account and full exposure does not necessarily represent current use.

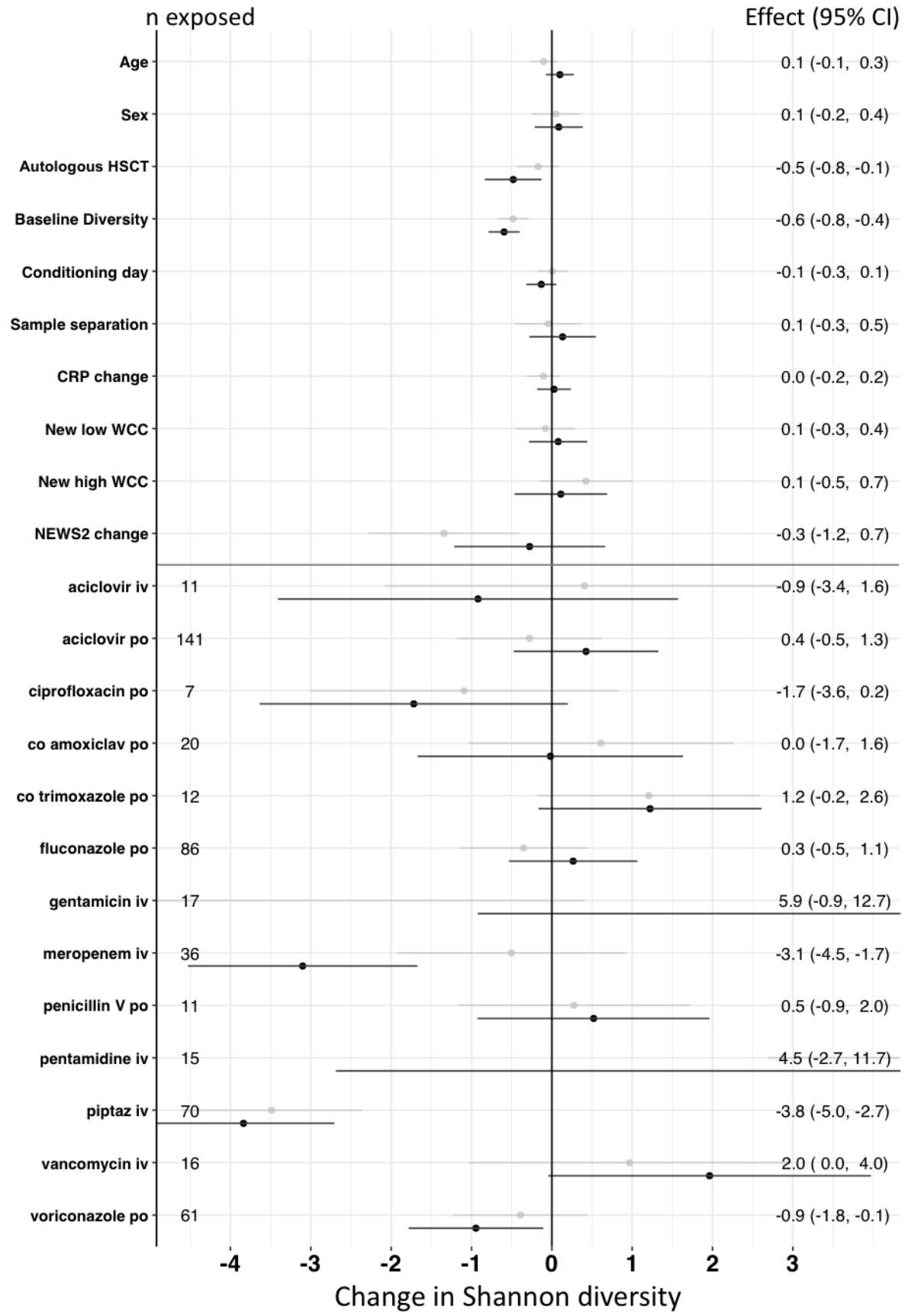

Figure 12.3 Sensitivity analysis: 21-day fading exposure

Longitudinal analysis

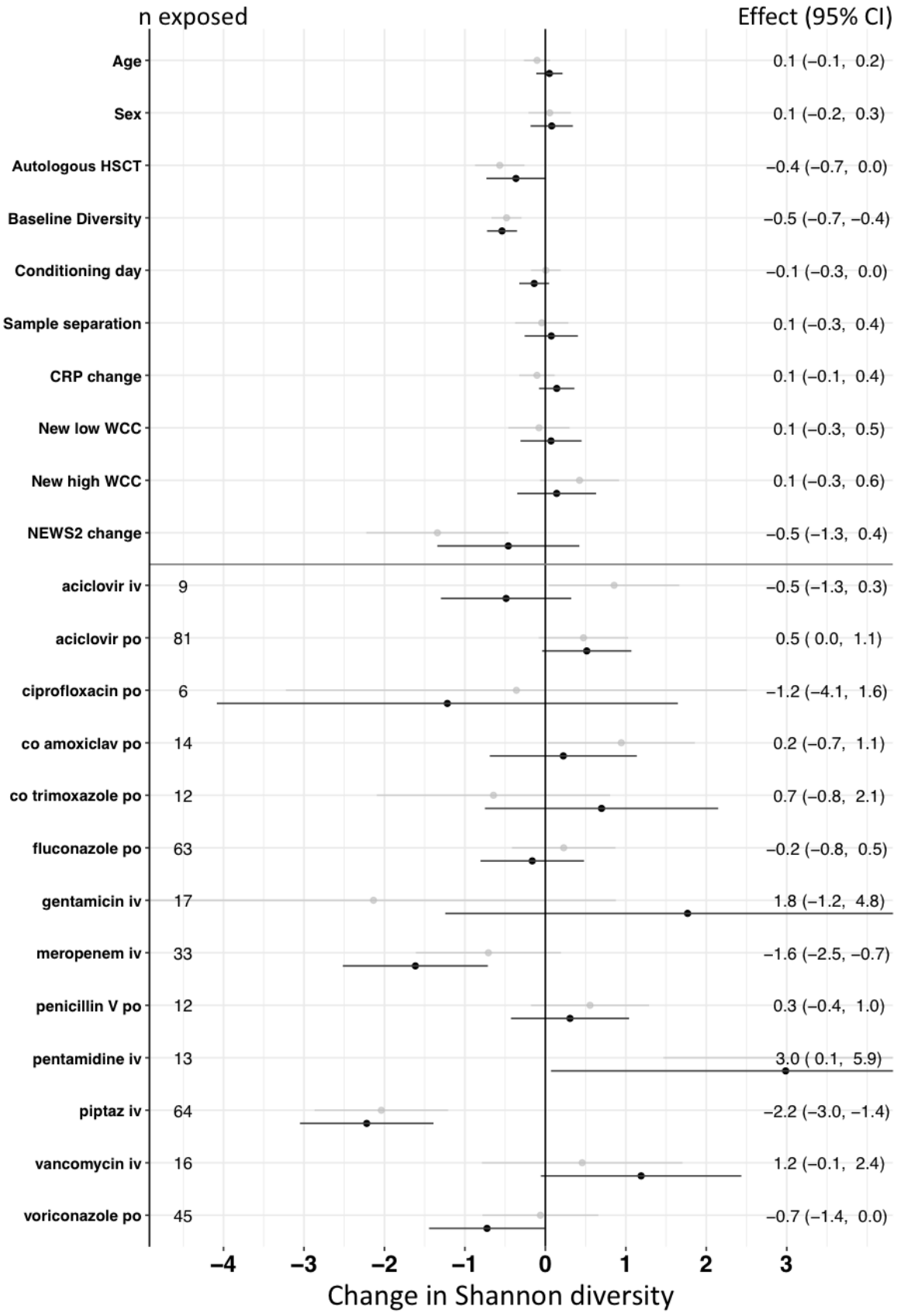

Figure 12.4 Sensitivity analysis: truncating negative antimicrobial exposures at zero

Longitudinal analysis, negative exposures truncated to 0 (i.e. exposure range is 0-1).